Isolation and Characterisation of CRTR-1 and altCP2: Negative Regulators of CP2 Transcription Factor Family Activity.

A thesis submitted to The University of Adelaide for the degree of Doctor of Philosophy

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SUMMARY

Mouse CP2 is the founding member of a group of highly conserved proteins in mouse, human, chicken and *Drosophila* collectively referred to as the CP2 family of transcription factors. CP2 was originally identified in murine erythroleukemia (MEL) cell nuclear extracts as an activator of the mouse α-globin gene, binding a promoter element overlapping the CCAAT box. In addition to CP2, the family includes mouse NF2d9, human LBP-1c and LBP1a, homologues of mouse CP2 and NF2d9 respectively, LBP-9, chicken CP2 and *Drosophila melanogaster* CP2. Mammalian CP2-related proteins have generally been shown to be activators of transcription and expressed ubiquitously. There is little knowledge of factors that regulate their activity.

This thesis describes CRTR-1, a novel member of the CP2 family, originally identified as a transcript differentially expressed between mouse ES and early primitive ectoderm-like (EPL) cells *in vitro*. *In vivo* expression analysis showed CRTR-1 to be expressed by the pluripotent inner cell mass cells of the blastocyst, but not in the later pluripotent cells of the primitive ectoderm. Analysis during later stages of development and in the adult mouse showed CRTR-1 expression to be developmentally and spatially regulated. Greatest levels of CRTR-1 expression were detected in the embryonic and adult kidneys with CRTR-1 specifically expressed in the branching ureteric buds of the developing kidney with expression becoming restricted to the epithelial lining of the distal convoluted tubules during later kidney development and in the adult mouse. These sites of CRTR-1 expression suggest distinct biological roles for CRTR-1 function in the maintenance of pluripotency in the early stage embryo and in the development and function of the kidney.

Conservation in nucleotide and amino acid sequence defined CRTR-1 as a novel member of the mouse CP2 family of transcription factors. Consistent with this,

CRTR-1 was shown to interact with all other members of the mouse CP2 family, forming protein complexes competent to bind a CP2 family consensus DNA response element. However, unlike other CP2 family members, CRTR-1 was shown to act as a transcriptional repressor with the ability to repress transcription localised to a novel 52 amino acid N-terminal repression domain. Furthermore, the ability of CRTR-1 to repress transcription was shown to be dominant over CP2 mediated transcriptional activation. CRTR-1 is therefore distinct from other family members in two respects, CRTR-1 expression is spatially and temporally regulated and CRTR-1 acts as a dominant transcriptional repressor of CP2 family mediated transcriptional activation.

This thesis also describes the identification, isolation and functional characterisation of an alternatively spliced isoform of CP2, altCP2. Similar to CRTR-1, altCP2 appears to be differentially expressed and was demonstrated to act as a dominant repressor of CP2 family mediated transcriptional activation by formation of heteromultimers with other CP2 family proteins that cannot bind DNA. Together, altCP2 and CRTR-1 provide mechanisms to achieve spatially and temporally regulated activity of ubiquitously expressed CP2 family transcriptional activators.

Possible mechanisms regulating the cellular localization and transcriptional regulatory ability of CP2 family members were investigated by identification of non-related binding proteins. Yeast-2-hybrid analysis identified Ubc9, PIAS1 and FKBP4 as CRTR-1 binding proteins. Ubc9 and PIAS1 function as regulators of post-translational modification by sumoylation. These have been shown to regulate the cellular localization and transcriptional regulation of other transcription factors, and were shown here to interact with other mouse CP2 family members. In contrast the immunophilin FKBP4 was determined to be a CRTR-1 specific binding protein suggesting that alternate mechanisms regulate CRTR-1 and other family members.

Identification of CP2 family member specific binding proteins suggest mechanisms for independent regulation of CP2 family members through distinct cellular pathways.

Finally, identification of the sumoylation enhancer PIAS3 and transcription factors Rex1 and YY1 as CRTR-1 binding proteins provide further mechanisms for the regulation of CRTR-1 activity through the control of cellular localization, transcriptional regulation and promoter specificity and together suggest mechanisms for the regulation of CRTR-1 activity through signaling pathways important for pluripotence and kidney development.

Declaration:

This work contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution and, to the best of my knowledge and belief, contains no material that has previously been published or written by another person, except where due reference is made in the text of the thesis.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Signed: _____ **Dated:** 18-3-03

This thesis is dedicated to my Mother, Judith Evelyn Rodda, and Father, Brenton James Rodda.

You have incredible strength, tolerance and an unconditional love for your family, which together make you amazing people that I admire greatly. Your support for me over the years has been truly appreciated. Without the opportunities provided to me through the hard work of you both, this would never have been possible.

You have inspired me to be the best person I can.

Whatever life's challenges you may face, remember always to look to the mountaintop, for in so doing you look to greatness.

Remember this, and let no problem, no matter how great it may seem, discourage you, nor let anything less than the mountaintop distract you.

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CHAPTER 1

Introduction.

1.1 REGULATION OF GENE EXPRESSION.

The identity and function of a cell is determined by the repertoire of genes expressed. Control of gene expression is also critical for the processes of cell differentiation, specification, establishment of a body plan and the patterning and function of tissues. Regulation of gene expression can be achieved through the action and differential expression of transcription factors, which activate or repress transcription by associating with sequence specific, *cis* acting, DNA elements located in the promoter of responsive genes. Transcription factor expression and function is therefore critical for successful development of complex organisms. An understanding of the mechanisms involved in the expression of transcription factors and the molecular pathways involved in the regulation of their function is fundamental to our understanding of the processes of development, cell specification and tissue function.

Activation or repression of gene expression is achieved by promoting or inhibiting interaction of the basal transcriptional machinery, containing RNA polymerase, with the promoter region of target genes. Transcription factors contain domains that are typically classified based on their structure and include the homeobox, helix-turn-helix, zinc finger, leucine zipper, POU and RING domains. These domains enable transcription factors to bind DNA specifically and/or interact with the basal transcriptional machinery or with other proteins, co-activators or co-repressors, which regulate association of the basal transcriptional machinery with the promoter. Co-activator and co-repressor proteins typically modulate chromatin structure through the acetylation or de-acetylation of histones by histone acetyl transferase or histone deacetylase activity, which relaxes or tightens the DNA chromatin structure respectively (Ng and Bird, 2000). Other mechanisms used for the regulation of transcription include direct interference with the assembly of the transcriptional machinery (Koipally and Georgopoulos, 2000) and recruitment of methyltransferases (Chen *et al.*, 1999b).

Cellular localization and post-translation modification provide a means for regulating transcription factor activity. Ligand activated transcription factors such as the vitamin D (Dwivedi *et al.*, 2000) or dioxin (Lees and Whitelaw, 1999) receptors are located in the cytoplasm as latent complexes associated with partner factors and cellular chaperone proteins. Binding of ligand activates the receptor enabling it to translocate to the nucleus and regulate gene expression. The transcription factor Stat3 is localized to the cytoplasm associated with the intracellular domain of gp130 family cell transmembrane receptors (Heinrich *et al.*, 1998). Activation of the gp130 signaling pathway through binding of the receptor by interleukin-6 cytokine family proteins results in Stat3 phosphorylation, and enables Stat3 to translocate to the nucleus and regulate transcription. These mechanisms for the regulation of transcription factor activity permit the integration of changes in the external cellular environment with changes in gene expression.

In contrast, many transcription factors are expressed in a spatially and temporally regulated fashion enabling the specific regulation of target genes important for a variety of developmental and cellular processes. One such group of proteins is the homeobox family of transcription factors (Wright *et al.*, 1989). These proteins and their genomic organization have been conserved from insects to vertebrates and their tightly regulated expression and function is critical for establishment of the anterior/posterior patterning of the embryo during development. Members of other conserved transcription factor families such as the forkhead (Carlsson and Mahlapuu, 2002), POU (Rosner *et al.*, 1990; Ryan and Rosenfeld, 1997) and T-box (Wilson and Conlon, 2002) families, are specifically expressed and play diverse roles important for the successful development of the mammal.

Transcription factors are typically classified into families based on conservation in amino acid sequence, structural domains, expression and/or function. The identification and study of transcription factor families that are expected to have important biological

functions is critical to our understanding of mammalian development, tissue function and our overall understanding of general cell biology.

1.2 THE CP2 FAMILY OF TRANSCRIPTION FACTORS.

Mouse CP2 is the founding member of a group of highly conserved proteins in mouse, human, chicken and *Drosophila* collectively referred to as the CP2 family of transcription factors (Sueyoshi *et al.*, 1995). CP2 was identified in murine erythroleukemia (MEL) cell nuclear extracts (Barnhart *et al.*, 1988; Barnhart *et al.*, 1989; Kim *et al.*, 1990) as an activator of the mouse α-globin gene, binding a promoter element overlapping the CCAAT box (Kim *et al.*, 1990; Lim *et al.*, 1993; Lim *et al.*, 1992). Studies of mammalian CP2-related proteins have generally shown them to be activators of transcription and expressed ubiquitously.

Other members of the CP2 family include NF2d9 (Sueyoshi *et al.*, 1995) in mice, while chicken (Murata *et al.*, 1998) and *Drosophila melanogaster* CP2 (dCP2) (Wilanowski *et al.*, 2002) homologues have been described. In humans, multiple cDNAs encoding CP2-related proteins have been identified including human CP2 ((Jane *et al.*, 1995; Lim *et al.*, 1992); also referred to as LSF and LBP-1c (Kim *et al.*, 1987; Shirra *et al.*, 1994; Yoon *et al.*, 1994)), LBP-1d ((Yoon *et al.*, 1994); also known as LSF-ID (Shirra *et al.*, 1994)), an alternatively spliced form of LBP-1c (Yoon *et al.*, 1994), LBP-1a (Yoon *et al.*, 1994), LBP-1b, ((Yoon *et al.*, 1994); an alternatively spliced form of LBP-1a (Yoon *et al.*, 1994)) and LBP-9 (Huang and Miller, 2000).

CP2 family members share amino acid sequence conservation with *grainyhead*-like proteins across regions of functional importance. The *grainyhead*-like family of proteins includes the *D.melanogaster* protein *grainyhead* (*GRH*) (Bray *et al.*, 1989; Bray and Kafatos, 1991), also known as NTF-1 or ELF-1 (Huang *et al.*, 1995), and mammalian

proteins, mammalian GRH (MGR) and brother of GRH (BOM) have also been described recently (Wilanowski *et al.*, 2002).

1.3 SEQUENCE AND cDNA STRUCTURE OF *CP2* RELATED GENES.

Mouse *CP2* is encoded by a 4021 bp transcript with an 871 bp 5' untranslated region (UTR), an open reading frame (ORF) of 501 amino acids encoding a protein with a predicted molecular weight of 56,957 Da, and a 1644 bp 3' UTR (Lim *et al.*, 1992).

Human CP2, referred to in this thesis as LBP-1c, was originally identified in HeLa cells as a protein able to bind the simian virus 40 (SV40) late promoter (Huang *et al.*, 1990; Shirra and Hansen, 1998; Shirra *et al.*, 1994). LBP-1c has also been characterized to bind promoter regions of the human immunodeficiency virus-1 (HIV-1) from HeLa cells (Wu *et al.*, 1988; Yoon *et al.*, 1994) and a CD4+ lymphocyte cell line, CEM, (Romerio *et al.*, 1997). *LBP-1c* is encoded by a 2277 bp transcript consisting of a 363 bp 5'UTR, an ORF of 502 amino acids and a 411 bp 3'UTR, and shares 96.4% amino acid sequence identity with CP2 (Lim *et al.*, 1992). An alternatively spliced variant of LBP-1c, LBP-1d, lacks 51 amino acids, equivalent to amino acids 189-239 of LBP-1c (Shirra *et al.*, 1994; Yoon *et al.*, 1994).

LBP-1a, originally identified in HeLa cell extracts by its ability to bind promoter elements present in HIV-1, is encoded by a 3843 bp transcript consisting of a 174 bp 5'UTR, a 504 amino acid ORF and a 2046 bp 3'UTR (Yoon *et al.*, 1994). LBP-1a shares 72% amino acid sequence identity with CP2 (Sueyoshi *et al.*, 1995). An alternatively spliced variant of LBP-1a, LBP-1b, contains an additional exon encoding 36 amino acids in frame between amino acids 275 and 276 of LBP-1a (Yoon *et al.*, 1994).

LBP-9 is the most recent CP2 family member identified in humans (Huang and Miller, 2000). The LBP-9 cDNA is 5019 bp long with a 91 bp 5' UTR, an ORF of 479 amino acids and an incomplete 3578 bp 3' UTR. LBP-9 was identified in human placental

JEG3 cells as a protein able to bind the cytochrome P450scc promoter and shows 71% amino acid similarity to LBP-1c and 83% amino acid similarity to LBP-1a (Huang and Miller, 2000).

NF2d9, identified as a protein in mouse liver nuclear extracts able to bind the promoter of the male-specific P450 (*Cyp* 2d-9) gene, is encoded by a 3733 bp cDNA with an ORF of 504 amino acids and a 706 bp 3' UTR (Sueyoshi *et al.*, 1995). Only 2 bp of the NF2d9 5' UTR has been described indicating that the cDNA is incomplete. NF2d9 shares 72% and 94% amino acid sequence identity with CP2 and LBP-1a, respectively and is recognised as the LBP-1a human homologue (Sueyoshi *et al.*, 1995).

dCP2 shares 35% amino acid sequence identity to LBP-1c and was identified by cxpressed sequence tag (EST) database searches using the LBP-1c sequence (Wilanowski *et al.*, 2002).

GRH is a 1063 amino acid protein initially identified as a factor purified from crude nuclear extracts of *Drosophila* embryos able to bind the *Ultrabithorax* (*Ubx*) promoter (Dynlacht *et al.*, 1989). MGR (618 amino acids) and BOM (625 amino acids) were identified by EST database searches using the GRH sequence (Wilanowski *et al.*, 2002) and share 37% and 35% amino acid sequence identity with GRH, respectively.

1.4 EXPRESSION OF CP2 RELATED PROTEINS.

Northern and RT-PCR analysis on RNA and Western analysis on protein isolated from adult mouse tissues (Swendeman *et al.*, 1994), Northern analysis on RNA isolated from human tissues (Jane *et al.*, 1995), *in situ* hybridization analysis on sectioned 9.5 d.p.c. mouse embryos (Swendeman *et al.*, 1994) investigating *CP2* expression and Northern analysis on RNA isolated from adult mouse tissues investigating *NF2d9* expression (Sueyoshi *et al.*, 1995) has shown CP2 family members to be expressed ubiquitously (Jane *et al.*, 1995; Murata *et al.*, 1998; Sueyoshi *et al.*, 1995; Swendeman *et al.*, 1994).

The expression of *LBP-9* has not been mapped in detail *in vivo*. However, RT-PCR analysis of *LBP-9* expression in cultured cell lines (placental (JEG-3), adrenal (NCI-H295A), cervical (HeLa), hepatic (HepG2), and kidney (COS-1) origin) and human adrenal tissue suggests regulated expression of this gene (Huang and Miller, 2000). Specifically, *LBP-9* expression was detected at highest levels in JEG-3 cells, at lower levels in COS-1, HepG2 cells and HeLa cells, and was not detected in NCI-H295A cells or human adrenal tissue (Huang and Miller, 2000).

In situ analysis of *GRH* on drosophila embryos showed developmentally regulated expression, restricted to ectodermally-derived tissues including the central nervous system and the epidermis (Bray *et al.*, 1989; Dynlacht *et al.*, 1989). *MGR* and *BOM* are also expressed in a developmentally and spatially regulated fashion. In mice *in situ* analysis on embryo sections demonstrated that *MGR* expression mirrors *GRH*, being predominantly in the epidermis, while *BOM* is expressed in the skin, lung, esophagus and kidney (Wilanowski *et al.*, 2002). In humans RT-PCR analysis demonstrated *MGR* expression in the pancreas, brain, tonsils, placenta and kidney while *BOM* is expressed in the pancreas, brain placenta and kidney (Wilanowski *et al.*, 2002). Characterisation of the *GRH* mRNA has demonstrated the existence of a nuroblast-specific isoform generated by tissue-specific splicing contributing two additional exons to the *GRH* ORF, which have been reported not to affect the activity of the protein in comparison to GRH (Uv *et al.*, 1997). Characterisation of mouse *MGR* has identified an alternatively spliced isoform lacking the first three exons of *MGR* but containing a unique first exon (Wilanowski *et al.*, 2002). Human MGR has also been shown to be alternatively spliced, generating a *MGR* isoform lacking the first three exons. Expression analysis of the human *MGR* isoform demonstrated higher levels of expression in the brain and unique expression in fetal liver and liver compared with *MGR* (Wilanowski *et al.*, 2002). These observations suggest that

differential expression of alternatively spliced isoforms of *GRH*, *MGR* and *BOM* are an important feature of these proteins.

1.5 DOMAIN STRUCTURE OF CP2 RELATED PROTEINS.

Comparison with known proteins in the BlastP database and various internet based motif search databases has demonstrated that CP2 family members do not contain domain structures characteristic of other transcription factor families such as helix-loop-helix, leucine zipper, zinc finger or homeobox DNA binding motifs (Lim *et al.*, 1993). Despite this, functional characterization of alternative splice variant LBP-1d together with sequence truncation studies have broadly defined regions of functional importance described below.

1.5.1 Protein/DNA Interactions of CP2 Related Proteins.

Members of the CP2 family of transcription factors bind a consensus DNA sequence consisting of a direct bipartite repeat sequence, CNRG-N_6-CNRG (Lim *et al.*, 1993; Murata *et al.*, 1998). N- and C-terminal truncation studies have defined the minimum DNA binding region of LBP-1c between amino acids 65 and 383 (Shirra and Hansen, 1998) (Figure 1.1) although DNA binding comparable to the full-length protein was only observed with amino acids 65-502. Amino acids 63-270 of CP2 share 32% identity and 52% sequence similarity with the region required for GRH DNA binding, amino acids 631-833, (Lim *et al.*, 1992; Wilanowski *et al.*, 2002) and are highly conserved within other CP2 family members. The significance of this sequence conservation is highlighted by the fact that GRH can bind the LBP-1c response element present in the SV40 promoter (Shirra and Hansen, 1998). Consistent with this, LBP-1d, which lacks amino acids 189-239 of LBP-1c, is unable to bind the LBP-1c DNA binding sequence (Shirra *et al.*, 1994; Yoon *et al.*, 1994).

FIGURE 1.1

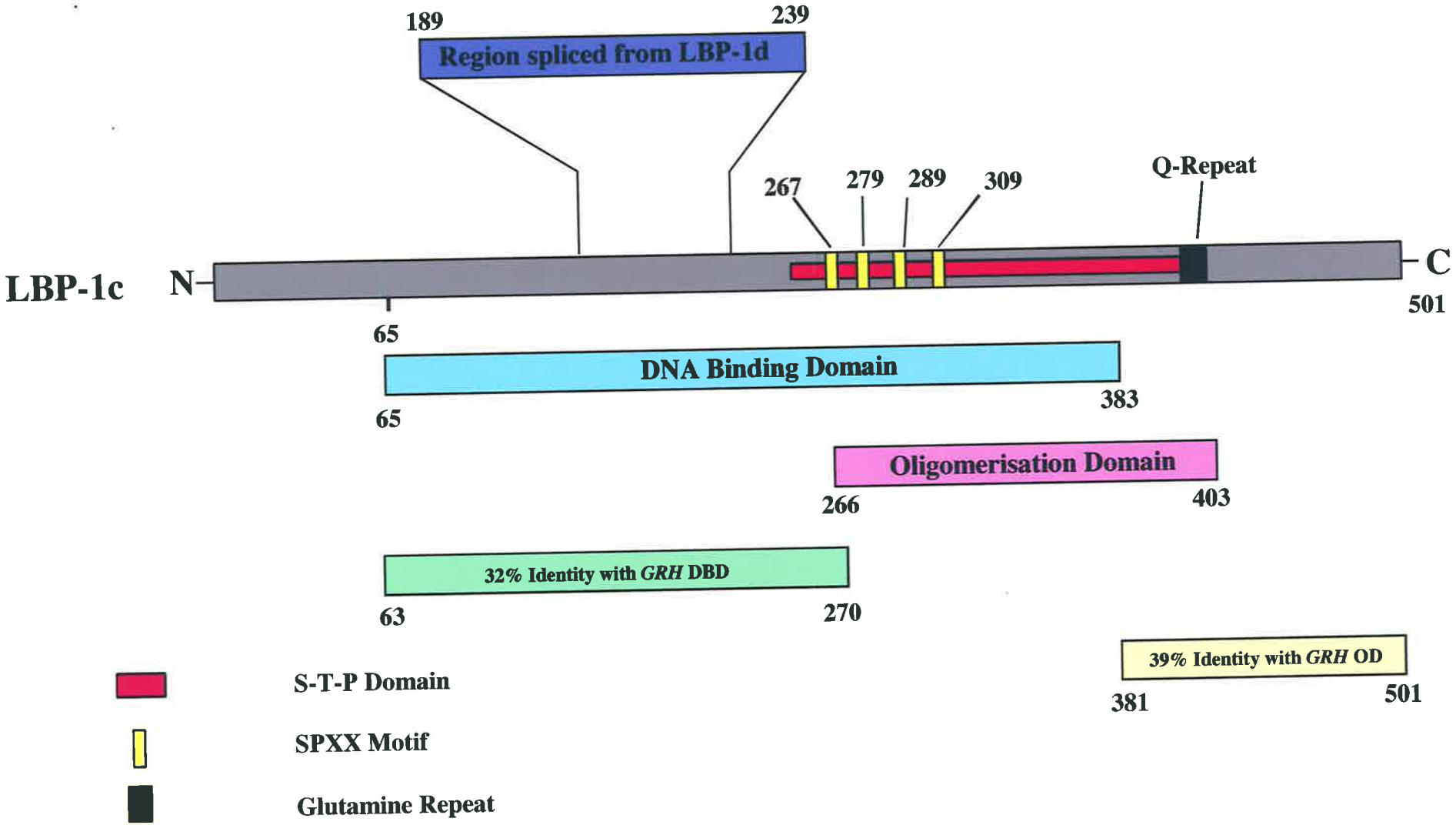
Schematic summary of conserved, functionally important, regions in LBP-1c.

Regions in LBP-1c conserved with grainyhead (GRH) and regions that have been shown to be functionally important by truncation studies are indicated. These regions are conserved in all CP2 family members. Also shown is the region alternatively spliced from LBP-1d and the location of the STP domain, SPXX motifs and the glutamine repeat.

DBD, DNA binding domain

OD, oligomerisation domain.

Q, glutamine


189
239
Region spliced from LBP-1d
267
279
289
309
Q-Repeat
LBP-1c
N
C
501
65
DNA Binding Domain
65
383
Oligomerisation Domain
266
403
32% Identity with GRH DBD
63
270
39% Identity with GRH OD
381
501
S-T-P Domain
SPXX Motif
Glutamine Repeat

Binding sites for the CP2 family of transcription factors fitting the consensus direct bipartite repeat sequence have been described in a wide range of cellular and viral promoters and are summarised in table 1.1.

1.5.2 CP2 Family Protein Interactions.

LBP-1c has been reported to bind DNA as a dimer (Shirra *et al.*, 1994; Zhong *et al.*, 1994) although other reports have shown LBP-1c (Shirra and Hansen, 1998) and chicken CP2 (Murata *et al.*, 1998) to bind DNA as tetramers. Truncation studies have localised a region of LBP-1c required for oligomerisation to amino acids 266-403 (Shirra *et al.*, 1994) (Figure 1.1), although oligomerisation comparable to the full-length protein was only observed with amino acids 266-502. This is consistent with an overlapping region of similarity with *GRH* from amino acids 983-1063 which shares 29% identity and 47% similarity with a region of CP2 spanning amino acids 381-501 (Uv *et al.*, 1994; Wilanowski *et al.*, 2002). Despite conservation of amino acid sequence across this region, GRH, MGR or BOM do not form heteromeric protein complexes with LBP-1c (Uv *et al.*, 1994; Wilanowski *et al.*, 2002) demonstrating that this level of amino acid sequence conservation is not functionally significant.

Hetero-multimers between CP2 family members LBP-1a, b, and c have been demonstrated in DNA binding protein complexes *in vitro* (Yoon *et al.*, 1994). The biological consequence of these interactions *in vivo* have not been analysed.

CP2 family members can also form complexes with unrelated cellular proteins. Published examples include;

YY1: Ying and Yang 1, YY1, is a transcription factor that binds DNA through four C_2H_2 zinc fingers and has been shown to be capable of activating (Seto *et al.*, 1991) or repressing

TABLE 1.1 Consensus CP2 family DNA binding sites from cellular and viral promoters.

Origin	Protein	Target gene promoter	Binding sequence	Location	Reference
Chicken	CP2	αA-crystallin	ggcg CTGG ttccca CCAG actgtc	-153/-134	Murata *et al.*, 1998
	LBP-1#	type III collagen	ccaacc CAGA agagct CAGG tacat	-73/-56	Zhang *et al.*, 1999
Mouse	CP2	α-globin	ttta CTGG gtagag CAAG cacaaa	-117/-98	Lim *et al.*, 1993
			gag CAAG cacaaa CCAG ccaa	-107/-88	Lim *et al.*, 1993
	NF2d9	Cyp 2d-9	tggct CCTC cctatt CCGG gccgg	-108/-89	Sueyoshi *et al.*, 1995
Human	LBP-1c	γ-Fibrinogen	tga CCAG ttccag CCAC tctt	-62/-43	Chodosh *et al.*, 1988
		HIV-1	gta CTGG gtctct CTGG ttag	-6/+15	Yoon *et al.*, 1994
		SV40	acag CTGG ttcttt CCGC tcaga	+273/+292	Huang *et al.*, 1990
			CCGC ccagtt CCGC	+68/+49	Shirra *et al.*, 1994
		serum amyloid A3^	cacattt CTGG aaatgc CTAG at	-169/-147	Bing *et al.*, 1999
		Interleukin-4	gt CTGA tttca- CAGG aa	-176/-160	Casolaro *et al.*, 2000
		thymidylate synthase	ccag CAGG aagagg CGGG c	-93/-75	Powell *et al.*, 2000
		Uroporphyrinonen III synthase	taa CATG ctcttt CTTG gcc	-90/-76	Solis *et al.*, 2001
		CP2 Consensus DNA Binding Sequence	CNRG nnnnnn CNRG		
Human	LBP-1c	P450scc*	gatctcgctgcagaaattccagactgaaccggatc	-155/-131	Huang *et al.*, 2000
		Ig switch region, Sμ**	CTGG gctgag CTGG gctggg CTGG gctgag CTAG	+801/+842	Drouin *et al.*, 2002
		a_2-macroglobulin*	gcagtaactggaaagtccttaat	N/D	Bing *et al.*, 1999
		Aa-fibrinogen*	gagcaagaatttctgggatgccgtggtt	N/D	Bing *et al.*, 1999
		Wilm's Tumor 1*^	cggccgcccgcgtctgcgatagggttgcct	N/D	Bing *et al.*, 1999

^ Gene enhancer region #LBP-1 gene and isoform not determined *Actual DNA binding sequence not determined **Actual half site sequence not determined

(Shi *et al.*, 1991) transcription from a variety of cellular and viral promoters important for cell growth and differentiation, including c-Myc, c-Fos, p53, α-actin, γ-interferon, adeno-associated virus P5, E6 and E7 of human papilloma virus (Romerio *et al.*, 1997; Yao *et al.*, 2001). YY1 was identified as an LBP-1c binding protein by co-purification with YY1 from CEM cell nuclear extracts and has been shown to be recruited to the long terminal repeats of HIV-1 by interaction with LBP-1c (Romerio *et al.*, 1997). The consequence of this interaction is repression of the LTR promoter by the recruitment of histone deacetylase 1 (HDAC1) through direct interaction with YY1 (Coull *et al.*, 2000). Recent studies investigating the effect of interleukin-2 administration to HIV-infected individuals has demonstrated a reduced ability of LBP-1c/YY1 to bind DNA (Bovolenta *et al.*, 1999).

NF-E4: The preferential expression of human γ-globin genes in fetal-erythroid cells is directed by the heterogeneous stage selector protein (SSP) complex (Gumucio *et al.*, 1992; Jane *et al.*, 1992; Jane *et al.*, 1995). The SSP consists of LBP-1c and a tissue specific factor NF-E4 that is expressed only in fetal liver, cord blood, bone marrow and K562 and HEL cells (*in vitro* cell lines that constitutively express fetal globin genes) (Zhou *et al.*, 2000). NF-E4 was identified as an LBP-1c binding protein expressed from a K562 cell yeast-2-hybrid cDNA library screened using the C-terminal dimerisation domain, amino acids 260-502 of LBP-1c, as bait. The formation of the NF-E4/CP2 SSP complex is expected to play an important role in hemoglobin switching during development and in hemoglobinopathies (Ciavatta *et al.*, 1995; Zhou *et al.*, 2000).

FE65: FE65 is a neural-specific protein that remains anchored to the cell membrane by interaction with the Alzheimers disease associated protein, β-amyloid precursor protein (Fiore *et al.*, 1995). LBP-1c interacts with FE65 and LBP-1c/FE65 protein complexes have been shown to localise within the nucleus (Zambrano *et al.*, 1997). FE65, used as bait in a

yeast-2-hybrid screen, interacted with the C-terminal 195 amino acids of LBP-1c expressed from a human brain cDNA library (Zambrano *et al.*, 1998). The mechanism for dissociation of the LBP-1c/FE65 complex from β-amyloid precursor protein or the ability of the LBP-1c/FE65 complex to bind DNA and regulate transcription have not been elucidated (Zambrano *et al.*, 1998).

RING1: Polycomb group proteins are able to repress transcription by assembling protein complexes on promoter elements. While the polycomb proteins them selves have not been shown to bind DNA directly, RING1 and the human homologue, dinG, bind LBP-1c and are able to negate CP2 mediated transcriptional activation (Tuckfield *et al.*, 2002b). This mechanism is conserved by the *D.melanogaster* homologue of dinG, *dring*, that is able to bind *grh* and mediate transcriptional repression (Tuckfield *et al.*, 2002b). drinG was identified as an LBP-1c binding protein expressed from a K562 cell yeast-2-hybrid cDNA library screened using the C-terminal dimerisation domain, amino acids 260-502 of LBP-1c as bait (Tuckfield *et al.*, 2002b).

1.5.3 Transcriptional Regulation by CP2 Related Proteins.

Members of the CP2 family of transcription factors are generally described as transcriptional activators. Characterisation of CP2 and LBP-1c demonstrated the presence of a conserved serine, threonine and proline (S-T-P) rich region spanning amino acids 250-405 (Lim *et al.*, 1992) (Figure 1.1). Within this region are serine/proline/X/X (SPXX) motifs present in many DNA binding proteins, which have a role in sequence specific DNA binding (Suzuki, 1989a; Suzuki, 1989b). C-terminal to the SPXX motifs is a glutamine/proline repeat in CP2 and a 10 amino acid glutamine repeat in LBP-1c. Related motifs have been shown for other transcription factors such as Sp1 (Xiao and Jeang, 1998)

to form part of a transactivation domain (Courey *et al.*, 1989; Mitchell and Tjian, 1989). These conserved regions in the CP2 family have not yet been functionally investigated.

Experiments investigating the effect of CP2 on the α-globin promoter used both *in vitro* and *in vivo* approaches. Addition of bacterially expressed and purified CP2 to *in vitro* transcription assays demonstrated that CP2 can activate transcription 3-4 fold from the α-globin promoter CP2 binding site (Lim *et al.*, 1993). Consistent with *in vitro* experiments, *in vivo* expression of CP2 in transiently or stably transfected K562 cells activated the α-globin promoter 2.5-5.5 fold (Lim *et al.*, 1993). Similarly, expression of chicken CP2 in transfected lens and lung cultures activated transcription from CP2 response elements from the αA-crystalin gene 6 and 10 fold respectively (Murata *et al.*, 1998), while LBP-1c expression activated transcription 5 fold from the serum amyloid A3 enhancer region in HepG2 cells (Bing *et al.*, 1999), 5 fold from the interleukin-4 promoter in the human T cell line Jurkat (Casolaro *et al.*, 2000), and 7 fold from the thymidylate synthase promoter in NIH-3T3 cells (Powell *et al.*, 2000).

LBP-1c transcriptional activation of viral promoters has also been demonstrated by the identification of LBP-1c binding sites in the SV40 late promoter (Huang *et al.*, 1990). *In vitro* transcriptional analysis of these sites has shown LBP-1c purified from HeLa s-3 cells activates transcription 5 fold (Huang *et al.*, 1990).

LBP-1c interacts with two distinct sites on the HIV-LTR promoter, one with high affinity and one with lower affinity (Jones *et al.*, 1988; Kato *et al.*, 1991; Wu *et al.*, 1988). Mutation of the high affinity site reduced *in vitro* transcription of HIV-1 in the presence of LBP-1c (Jones *et al.*, 1988), while LBP-1c, LBP-1a and LBP-1b activate transcription 30 fold in transiently transfected HeLa cells. LBP-1c binding to the low affinity site abrogates HIV-1 transcription by blocking the binding of TFIID to the TATA box (Kato *et al.*, 1991; Yoon *et al.*, 1994). Consistent with the inability of LBP-1d to bind DNA, this protein failed to affect transcription from the high or low affinity LBP-1 binding sites (Yoon *et al.*, 1994).

In contrast to these observations, an independent report claims that LBP-1c plays little or no role in the transcriptional regulation of HIV-1 (Zhong *et al.*, 1994). Specifically, over expression of LBP-1c or a dominant negative LBP-1c did not affect HIV-1 transcription in T cells infected with HIV-1 or in cells superinfected with HSV-1, known to rapidly induce HIV-1 transcription (Zhong *et al.*, 1994).

The ability to activate transcription has also been demonstrated for other CP2 family members. LBP-1b has been shown to activate transcription from the -155/-131 region of the human P450scc promoter 21 fold in transfected JEG-3 cells (Huang and Miller, 2000). Despite these examples described, the specific molecular mechanisms by which CP2 family members activate transcription have not determined.

LBP-9, identified on the basis of its ability to bind the -155/-131 region of the human P450scc promoter, did not activate transcription in JEG-3 cells (Huang and Miller, 2000). Transfection of JEG-3 cells with increasing amounts of LBP-9 suppressed LBP-1b-mediated activation to basal levels (Huang and Miller, 2000). The mechanism of inhibition was not resolved, and could result from direct repression of transcription, steric exclusion of LBP-1b from the DNA-binding site, or displacement of LBP-1b from the promoter by formation of non-DNA binding complexes with LBP-9.

GRH has been demonstrated to act as a transcriptional activator binding to *cis* elements essential for activation of the *Ultrabithorax* and *Dopa decarboxylase* genes (Bray *et al.*, 1989; Bray and Kafatos, 1991; Dynlacht *et al.*, 1989; Johnson *et al.*, 1989). Sites where *GRH* acts as a transcriptional repressor have been identified in the dorsal-ventral patterning genes *decapentaplegic* and *zerknullt* (Huang *et al.*, 1995). Putative binding sites have been identified in the promoters of *fushi tarazu* and *engrailed* but the effect on transcriptional regulation has not been detailed (Dynlacht *et al.*, 1989; Soeller *et al.*, 1988).

The region required for GRH transcriptional regulation has been mapped to an N-terminal minimal region of 56 amino acids (Attardi and Tjian, 1993). Consistent with this is the identification of the N-terminal 93 amino acids required for transcriptional activation mediated by MGR (Wilanowski *et al.*, 2002). Recently, it has been shown that the N-terminal 40 amino acids of CP2 are required and sufficient for transcriptional activation ability of LBP-1c (Ramamurthy *et al.*, 2001; Tuckfiled, 2002a). This region is highly conserved between CP2 family members with the exception of LBP-9. The mechanisms used to regulate transcription have not been determined.

1.6 ACTIVATION OF LBP-1c AND *GRH* BY SERINE PHOSPHORYLATION.

Phosphorylation of LBP-1c at serine 291 by the kinase pp44 (ERK1) occurs in response to mitogenic stimulation of human peripheral T lymphocytes (Volker *et al.*, 1997). Phosphorylation increased the DNA binding ability of LBP-1c 5 fold, resulting in transformation of T cells from resting into an actively replicating state (Volker *et al.*, 1997). Increased DNA binding of LBP-1c by phosphorylation could explain an increase in LBP-1c directed transcriptional activation from 5 to 31 fold after interleukin-1 treatment of HepG2 cells (Bing *et al.*, 1999). Serine 291 is conserved in all CP2 family members, however, the functional significance of this amino acid has not been studied for CP2 family members other than LBP-1c.

Regulation of transcriptional activation mediated by GRH also appears to be at the level of phosphorylation. Activation of *Torso*, a receptor tyrosine kinase, initiates a signal transduction pathway resulting in the phosphorylation of *GRH* by MAPK resulting in transcriptional activation of the *tailless* gene (Liaw *et al.*, 1995). The mechanism of transcriptional activation and the elucidation of specific amino acid phosphorylation have not been determined.

1.7 A ROLE FOR *LBP-1c* IN DISEASE STATES.

The importance of LBP-1c in mammalian biological processes is evidenced by the association of disease states with mutations in the LBP-1c mRNA and in the LBP-1c binding sites of promoters regulated by CP2.

The LBP-1c gene, located on chromosome 12 mapping to the region q13 (Cunningham *et al.*, 1995; Swendeman *et al.*, 1994), has been shown to be linked with Alzheimers disease (AD) as a genetic determinant. (Lambert *et al.*, 2000; Taylor *et al.*, 2001). An A for a G nucleotide polymorphism present in the LBP-1c 3'UTR, 15 bp from the stop codon, appears to be protective of the AD phenotype in French, United Kingdom and North American populations (Lambert *et al.*, 2000; Taylor *et al.*, 2001).

Congenital erythropoietic porphyria is an autosomal recessive disease affecting heme biosynthesis. Specifically, the disease state results from a deficiency in the activity of uropophyyrinogen III synthase (URO-synthase) (Desnick *et al.*, 1998). Analysis of the URO-synthase promoter in congenital erythropoietic porphyria patients identified a C to an A nucleotide exchange mutation in a putative CP2 binding site located at position -90/-76 (Table 1.1) (Solis *et al.*, 2001). *In vitro* studies investigating the effect of this mutation have demonstrated that LBP-1c in unable to bind the mutant DNA sequence and suggests a role for LBP-1c in erythroid-specific heme biosynthesis (Solis *et al.*, 2001).

1.8 FUNCTIONAL ANALYSIS OF CP2 RELATED PROTEINS.

Mice null for CP2 observed over 18 months display no phenotype (Ramamurthy *et al.*, 2001). Investigations into the null mice revealed that the ubiquitously expressed CP2 family member, NF2d9, could compensate for the loss of CP2 function as NF2d9 was capable of binding to CP2 response elements and binds known protein interactors of CP2 (Ramamurthy *et al.*, 2001). Analysis of CP2 null mice focused on the effect of CP2 loss of function in the regulation of hemotopoietic development, globin gene expression and T- and B-cell mitogen stimulated immunological response. Association of LBP-1c with AD

and congenital erythropoietic porphyria in humans suggest that LBP-1c plays biological roles that can not be compensated for by LBP-1a, the human homologue of NF2d9. However, the occurrence of AD or congenital erythropoietic porphyria like phenotypes was not investigated in CP2 null mice.

The developmental importance of *GRH* is demonstrated by embryonic lethality of flies mutant for *grh* (Bray and Kafatos, 1991), consistent with the embryonic lethality observed in flies expressing a dominant negative form of GRH (Attardi and Tjian, 1993). Furthermore, flies mutant for the alternatively spliced GRH isoform either die at the pupal (>75%) or adult stage where adults appear incapable of coordinated movement prior to death (Uv *et al.*, 1997).

1.9 PHYLOGENETIC DIFFERENCE BETWEEN CP2-LIKE AND *GRH*-LIKE PROTEINS.

CP2, NF2d9, LBP-1a, LBP-1c and LBP-9 were identified and described as related to GRH (Lim *et al.*, 1992; Sueyoshi *et al.*, 1995; Yoon *et al.*, 1994). The identification of the mammalian forms of *GRH*, MGR and BOM, and the *Drosophila* form of CP2 suggest that these two groups of proteins may in fact be divergent. Phylogenetic analysis carried out on the CP2 and GRH related proteins has demonstrated GRH-like and CP2-like proteins to form separate branches on the phylogenetic tree and suggest that the two protein groups may have been derived from the one common precursor as long as 1 billion years ago (Wilanowski *et al.*, 2002) (Figure 1.2).

Despite the phylogenetic separation observed between *GRH* related proteins and CP2 related proteins, both protein families share sequence identity that is functionally important for DNA binding as well as similarity in linear structure with an N-terminal transcriptional regulatory region, a central DNA binding region and a C-terminal protein/protein interaction region (Figure 1.1).

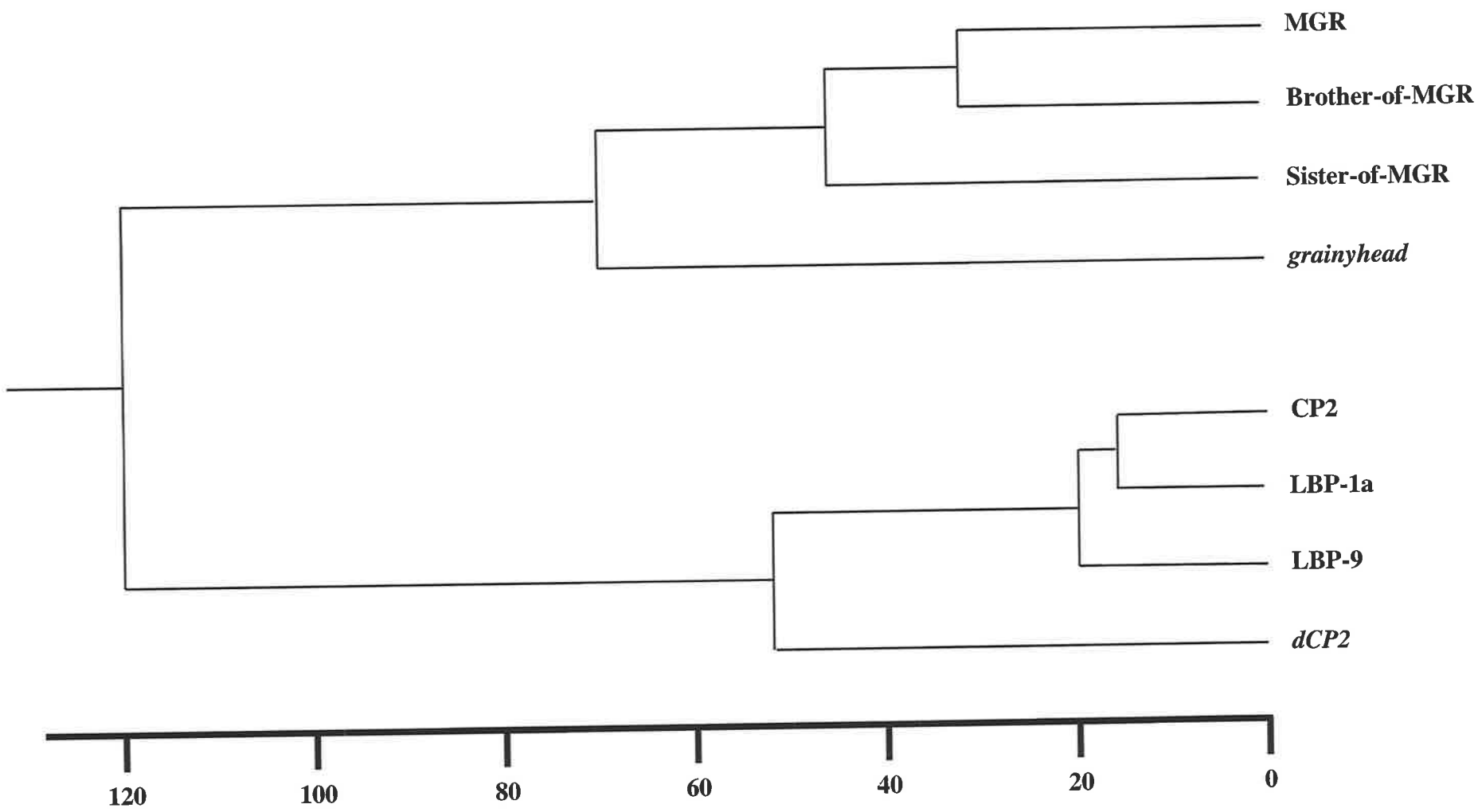
FIGURE 1.2

Phylogenetic analysis of the GRH- and CP2-like protein families.

Protein sequences from Grainyhead-like and CP2-like family members were aligned using the Clustal method (Higgins and Sharp, 1989), revealing the evolutionary derivation of members from both protein groups.

Axis, PAM units, which refers to the number of point mutations in protein sequence over an evolutionary period (Saitou and Nei, 1987).

Taken from Wilanowski *et al.*, 2002.


MGR
Brother-of-MGR
Sister-of-MGR
grainyhead
CP2
LBP-1a
LBP-9
dCP2
120
100
80
60
40
20
0

1.10 IDENTIFICATION OF A NOVEL *CP2* FAMILY cDNA, *CRTR-1*, BY ddPCR.

A novel member of the CP2 family of transcription factors was isolated during a differential display PCR (ddPCR) (Pelton *et al.*, 2002) screen carried out using RNA isolated from pluripotent mouse embryonic stem (ES) and early primitive ectoderm-like (EPL) cells. EPL cells are formed by the culture of ES cells in the presence of a HepG2 cell line conditioned medium, MEDII, *in vitro* and are representative of the inner cell mass to primitive ectoderm transition during early mouse development (Rathjen *et al.*, 1999).

CRTR-1 was isolated as a 736 bp ddPCR fragment showing highest expression in ES cells which was then rapidly down regulated upon conversion and passaging of EPL cells for 2 days, and further down regulated with continued EPL cell passaging (Figure 1.3a). The *CRTR-1* expression pattern across the ES to EPL cell conversion was confirmed by DIG-labeled *in situ* hybridisation (Figure1.3b-e), Northern analysis (Figure 1.4) and ribonuclease protection analysis (Figure 1.5). Furthermore, Northern analysis demonstrated that the full length *CRTR-1* transcript is approximately 9.4 kb in length.

1.11 ISOLATION AND ANALYSIS OF THE *CRTR-1* OPEN READING FRAME.

CRTR-1 cDNA clones were isolated by four complete rounds of screening from a D3 ES cell ZAP II cDNA library (Clontech Inc; (Thomas *et al.*, 1995) using successively more 5' *CRTR-1* specific probes (Figure 1.6). Clones were confirmed to be *CRTR-1* specific using Southern, sequence data and expression analysis (data not shown) (Rodda, 1998).

The collated sequence of cDNA clones isolated from the four rounds of library screening provided 9290 bp of the predicted 9.4 kb crtr-1 sequence (Rodda *et al.*, 1999). Analysis of the sequence revealed a 7868 bp 3'untranslated region (UTR) which contained a poly(A)-tail 25 bp downstream of a consensus polyadenylation signal (AATAAA), consistent with the typical positioning of the polyadenylation signal 10-30 bp upstream of the poly(A)-tail (Goulding *et al.*, 1991). An incomplete open reading frame of 1422 bp

FIGURE 1.3

Differential display PCR analysis of ES and EPL cells.

A) 2 μg of cytoplasmic RNA isolated from ES cells, EPL cells grown for 2-6 days in the presence (+) or absence (-) of LIF, and spontaneously differentiated ES cells (S), was reverse transcribed with a 3'-oligo d(T) primer, before dilution 1/20 for dd-PCR, incorporating (^{33}P) dATP, using OPA-01 5' primer (Operon technologies inc.) and the 3'-oligo d(T) primer. Reactions were resolved by electrophoresis on a 6% (v/v) denaturing polyacrylamide gel (Pelton *et al.*, 2002). The *CRTR-1* band is indicated.

5' OPA-01 primer: CAGGCCCTTC

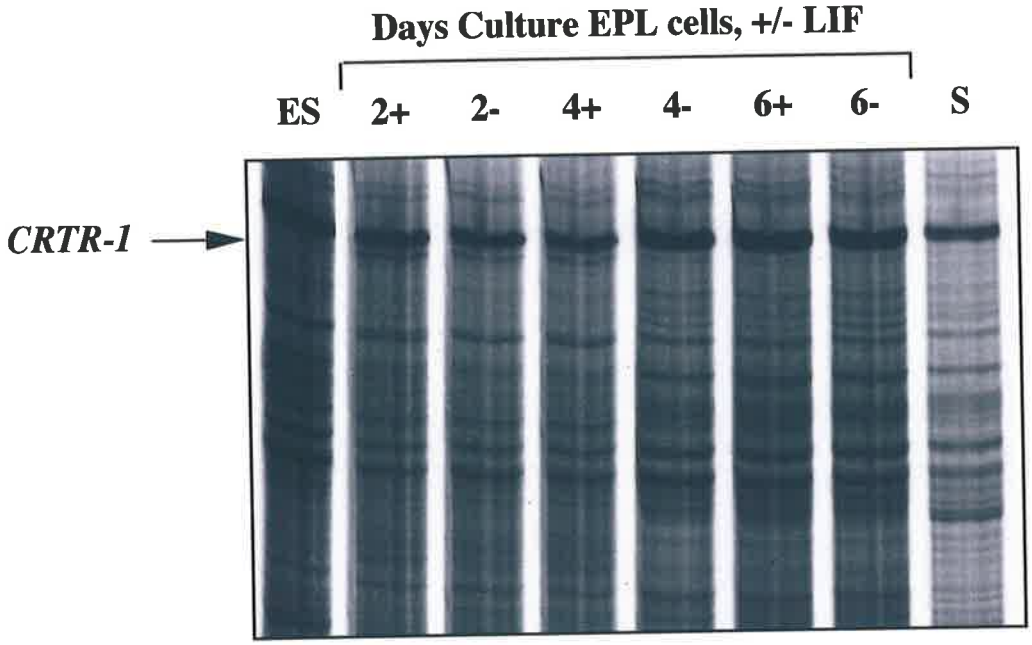
3' oligo d(T) primer: TTTTTTTTTTTCA

In situ hybridisation of ES cells **(B and C)**, and EPL cells grown for 4 days in the presence of LIF **(D and E)**. Cells were probed with a DIG-labelled anti-sense *CRTR-1* specific riboprobes generated from the cloned CRTR-1 ddPCR product **(A)** and detected with an alkaline phosphatase conjugated anti-DIG antibody. CRTR-1 expressing cells are stained purple and were photographed on a Nikon Eclipse TE300 inverted microscope under phase contrast at 100x magnification.

d: differentiated cells.

Taken from Pelton *et al.*, 2002

A



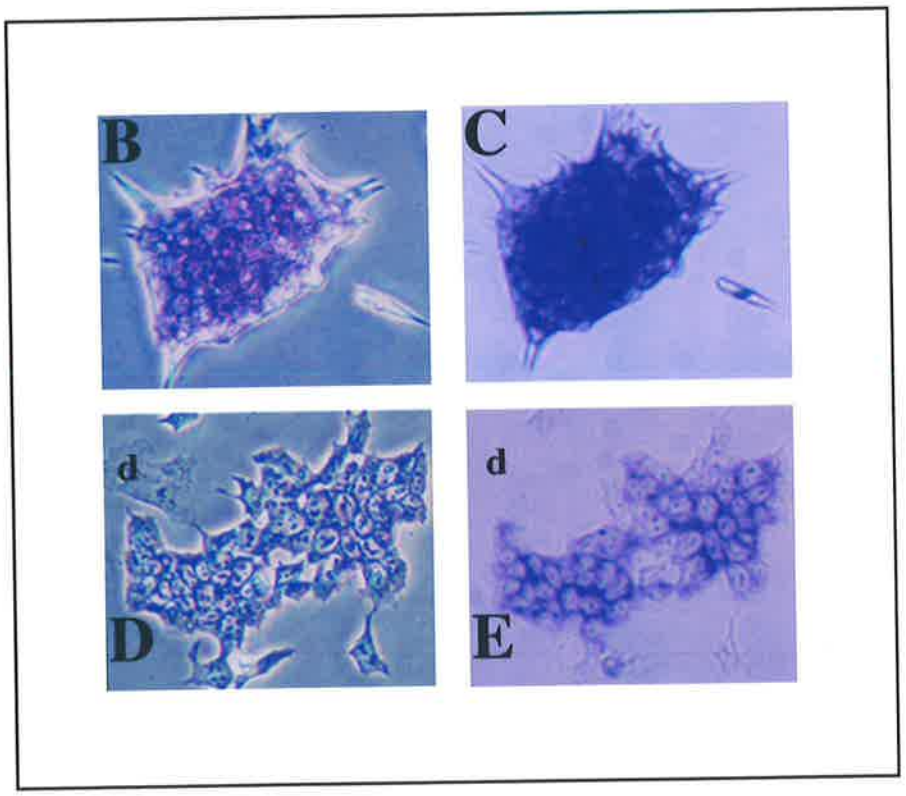


FIGURE 1.4

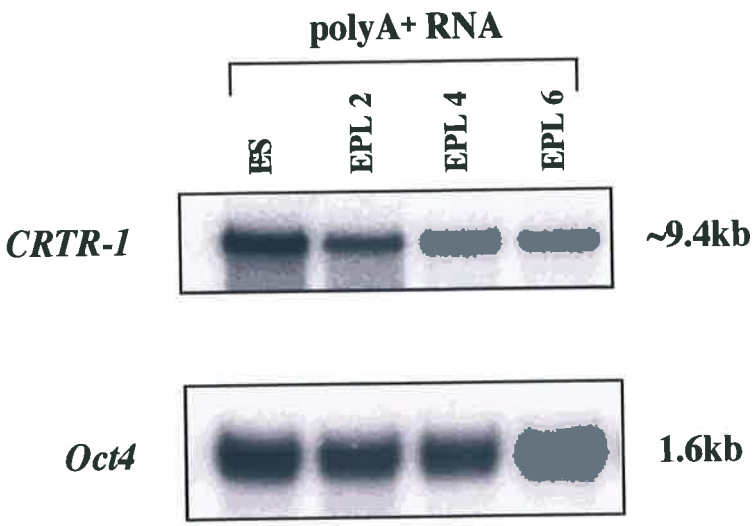
Northern analysis of *CRTR-1* expression in pluripotent cells.

A) 5 μg of poly-$(A)^+$ RNA isolated from ES and EPL cells cultured in 50% MEDII in the presence of LIF for 2, 4 and 6 days was separated by 1% agarose gel electrophoresis and transferred for northern analysis (2.3.5.5). The filter was probed with an α-^{32}P-dATP 736 bp ddPCR *CRTR-1* fragment (1.10), stripped and re-probed with an *Oct*4 (2.2.6.2) specific probe. The filters was visualised by phosphor imaging (2.5). The approximate size of the *CRTR-1* transcript is indicated.

B) Quantitation of data presented in **(A)**. *CRTR-1* band intensity was normalised against *Oct4* band intensity for each RNA sample using ImageQuant™ image analysis software (molecular Dynamics).

Taken from Pelton *et al.*, 2002

A


polyA+ RNA
ES
EPL 2
EPL 4
EPL 6
CRTR-1
~9.4kb
Oct4
1.6kb

B

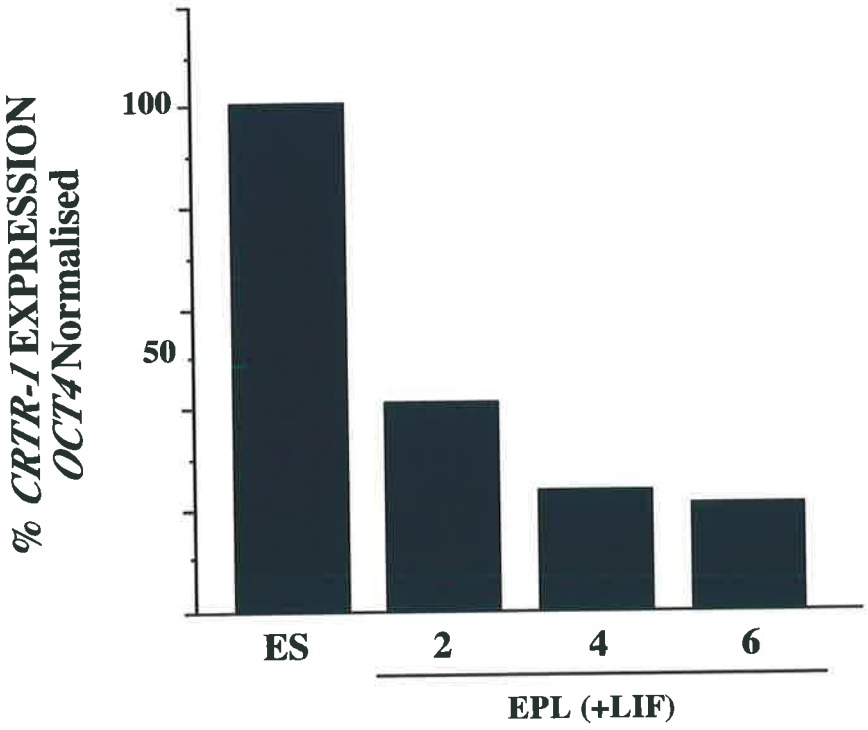

% CRTR-1 EXPRESSION
OCT4 Normalised
100
50
ES
2
4
6
EPL (+LIF)

FIGURE 1.5

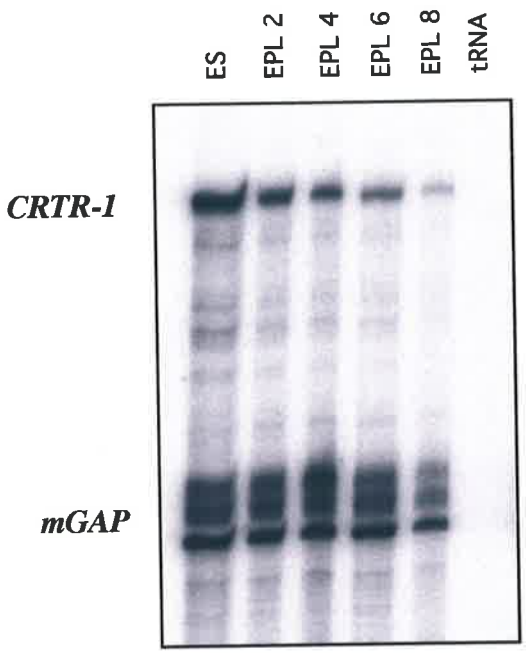
Ribonuclease protection analysis of *CRTR-1* expression in pluripotent cells.

A) *CRTR-1* expression was detected using an α-^{32}P-UTP labelled 460 bp anti-sense *CRTR-1* riboprobe (2.3.5.4). Ribonuclease protection analysis was performed using 10 µg total RNA isolated from ES and EPL cells cultured in 50% MEDII in the presence of LIF for 2, 4, 6, and 8 days and 10 µg of tRNA (Roche). A *mGAP* specific anti-sense riboprobe was used as a loading control. Protected bands were visualised by phosphor imaging (2.5).

B) Quantitation of ribonuclease protection analysis presented in **(A)**. The expression of *CRTR-1* was normalised against the *mGAP* loading control. The standard mean error is calculated from three independent ribonuclease protection assays using three different *CRTR-1* specific anti-sense riboprobes generated from independent *CRTR-1* cDNA library clones.

Taken from Rodda *et al.*, 2001

A


ES
EPL 2
EPL 4
EPL 6
EPL 8
tRNA
CRTR-1
mGAP

B

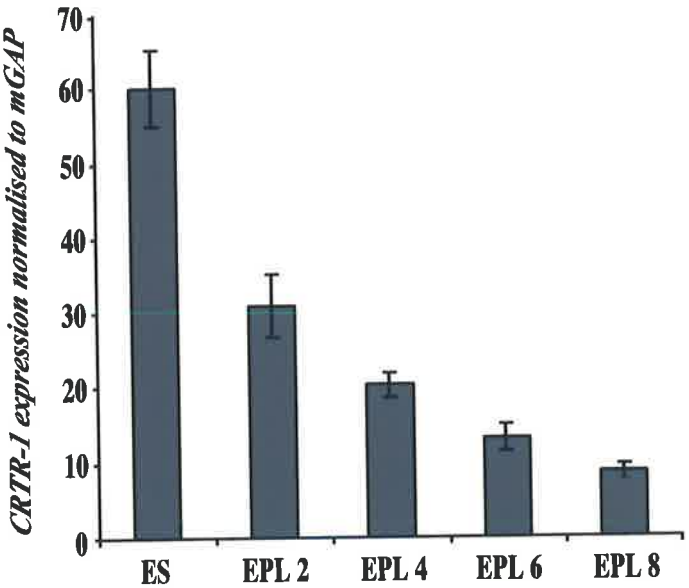

CRTR-1 expression normalised to mGAP
70
60
50
40
30
20
10
0
ES
EPL 2
EPL 4
EPL 6
EPL 8

FIGURE 1.6

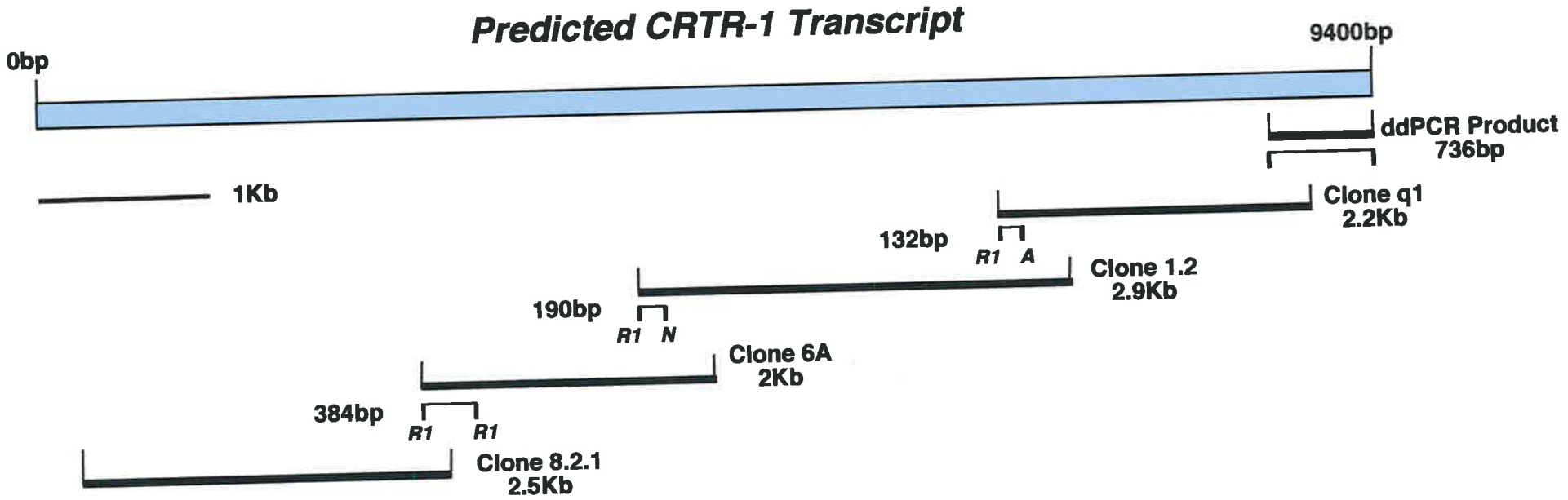
Schematic map and alignment of *CRTR-1* cDNA clones.

The *CRTR-1* transcript is predicted to be approximately 9.4Kb based on Northern analysis (Figure 1.4). Four library screens using probes to isolate successively more 5' clones isolated 9294bp of *CRTR-1* sequence. Probes used for the isolation of clones are indicated together with the restriction sites used for their excision.

RI; *Eco*RI

A; *Acc*I

N; *Nco*I


Predicted CRTR-1 Transcript
0bp
9400bp
ddPCR Product
736bp
1Kb
Clone q1
2.2Kb
132bp
R1
A
Clone 1.2
2.9Kb
190bp
R1
N
Clone 6A
2Kb
384bp
R1
R1
Clone 8.2.1
2.5Kb

extended upstream of the 3'UTR, but a 5' UTR, start codon and consensus Kozak sequence were not identified.

NCBI, BlastN and BlastP database searches (Altschul *et al.*, 1990), carried out using the compiled incomplete *CRTR-1* cDNA sequence and predicted CRTR-1 amino acid sequence, revealed that the most 5' cDNA sequence (encompassing the predicted open reading frame) and the predicted amino acid sequence shared significant homology with members of the CP2 transcription factor family (Lim *et al.*, 1992). Direct alignment of the isolated incomplete *CRTR-1* nucleotide sequence with *CP2* showed that *CRTR-1* shares 64% identity over the *CP2* coding region (1506 bp). Further *CP2* family members showing sequence identity included the human transcription factors, *LBP-1c* (63%) and the *LBP-1c* splice variant, *LBP-1d* (54%) (Shirra *et al.*, 1994; Yoon *et al.*, 1994).

Sequence similarity with members of the CP2 family of transcription factors did not extend past the stop codon of the predicted open reading frame into the 3'UTR. Direct sequence alignments of the 3' UTR showed no significant sequence similarity and as such provided no indication of the potential function of the 3' UTR (Rodda, 1998).

Searches of the BlastN EST database using the *CRTR-1* 3' UTR sequence revealed EST's showing identical alignment to *CRTR-1* (Rodda, 1998). One EST isolated by the RIKEN group from adult mouse kidney aligned with nucleotides 4818-5201 of *CRTR-1* while ESTs isolated by the NIA group from the 4.5 d.p.c mouse blastocyst aligned with nucleotides 7339-7665 and 7722-7874 of *CRTR-1*, consistent with isolation of the *CRTR-1* cDNA from mouse ES cells, the *in vitro* equivalent of the inner cell mass of the blastocyst stage mouse embryo.

1.12 DEVELOPMENTAL, SPATIAL AND TEMPORAL EXPRESSION OF *CRTR-1*.

1.12.1 *CRTR-1* Expression *In vivo* during Pre- and Early Post-implantation Mouse Development.

Whole mount *in situ* hybridisation analysis carried out on pre-and early post-implantation mouse embryos (Pelton *et al.*, 2002) (Figure 1.7b) showed specific *CRTR-1* expression in all ICM cells of 3.5 d.p.c. embryos, which was maintained within the epiblast of the expanded blastocyst at approximately 4.5 d.p.c. *CRTR-1* expression was down regulated during epiblast proliferation at 4.75 d.p.c., with expression of the transcript being variable at this stage (Figure 1.7c). *CRTR-1* expression was not detected in any cell type in 5.0, 5.25 or 5.5 d.p.c. embryos (Figure 1.7b). Expression of the pluripotent cell marker gene *Oct4* is maintained across these time points demonstrating the maintenance of pluripotence (Figure 1.7a).

CRTR-1 expression was compared with expression of other genes identified as differentially expressed between ES and EPL cells by ddPCR, *Psc-1*, a novel developmentally regulated SR protein (Kavanagh *et al.*, 2003), and *PRCE*, a novel developmentally regulated mammalian separin (Pelton, 2000), together with the inner cell mass marker gene, *Rex1* (Rogers *et al.*, 1991), a zinc finger protein regulated by *Oct4* expression (Ben-Shushan *et al.*, 1998), and the primitive ectoderm marker, *Fgf5* (Haub and Goldfarb, 1991), (Pelton *et al.*, 2002; Rodda *et al.*, 2002) (Figure 1.8). Expression of *CRTR-1* is coordinately regulated with *Rex1*, expressed in ES cells and ICM and down regulated *in vitro* in EPL cells cultured for 2 days and *in vivo* between 4.5 d.p.c. and 4.75 d.p.c., consistent with the onset of accelerated pluripotent cell division in the embryo. Down regulation of *CRTR-1* and *Rex1* coincided with up regulation of *PRCE* expression *in vitro* and *in vivo*. *Psc1* is expressed in ES cells and ICM and down regulated *in vitro* in EPL cells cultured for 4 days and *in vivo* between 5.0 and 5.25 d.p.c.. *Fgf5,* which is not expressed in ES cells or ICM, is up regulated to high levels in EPL cells cultured for 4 days

FIGURE 1.7

Whole mount *in situ* hybridisation analysis of *Oct4* and *CRTR-1* expression during early mouse development.

A) Analysis of *Oct4* expression during peri-implantation mouse development by whole mount *in situ* hybridisation using an *Oct4* specific anti-sense DIG labelled riboprobe (2.3.5.6.1). *Oct4* expressing cells can be identified by the purple staining.

B) Analysis of *CRTR-1* expression during peri-implantation mouse development by whole mount *in situ* hybridisation using a *CRTR-1* specific anti-sense DIG labelled riboprobe (2.3.5.6.1). *CRTR-1* expressing cells can be identified by the purple staining.

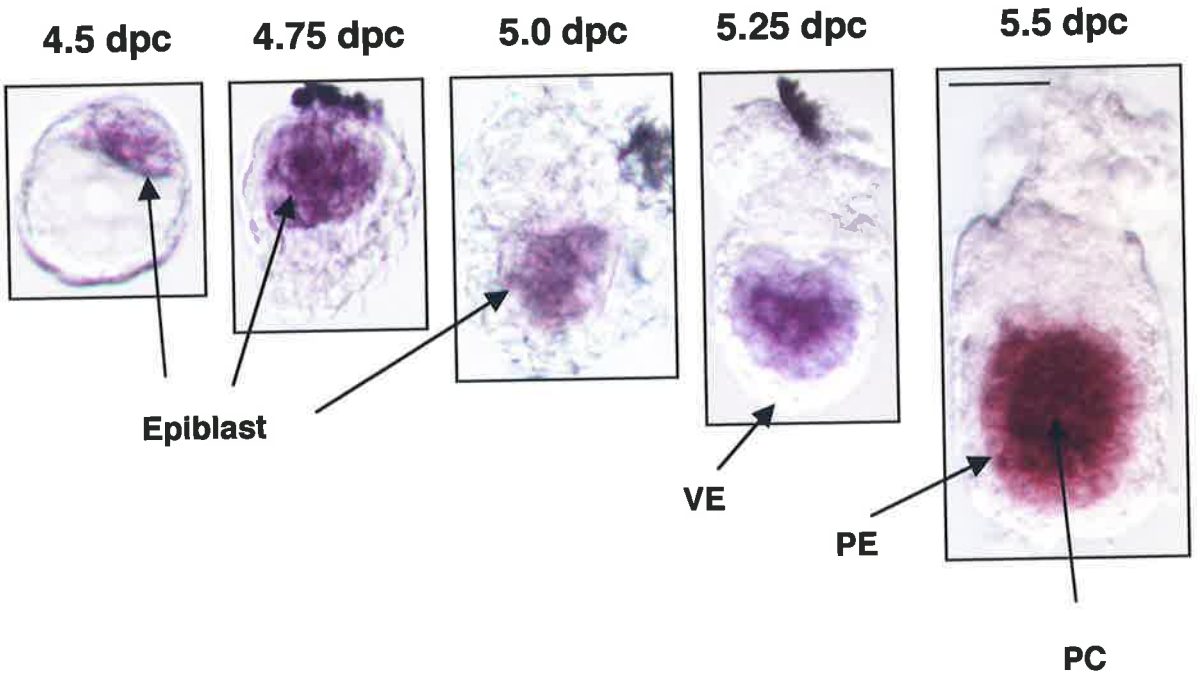
C) 4.75 d.p.c. embryo stained with a *CRTR-1* specific anti-sense DIG labelled riboprobe showing no *CRTR-1* expression highlighting the variability of *CRTR-1* expression at this time point **(compare with B)**.

Photography was carried out on a Nikon Eclipse TE300 inverted microscope using the Hoffman modulation contrast system. Bar represents 50 μm.

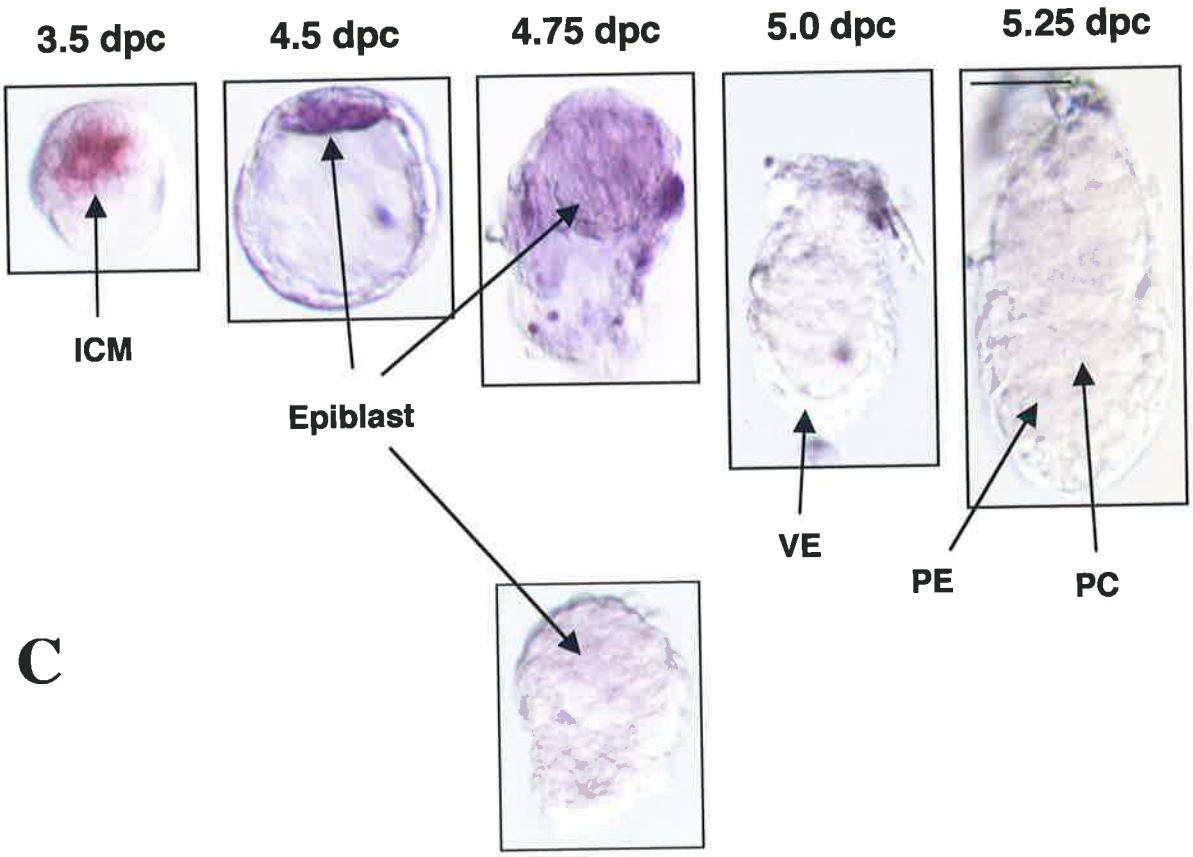
Abbreviations: ICM = inner cell mass; VE = visceral endoderm; PE = primitive ectoderm; PC = proamniotic cavity.

Taken from Pelton *et al.*, 2002

A


4.5 dpc
4.75 dpc
5.0 dpc
5.25 dpc
5.5 dpc
Epiblast
VE
PE
PC

B


3.5 dpc
4.5 dpc
4.75 dpc
5.0 dpc
5.25 dpc
ICM
Epiblast
VE
PE
PC

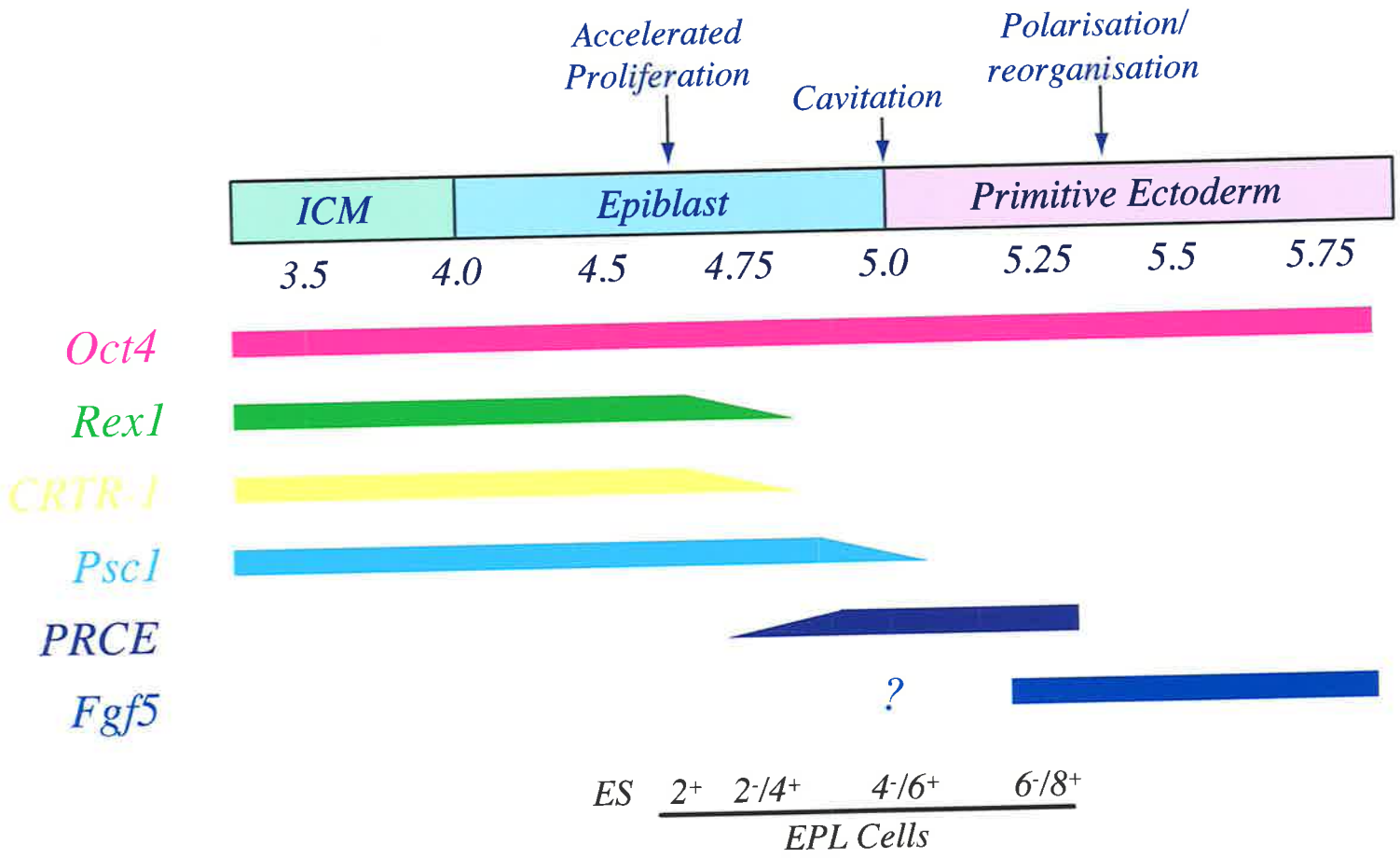
C

FIGURE 1.8

Summary of pluripotent cell gene expression *in vivo* and *in vitro*.

The pluripotent cell populations present within the early mouse embryo are aligned with approximate time points in days post coitum (d.p.c.), the embryonic events characteristic of this developmental stage and gene expression in pluripotent cell populations *in vivo*. ES cells and EPL cells grown for 2, 4 or 6 days in MEDII in the presence ($^{+}$) or absence ($^{-}$) of LIF are aligned on the basis of gene expression *in vitro*. *Fgf5* expression has not been determined in 5.0 d.p.c. embryos.

Taken from Rodda *et al.*, 2002.


Accelerated Proliferation
Cavitation
Polarisation/ reorganisation
ICM
Epiblast
Primitive Ectoderm
3.5
4.0
4.5
4.75
5.0
5.25
5.5
5.75
Oct4
Rex1
CRTR-1
Psc1
PRCE
Fgf5
?
ES
2+
2-/4+
4-/6+
6-/8+
EPL Cells

and throughout the embryonic primitive ectoderm. These observations demonstrated that ES to EPL cell differentiation *in vitro* parallels the ICM to primitive ectoderm differentiation event *in vivo*, and indicates that changes in CRTR-1 gene expression throughout coincides with key events during mammalian embryogenesis.

1.12.2 Regulated Expression of *CRTR-1* During Later Development and in Adult Mouse Tissues.

Ribonuclease protection analysis was used to investigate *CRTR-1* expression during later mouse embryogenesis from 10.5-17.5 d.p.c. (Rodda *et al.*, 2001). *CRTR-1* was expressed at low levels in 10.5 and 11.5 d.p.c embryos and highest between 14.5 and 17.5 d.p.c. (Figure 1.9a) Of the 16.5 d.p.c embryonic tissues analysed, low levels of *CRTR-1* expression were detected in 16.5 d.p.c embryonic intestine, limb, lung and skin, with highest expression in 16.5 d.p.c embryonic kidney (Figure 1.9b).

Expression analysis of *CRTR-1* in adult mouse tissues demonstrated low levels of *CRTR-1* expression in lung, mesenteric lymph nodes, muscle, ovary, and thymus, elevated levels of expression in placenta, testis and small intestine with highest levels of expression in adult kidney and stomach (Figure 1.9c) (Rodda *et al.*, 2001). Maintenance of *CRTR-1* expression from embryogenesis to the adult in the kidney and intestine suggests that *CRTR-1* may have an important role in both the development and physiological function of these tissues.

1.12.3 Specific Expression of *CRTR-1* in Embryonic and Adult Kidney Distal Convoluted Tubules.

Whole mount *in situ* hybridisation carried out using kidneys isolated from 16.5 d.p.c embryos showed specific staining representing *CRTR-1* expression in the epithelial monolayer lining a subset of tubules in the embryonic kidney cortex (Figure 1.10b and c) (Rodda *et al.*, 2001). *CRTR-1* expressing tubules were identified as distal convoluted

FIGURE 1.9

***CRTR-1* expression during later mouse development and in the adult mouse.**

CRTR-1 expression was detected using an α-^{32}P-UTP labelled 460 bp anti-sense *CRTR-1* riboprobe (2.3.5.4). Ribonuclease protection analysis was performed using 10 µg total RNA isolated from 10.5-17.5 d.p.c mouse embryos **(A)**, tissues from the 16.5 d.p.c mouse embryo **(B)**, tissues from adult mice **(C)** and 10 µg of tRNA (Roche) as indicated. A *mGAP* α-^{32}P-UTP labelled anti-sense riboprobe was used as a loading control. Protected bands were visualised by phosphor imaging (2.5).

SI; small intestine.

Taken from Rodda *et al.*, 2001.

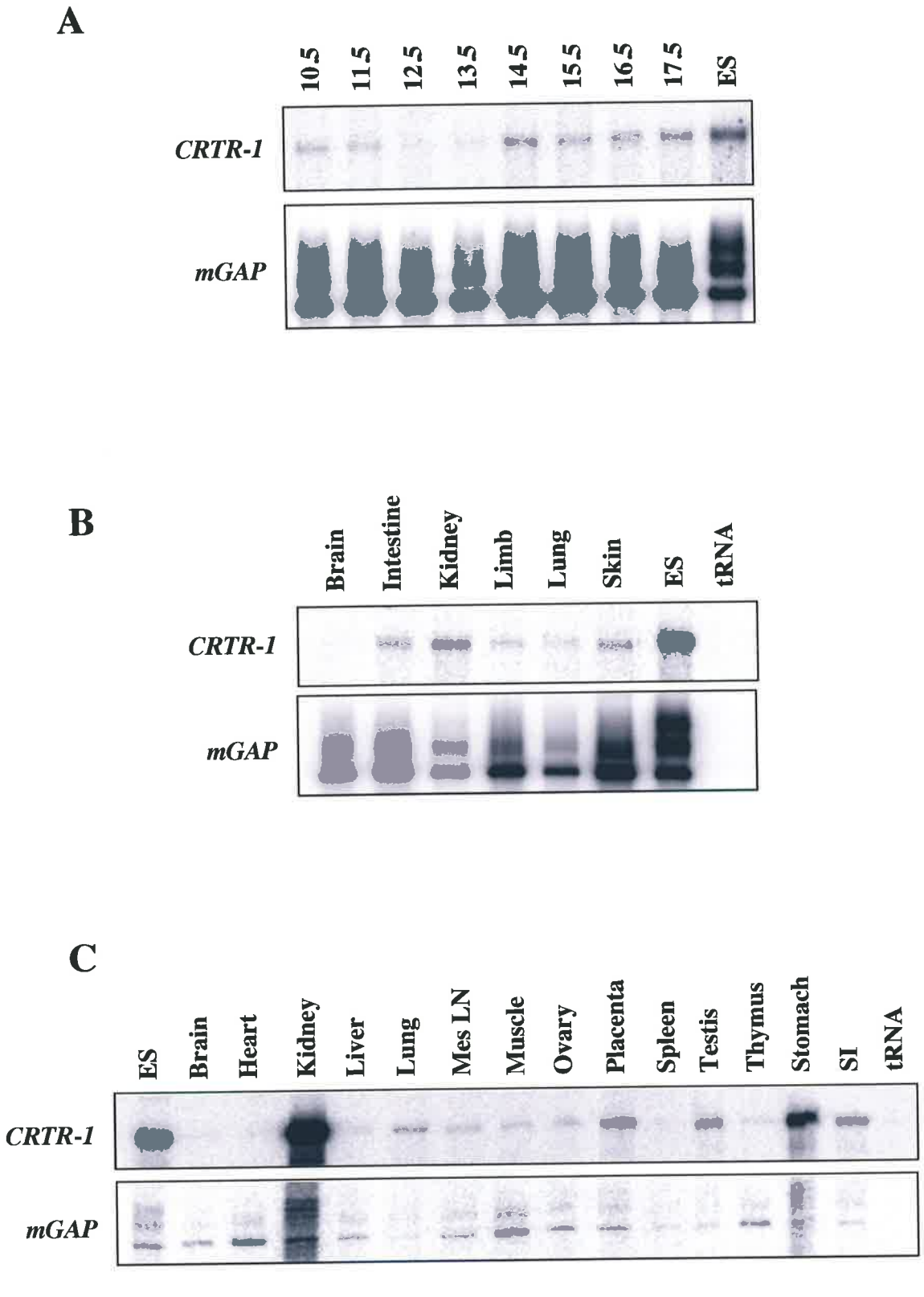

A
10.5
11.5
12.5
13.5
14.5
15.5
16.5
17.5
ES
CRTR-1
mGAP
B
Brain
Intestine
Kidney
Limb
Lung
Skin
ES
tRNA
CRTR-1
mGAP
C
ES
Brain
Heart
Kidney
Liver
Lung
Mes LN
Muscle
Ovary
Placenta
Spleen
Testis
Thymus
Stomach
SI
tRNA
CRTR-1
mGAP

FIGURE 1.10

***CRTR-1* expression in embryonic and adult mouse kidneys.**

Whole mount *in situ* hybridisation of 16.5 d.p.c mouse kidney probed with 736 bp ddPCR *CRTR-1* specific sense **(A)** and anti-sense **(B and C)** digoxygenin (DIG)-labelled riboprobes. Probed kidneys were cut into 7 μm sections then viewed and photographed on a Zeiss axioplan microscope.

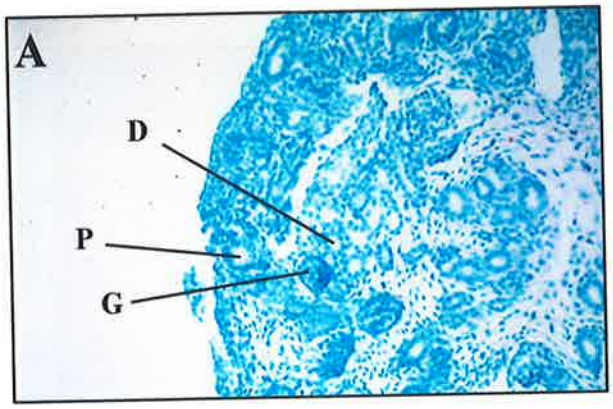
Radiolabelled *in situ* hybridisation on 7μm adult kidney sections using 460 bp *CRTR-1* sense **(D and E)** and anti-sense **(F and G)** α-[^{33}P]-rUTP labelled riboprobes. Developed slides were viewed and photographed under light **(D and F)** and dark field **(E and G)** condensers on a Zeiss axioplan microscope.

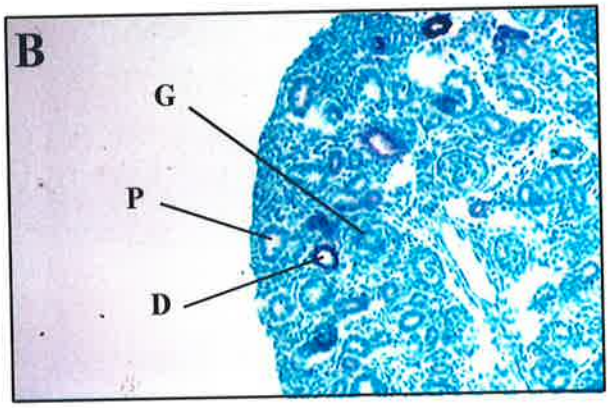
D; distal convoluted tubule, *G*; glomerulous, *P*; proximal convoluted tubule.

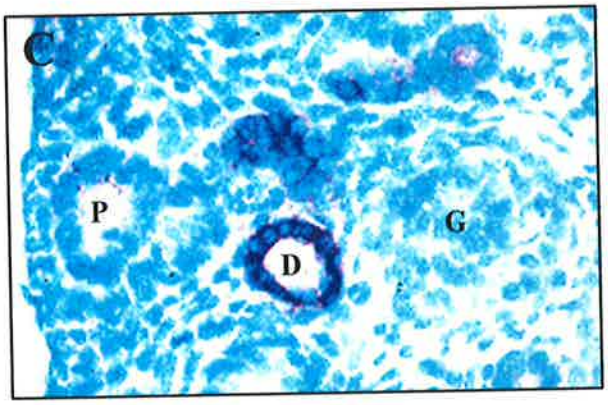
Magnifications are as follows;

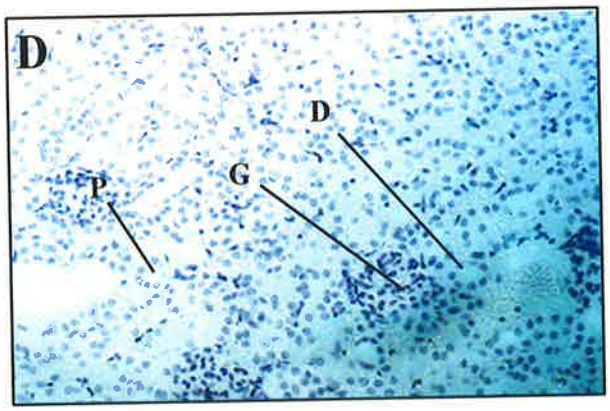
(A) 20X, **(B)** 20X, **(C)** 40X, **(D)** 10X, **(E)** 10X, **(F)** 20X, **(G)** 20X.

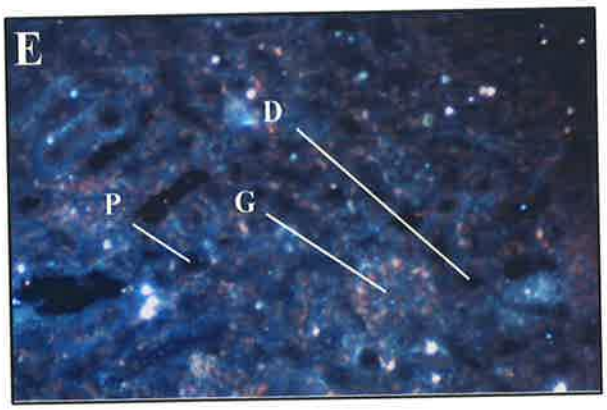
Taken from Rodda *et al.*, 2001.

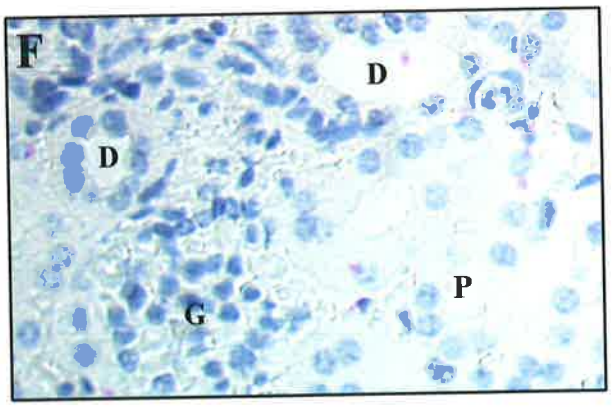

A
D
P
G

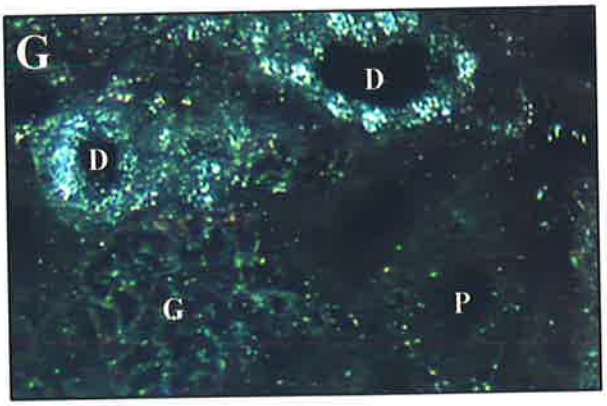

B
G
P
D


C
P
G
D


D
D
G
P


E
D
P
G


F
D
D
P
G


G
D
D
G
P

tubules (DCTs) based on several criteria. Firstly, they were located adjacent to glomeruli, consistent with the location of DCTs within the kidney cortex (Potter, 1972). Secondly, only a small proportion of the tubules present in any cortical section expressed *CRTR-1*, consistent with the greater relative representation of proximal convoluted tubules (PCTs) in this region of the kidney (Burkitt, 1993; Fawcett, 1997; Junqueria *et al.*, 1995; Saxen, 1987). Finally, the morphology of *CRTR-1* expressing tubules was clear and open, consistent with the morphology of DCTs but distinct from that of PCTs in which the epithelium forms a brush border consisting of microvilli that project into the lumen of the tubule (Fawcett, 1997; Junqueria *et al.*, 1995).

Radiolabelled *in situ* hybridisation to adult kidney sections showed specific localisation of *CRTR-1* expression to the epithelial monolayer lining a subset of tubules in the adult kidney cortex (Figure 1.10f and g) (Rodda *et al.*, 2001). Consistent with the expression in embryonic kidneys, these tubules were identified as DCTs.

Expression of *CRTR-1* in the embryonic and adult kidney, an heterogeneous cell population, is at similar or higher levels to that seen in ES cells, a homogeneous cell population (Figure 1.9b and c) indicating a high level of expression in kidney DCTs (Figure 1.10). DCTs form part of the functional nephron, which arises arise from the metanephric mesenchyme located at the tip of the branching ureteric buds (formed from the mesonephric duct) near the cortical periphery after 13 d.p.c. and is induced by molecular interaction between the ureteric bud and the metanephros (Davies and Bard, 1998). Although *CRTR-1* expression in the embryo kidney may be associated with induction of these tubules by response to signaling during development, continued high level expression during later stages of embryogenesis and in the adult is suggestive of a role for CRTR-1 in DCT function and physiology.

DCTs form part of the nephron, the basic filtration unit of the kidney, and become functional at around 16.0 d.p.c. (Davies and Bard, 1998). These tubules control blood pH through regulated ion channels which direct the reabsorption of Na^+ and HCO_3^- ions from kidney filtrates, and the secretion of K^+ and H^+ ions (Oberleithner *et al.*, 1987; Stanton, 1986). This process is regulated by the signaling molecule aldosterone, a ligand for the mineralcorticoid receptor that is associated with regulation of genes required for Na^+ and H^+ exchange (Lombes *et al.*, 1990; Roland *et al.*, 1995).

Other sites of *CRTR-1* expression such as stomach, intestine, placenta and testis, have not been investigated at the cellular level.

The detailed expression analysis demonstrates that expression of *CRTR-1* is spatially and temporally regulated. Sites of *CRTR-1* expression *in vivo* suggest at least two functions for *CRTR-1* in the mouse, in pluripotent cells during early mouse development, and in the development and function of kidney DCTs. While both pluripotent cells and the DCT lining are epithelial in origin, other epithelial cells including the lining of kidney proximal convoluted tubules did not express detectable *CRTR-1*, excluding a general role for the CRTR-1 protein in cells of this type.

1.13 AIMS.

CRTR-1 is a novel member of the CP2 family of transcription factors. Gene expression analysis demonstrated that *CRTR-1* expression is spatially and temporally regulated in contrast to the ubiquitous expression generally reported for CP2 family members. The purpose of this work was to understand the specific function of CRTR-1, achieved via investigation of the following aspects of transcription factor action;

1) Isolation and analysis of the complete *CRTR-1* cDNA and open reading frame.

Isolation of the full *CRTR-1* cDNA is required to obtain the whole ORF and 5'UTR. Full length cDNA will enable a comprehensive analysis of the sequence similarities with CP2 family members, thereby providing clues to biological function. Further, the availability of a complete ORF is a prerequisite for several aspects of functional CRTR-1 studies.

2) Determination of CRTR-1 protein binding partners.

CP2 family members have been shown to form homomeric complexes with each other, heteromeric complexes between CP2 family members and heteromeric complexes with unrelated cellular proteins. The identification of homomeric and heteromeric complexes formed by CRTR-1 may give insight to the possible biological role this protein, in particular, promoters that are potentially regulated by CRTR-1 and signaling systems that may regulate or require CRTR-1 function.

3) Ability of CRTR-1 to bind DNA.

Members of the CP2 family have been shown to bind a consensus DNA response element, CNRG-N_6-CNRG, with the exception of LBP-1d that lacks the DNA binding region conserved in other CP2 family members. Demonstrating the DNA binding ability of CRTR-1 and identification of the DNA response element sequence will give insight to potential downstream targets for CRTR-1 regulation.

4) Determination of the transcription regulatory ability of CRTR-1.

CP2 family members are described as transcriptional activators, with the exception of LBP-9 that can negate LBP-1b transcriptional activation by an unknown mechanism. The transcription activating ability of CP2 resides in the N-terminal 40 amino acids.

Investigation into the transcriptional regulatory ability of CRTR-1 will determine whether action of CRTR-1 is likely to be positive or negative, identify the region of CRTR-1 required for transcriptional regulation and the cell type restriction of this activity.

5) Generation of homozygous *CRTR-1* mutant mice and gene expression mapping.

The biological role of CRTR-1 can be investigated by detailed expression mapping during mouse embryogenesis, in particular in the pluripotent cells and developing kidney, and by generation of homozygous *CRTR-1* mutant mice. These experiments would provide information about the specific sites of *CRTR-1* expression and how this reflects biological function of CRTR-1 in pluripotent cell biology and development and physiology of the kidney.

CHAPTER 2

Materials and methods.

2.1 ABBREVIATIONS

Ac	acetate
APS	ammonium persulphate
rATP	adenosine triphosphate
BCIG	5-bromo-4-chloro-3-indolyl-β-D-galactoside
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
bp	base pair
BSA	bovine serum albumin
Ci	curie
CIP	calf intestinal phosphatase
cpm	counts per minute
d.p.c.	days *post coitum*
DIG	digoxygenin
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's modified Eagle medium
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
DOC	sodium deoxycholate
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetra-acetic acid
EGFP	enhanced green fluorescent protein
EMSA	electrophoretic mobility shift assay
EtBr	ethidium bromide
FCS	foetal calf serum
FITC	fluorescein isothiocynate
FLB	formamide load buffer
G418	G418 sulphate
GLB	gel loading buffer

HEPES	N-2-hydroxyethyl piperazine-N-ethane sulphonic acid
HRP	horse radish peroxidase
IP	immunoprecipitation
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase pair
kDa	kilodalton
LB	luria broth
LiAc	Lithium acetate
LIF	leukaemia inhibitory factor
M	molar
mA	milliamperes
min.	minutes
mM	millimolar
MOPS	3-[N-morpholino]propane sulphonic acid
Mr.	relative molecular weight
MQ H_2O	reverse osmosis filtered water passed through a Milli-Q™ ion-exchange matrix
NBT	nitro blue tetrazolium chloride
NP-40	nonidet-P 40
OD_n	optical density at a wavelength of n nm
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBT	phosphate buffered saline + 0.1% Tween-20
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFA	paraformaldehyde
PFU	plaque forming units
PMSF	phenylmethylsulfonyl fluoride
PSB	phage storage buffer
RNA	ribonucleic acid
RNase	ribonuclease
RNAsin	ribonuclease inhibitor
rNTP	ribonucleotide triphosphate
rpm	revolutions per minute

SAP	Shrimp Alkalynie Phosphotase
SD Buffer	standard digest buffer
SD media	synthetic dropout media
SDS	sodium dodecyl phosphate
sec.	seconds
SSC	salt and sodium citrate
ssDNA	sheared herring sperm DNA
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TBS	tris buffered saline
TBST	tris buffered saline + Tween-20
TEMED	N, N, N', N'-teramethyl-ethenediamine
TFB	transformation buffer
TRITC	Tetramethylrhodamine B isothiocyanate
tRNA	transfer RNA
Tween-20	polyoxyethylenesorbitan monolaurate
U	units
UV	ultra violet
V	volts
v	volume
w	weight
YPD	yeast media
YPDA	yeast media with adenine
βME	β-mercaptoethanol

2.2 MOLECULAR MATERIALS

2.2.1 Chemicals and Reagents

Chemicals and reagents used during the course of this work were of analytical grade.

Sigma: agarose, ampicillin, BSA, CHAPS, coomassie brilliant blue, DOC, EtBr, EDTA, heparin, kanamycin, MOPS, PMSF, rATP, SDS, TEMED, Tris base, Hoechst-33258, PIPES, propidium iodide, Tween-20, PEG 3350, Lithium Acetate, Imidazole, Glutathione.

Roche: BCIG, BCIP, 10x DIG labelling mix, DTE, DTT, glycogen, ssDNA, IPTG, NBT, tRNA (from brewers yeast), and 10x transcription buffer, EDTA-Free complete™ protease inhibitor tablets.

BDH Chemicals: APS, DMF, NP-40, phenol, and PEG 6000

Difco: Bacto-tryptone, yeast extract, Bacto-agar, yeast nitrogen base, casamino acids

Merck: PFA

Pharmacia: Sepharose CL-6B, glutathione sepharose 4B, reduced glutathione, protein-G sepharose, sephadex G50,

Promega: dNTPs

ICN: Gluteraldehyde,

National Diagnostics: Sequagel 6, protogel and accugel.

Pierce: SuperSignal™ Chemilimunesence Substrate.

Clontech: TALON metal affinity resin

Bioserve: Glutathione agarose.

2.2.2 Radiochemicals

[α-^{32}P]dATP (3000 Ci/mmol), [α-^{32}P]dCTP (3000 Ci/mmol), [α-^{33}P]dATP (1500 Ci/mmol), [α-^{32}P]rUTP (3000 Ci/mmol), and [^{35}S]-Met (1175 Ci/mmol) were supplied by Perkin Elmer.

2.2.3 Kits

Gigaprime labelling kit: GeneWorks

Megaprime labelling kit: Amersham

Gel purification kit:	GeneWorks
Alkaline phosphatase kit:	Sigma
Miniprep kit:	Geneworks
Universal Riboclone cDNA synthesis kit:	Promega
RNAzol^{TM} B:	Tel-test
Superscript II first strand synthesis system for RT-PCR,	Invitrogen
Platinum Taq PCR Supermix	Invitrogen
TnT Quick coupled *in vitro*	Promega
transcription translation kit	Promega
Dual-Luciferase reporter assay system	Promega
pGEM-T Easy vector system I	Promega

2.2.4 Enzymes

Restriction endonucleases were supplied by Pharmacia, New England Biolabs and GeneWorks. Other enzymes were obtained from the following sources:

Roche:	CIP, DNAse 1, RNAse T1, T7 and T3 RNA polymerases
Geneworks Ltd:	*E. coli* DNA polymerase I (Klenow fragment), Taq DNA
Polymerase,	RNAsin, T4 DNA ligase, and SP6 RNA polymerase
Sigma:	RNAse A, RNAse T1, Colchicine, Lyticase.
Merck:	Proteinase K
Stratagene:	Pfu Turbo polymerase
Promega:	T7, T3 and SP6 RNA polymerases, T4 PNK.

2.2.5 Buffers and Solutions

Acetate solution:	3M KAc, 2M HOAc, pH 5.8
AP buffer:	100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris HCl pH 9.5, 0.1% (v/v) Tween-20
Annealing buffer:	20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and 25 mM NaCl
10x CIP buffer:	500 mM Tris HCl pH 8.5, 10 mM EDTA (stored at 4°C)
Coomassie Destain:	45% (v/v) methanol, 45% (v/v) MQ H_20, 10% (v/v) acetic acid
Coomassie stain:	25 5 (w/v) coomassie brilliant blue, dissolved in destain
Denaturing Solution:	0.5 M NaOH, 1.5 M NaCl

50X Denhardt's solution:	0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrolidone and 0.1% (w/v) BSA
EDTA free cell lysis buffer:	50mM Tris pH7.4, 150mM NaCl, 1% Triton X-100, 5 mM Imidazole and EDTA free complete protease inhibitor cocktail (Roche).
EMSA Binding buffer:	10mM Tris-HCl (pH 8.0), 10% glycerol, 2% polyvinyl alcohol, 0.1mM EDTA, 100mM KCl, 1mM DTT and 1µg poly-dI/dC.
FLB:	95% (v/v) deionised formamide, 20mM EDTA, 0.02% (w/v) bromophenol blue, 0.02% (w/v) xylene cyanol
10x GLB:	50% (v/v) glycerol, 0.1% (w/v) SDS, 500µg/µl bromophenol blue, 500µg/µl xylene cyanol
5x Hyb:	2 M NaCl, 200 mM PIPES pH 6.4, 5 mM EDTA
GEB:	10mM reduced glutathione in 1x TBS pH 8.
GTE:	50 mM glucose, 25 mM Tris-HCl pH 7.6 and 10 mM
EDTA.	
Pre-hyb solution:	5.85g NaCl, 40% (v/v) deionised formamide, 50 mM Tris HCl pH 7.4, 16.5% (v/v) PEG, 1% (v/v) SDS, 5% (v/v) Denhardts solution (2.2.5), 100 µg/ml ssDNA
IP Lysis Buffer:	50 mM Tris-HCl pH7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 10 mM EDTA, 200 µM Na-orthovanadate, 1 complete™ protease inhibitor tablet
In situ Buffer 1:	50% formamide, 5x SSC, 0.1% Tween-20, 0.5% (w/v) CHAPS
In situ Hyb:	50% (v/v) deionised formamide, 5x SSC pH 4.5, 50 µg/ml heparin, 0.1% (v/v) Tween-20, 100 µg/ml tRNA, 100 µg/ml ssDNA
In situ substrate mix:	1 ml AP buffer, 4.5 µl NBT (75 mg/ml in 70% (v/v) DMF), 3.5 µl BCIP (50 mg/ml in 100% (v/v) DMF)
10x Klenow buffer:	500 mM Tris HCl pH 7.6, 100 mM $MgCl_2$
5x Ligation Buffer:	250 mM Tris HCl pH 7.5, 25% (w/v) PEG 6000, 50 mM $MgCl_2$, 5 mM rATP, 5 mM DTT
10x Ligation Buffer:	500 mM Tris HCl pH 7.5, 100 mM $MgCl_2$, 10 mM rATP, 100 mM DTT

Lysis solution/ Solution II:	0.2 M NaOH, 1% (v/v) SDS
4x lower buffer:	1.5mM Tris pH 8.8, 0.4% SDS
10x MOPS:	23 mM MOPS pH 7.0, 50 mM NaAc, 10 mM EDTA
Megadeath:	0.1 M NaOH, 0.5% SDS, 10 mM Tris-HCl pH 8.0
and 1 mM EDTA	
Na-TES:	0.5 M NaCl, 10 mM Tris HCl pH 7.5, 1 mM EDTA, 0.1% (v/v) SDS
Neutralising solution:	0.5 M Tris HCl pH 8.0, 1.5 M NaCl
NETN buffer:	20 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP40
NP-40 Lysis Buffer:	50 mM Tris HCl pH 7, 150 mM NaCl, 1% (v/v) NP-40, 1 mM PMSF, 1 mM EDTA
PEG/LiAc:	40% PEG, from a 50% stock, 1x TE, from a 10x stock (0.1 M Tris-HCL, 10 mM EDTA pH 7.5) and 1x LiAc, from a 10x stock (1 M LiAc pH 7.5)
PBS:	136 mM NaCl, 2.6 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4` pH 7.4.
PSB:	10 mM Tris HCl pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 0.05% (w/v) gelatin
RNAse digestion buffer:	300 mM NaCl, 10 mM Tris HCl pH 7.5, 5 mM EDTA, 40 µg/ml RNAse A, 2 µg/ml RNAse T1
PBT:	PBS + 0.1% (v/v) Tween-20
RIPA:	150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl pH 8.0, 1% (v/v) NP-40, 0.5% (v/v) DOC, 0.1% (v/v) SDS
10x SD buffer:	30 mM Tris-HAc pH 7.8, 625 mM KAc, 100 mM MgAc, 40 mM spermidine, 5 mM DTE
2x SDS load buffer:	125 mM Tris HCl pH 6.8, 4% (v/v) SDS, 20% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 5% (v/v) β-mercaptoethanol
SDS-PAGE buffer:	25 mM Tris-Glycinc, 0.1% (w/v) SDS
Solution D:	4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7, 0.5% (v/v) sarcosyl, 0.1 M β-mercaptoethanol

Solution 1:	25 mM Tris HCl pH 8, 10 mM EDTA pH 8, 15% (w/v) sucrose
Solution I:	50 mM glucose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA
Solution III:	3 M KAc and 2 M HAc
SSC:	150 mM NaCl, 15 mM sodium citrate, pH 7.4
TAE:	40 mM Tris-acetate, 20 mM NaAc, 1 mM EDTA, pH8.2
TBE:	90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, pH 8.3
TBS:	25 mM Tris HCl pH 8, 150 mM NaCl
TBST:	TBS + 0.1% (v/v) Tween-20
TE/LiAc:	1x TE, from a 10x stock (0.1 M Tris-HCL, 10 mM EDTA pH 7.5) and 1x LiAc, from a 10x stock (1 M LiAc pH 7.5)
TTBS:	TBS + 0.1% (v/v) Triton X-100
TE:	10 mM Tris HCl pH 7.5, 1mM EDTA
TEN Buffer:	40 mM Tris HCl pH 7.4, 1 mM EDTA, 150 mM NaCl
TES:	10 mM Tris HCl pH 7.5, 1 mM EDTA, 0.1% (v/v) SDS
Tfb 1:	30 mM KAc, 100 mM RbCl, 10 mM $CaCl_2$, 15% (v/v) Glycerol, pH 5.8 (adjusted with 0.2 M Acetic Acid)
Tfb 2:	10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15% (v/v) Glycerol, pH 6.5 (adjusted with 1 M KOH)
TNM:	30 mM Tris HCl pH 7.6, 150 mM NaCl, 15 mM $MgCl_2$, 0.4% (v/v) NP40
Transcription buffer:	40 mM Tris HCl pH 8, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT
4x Tris-SDS buffer:	1.5 M Tris HCl pH 8.8, 0.4% (w/v) SDS
Western lysis buffer:	20 mM HEPES, 0.42 M NaCl, 0.5% NP40, 25% Glycerol, 0.2 mM EDTA, 1.5 mM $MgCl_2$, and 1mM PMSF.
Western Transfer buffer:	192 mM glycine, 25 mM Tris HCl pH 8.3, 0.1% (w/v) SDS, 20% (v/v) methanol.

2.2.6 Plasmids

2.2.6.1 Vectors

pBluescript II KS (Stratagene) was a kind gift from Dr. Gavin Chapman

pGEMT-Easy was obtained from Promega

pGEX-6P-1,2,3 (Pharmacia), pGADT7, pGBKT7, pCL1, pLAM5'-1, pVA3-1, pTD1-1 (Clontech) were a kind gift from Dr. Stephen Jane.

pXMT2 (Rathjen *et al.*, 1990a) was a kind gift from Prof. Peter Rathjen.

pSG5.HA (Chen *et al.*, 1999b) and pSG5.HA-VDR were a kind gift from Associate Professor Brian May.

pGalO (Kato *et al.*, 1990), pTK-MH100x4-LUC (Kang *et al.*, 1993), pRLTK (Promega) and pHRE-Luc (Ema *et al.*, 1997) were kind gifts from Dr. Murray Whitelaw

pGEX2-T was a kind gift from Dr. Grant Booker.

pCMX-mSMRTα-FLAG was a kind gift from Dr. Ronald Evans.

pCEP4F-YY1 was a kind gift from Dr. Edward Seto

pEF-6Myc-Rex1 was a kind gift from Dr. Paul Bello.

pACT2 cDNA library was constructed from $(dT)_{15}$-*Xho*I primed cDNA generated using mRNA isolated from normal, whole testis pooled from 200 BALB/c male mice, aged between 8-12 weeks cloned into the pACT2 vector digested with *Eco*RI/*Xho*I and was purchased from Clontech.

2.2.6.2 Cloned DNA sequences

mGap: The mouse *glyceraldehyde phosphate dehydrogenase (mGap)* cDNA clone in pGEM3Z was a kind gift from Prof. Peter Rathjen and contained a 300 bp *Hind*III/*Pst*I fragment from the 5' end of the mouse gene (Rathjen *et al.*, 1990b).

Oct4: The *Oct4* cDNA clone in pBluescript was a kind gift from Dr. Hans Schöler and contained a 462 bp *Stu*I cDNA fragment of positions 491 to 953 of the *Oct4* cDNA sequence (Scholer *et al.*, 1990).

2.2.7 Oligonucleotides

DNA primers were synthesised by Sigma or GeneWorks Ltd.

TABLE 2.1: Oligonucleotide names, sequence and specifications.

Name	Sequence	Length	Gene Specific Length	Tm	Gene Specific Tm	Purity	Manufacturer
Crtr-1							
SR 659/675/8.1	CGA ACA TCT ACA CTC T	16	16	41	40.7	crude	GeneWorks
SR 208/224/8.2	CTC CAG TCT TAT CAA G	16	16	41	40.7	crude	GeneWorks
SR1	ATA GTC GAC CAG CCA TGC TGT TCT GG	26	18	73	49	desalt	Sigma
SR2	ATA AAG CTT GAG CTC AGA GTC CAC ACT TCA G	31	18	71	48	desalt	Sigma
SR3	ATA GTC GAC TAC AGT ATG TGT TGT GT	26	18	60	43	desalt	Sigma
SR4	ATA GAG CTC ACA ACA CAT ACT GTA G	25	17	58	42	desalt	Sigma
SR5	GTG CTG GTT GTA GTG TTC G	19	19	59	59.3	desalt	Sigma
SR6	GAC TGA GAA GAT CGC CAG CC	20	20	66	66.3	desalt	Sigma
SR7	ATA GTC GAC CAG CCA TGG ACC GGG AAA AGA TGG AA	35	21	82	52	desalt	Sigma
SR7-2	ATA GTC GAC CAG CCA TGG ACC GGG AAA AGA TGG AA	35	21	82	52	desalt	Sigma
SR8	CAA CCC TGG CCG CCG TC	17	17	73	72.7	desalt	Sigma
SR9	GCT GGA GAA TCG GAA GC	17	17	61	61.1	desalt	Sigma
SR10	GCT TCC GAT TCT CCA GC	17	17	61	61.1	desalt	Sigma
SR11	GAG CAT CAT CCG TGT CG	17	17	62	61.5	desalt	Sigma
SR12	CGA CAC GGA TGA TGC TC	17	17	62	61.5	desalt	Sigma
SR13	CTG CAC TGG TAG AGG C	16	16	55	54.8	desalt	Sigma
SR14	GCT CGT GTG ACA TGC AG	17	17	60	60.2	desalt	Sigma
SR15	GAA GTA GGT CTG CGC TG	17	17	57	56.9	desalt	Sigma
SR16	GCC AGA GAC TGG AGC TAG	18	18	58	57.7	desalt	Sigma
S617	CTA GCT CAG AGT CAG CC	17	17	53	53	desalt	Sigma
SR18	AGG CCA TGG AGA ACA CTC	18	18	60	60.2	desalt	Sigma
SR19	ATA GAG CTC TAG GAC TAA AAT GGC CTT TGC ATT G	34	25	73	54	desalt	Sigma
SR20	ATA GAG CTC TAT AGG CCG TCA GGT GGG CGT GGC AT	35	26	82	64	desalt	Sigma
SR21	ATA GAT ATC GGA TCC AAA GGC GCC GGC CGC AGC CCA CTC AG	41	32	89	73	desalt	Sigma
SR22	ATA GAT ATC GAA GCC ACA CAA GCT AAG CCA CAG AAT CTG	39	30	77	63	desalt	Sigma
SR23	ATA GAT ATC CCA GGA CCT ACA TGA CTT CAC TGG GTA AAT	39	30	74	62	desalt	Sigma
SR24	ATA GAT ATC GGA TCC GAG GAT CTG ACT CCC TCT TCT GGA GTG	42	33	83	68	desalt	Sigma
SR25	ATA GGA TCC CCA GCC ATG GAC CGG GAA AAG ATG GAA	36	21	84	52	desalt	Sigma
SR26	ATA GGA TCC TCA GAG TCC ACA CTT CAG GAT G	31	19	73	51	desalt	Sigma
SR27	ACA GCT TGC AGG AGT CAT	18	18	58	58.4	desalt	Sigma
SR28	CTC GAG CCA GCC ATG CTG TTC TGG CAC ACG CAG	33	21	71	56	PCR/Seq	GeneWorks
SR29	TCT AGA GAG TCC ACA CTT CAG GAT GAT	27	21	58	52	PCR/Seq	GeneWorks
SR30	CTC GAG CTG TTC TGG CAC ACG CAG	24	18	62	53	PCR/Seq	GeneWorks
SR31	CTC GAG CTA CAG TAT GTG TTG TGT GCC	27	21	61	52	PCR/Seq	GeneWorks
SR32	CTC GAG CCA GCC ATG CAT CAT CAC CAC CAT CAC CTG TTC TGG CAC ACG CAG CCC GAA	57	24	78	63	PCR/Seq	GeneWorks
RACE1	GGG TCC AAG ATA CCA ACA GGC AGT GGA A	28	28	63	62.8	PCR/Seq	GeneWorks
RACE2	TGT CCA GGA TGC GGT CCC CAG GCC GAC TC	29	29	70	70	HPLC	GeneWorks
L17mic1	TTC CTG CTT GAA GAT AGG	18	18	46	46	RPC	GeneWorks
Mut1SR1	CTG GCT CTG CCT ATC TTC GCA CAG GAA GAG CCG CAG CTA	39	39	72	72	RPC	GeneWorks
Mut1SR2	TAG CTG CGG CTC TTC CTG TGC GAA GAT AGG CAG AGC CAG	39	39	72	72	RPC	GeneWorks
Mut2SR1	AAG AAG ACT GAC CGG GAA GCA ATG GAA AAA AGA ACG GCT	39	39	67	67	RPC	GeneWorks
Mut2SR2	AGC CGT TCT TTT TTC CAT TGC TTC CCG GTC AGT CTT CTT	39	39	67	67	RPC	GeneWorks

Name	Sequence	Length	Gene Specific Length	Tm	Gene Specific Tm	Purity	Manufacturer
CP2							
CP2SR1	ATA GTC GAC CAG CCA TGG CCT GGG CTC TGA AG	32	18	82	53	desalt	Sigma
CP2SR2	ATA AAG CTT GAG CTC CTA CTT GAG AAT GAC ATG	33	18	71	43	desalt	Sigma
CP2SR3	ATA GTC GAC CAG CCA TGG ATA GAG AGA AAA TGG AG	35	21	75	49	desalt	Sigma
CP2SR4	ATA AAG CTT GAG CTC ACA AAC TTG ACT CTT CTT G	34	18	71	43	desalt	Sigma
CP2SR5	GGT GAG TAT GGT TGT CTC	18	18	52	52	desalt	Sigma
CP2SR6	CAG ATT TAC AAG CAA GG	17	17	51	51	desalt	Sigma
CP2SR7	TGT GTA CTT TCT GTT GAG	18	18	48	48.2	desalt	Sigma
CP2SR8	ATA GAA TTC CCA GCC ATG GAT AGA GAG AAA ATG GAG	36	21	75	49	desalt	Sigma
CP2SR9	ATA GAA TTC CTA CTT GAG AAT GAC ATG	27	17	61	40	desalt	Sigma
CP2SR10	GAA TTC CCA GCC ATG CAT CAT CAC CAC CAT CAC GCC TGG GCT CTG AAG CTG CCG	54	21	95	62	desalt	Sigma
CP2SR11	GAA TTC TCA CTT GTC ATC GTC GTC CTT GTA GTC CTT GAG AAT GAC ATG ATA GCT	54	21	83	49	desalt	Sigma
NF2d9							
NF2d9SR1	ATA GTC GAC CAG CCA TGG CCT GGG TGC TC	29	15	81	47	desalt	Sigma
NF2d9SR2	ATA AAG CTT GAG CTC ACT TCA AAA TTA TGT G	33	18	69	39	desalt	Sigma
NF2d9SR3	ATA GTC GAC CAG CCA TGG ACC GAG AAA AGA TGG GAG	35	19	80	51	desalt	Sigma
NF2d9SR4	ATA AAG CTT GAG CTC AAA GAC TGG AAT CTT CTT G	34	18	72	43	desalt	Sigma
NF2d9SR5	GTG AGA ATG GTG GTG TCG	18	18	60	60.1	desalt	Sigma
NF2d9SR6	CAG GTT TAC AGA CAG GGC	18	18	58	58.1	desalt	Sigma
NF2d9SR7	ATG AGG CTT GAG CCT ATA ATT G	22	22	61	61	desalt	Sigma
NF2d9SR8	ACT GCC TGC AGC TTG TGG CTG	21	21	72	72.1	desalt	Sigma
NF2d9SR9							
NF2d9SR10	GAA TTC CCA GCC ATG CAT CAT CAC CAC CAT CAC GCC TGG GTG CTC AGT ATG GAC	54	21	94	58	desalt	Sigma
NF2d9SR11	GAA TTC TCA CTT GTC ATC GTC GTC CTT GTA GTC CTT CAA AAT TAT GTG AAT ACC	54	21	82	45	desalt	Sigma
PIAS1							
PIAS1SR1	GAA TTC CCA GCC ATG CAT CAT CAC CAC CAT CAC GCG GAC AGT GCG GAA CTA AAG	54	21	92	56	desalt	Sigma
PIAS1SR2	GAA TTC TCA CTT GTC ATC GTC GTC CTT GTA GTC GTC CAA TGA GAT AAT GTC TGG	54	21	84	50	desalt	Sigma
PIAS1SR3	AGC ACA TCT CTG CCA GCC	18	18	64	64.4	desalt	Sigma
PIAS1SR4	TAC AGC AGG ACT ACA GCC	18	18	56	56.3	desalt	Sigma
PIAS3							
PIAS3SR1	CTC GAG CCA GCC ATG GTG ATG AGT TTC CGA GTG TCT	36	21	69	52	PCR/Seq	GeneWorks
PIAS3SR2	CTC GAG TCA CTT GTC ATC GTC GTC CTT GTA GTC GTC CAA GGA AAT GAC GTC TGA	54	21	71	52	PCR/Seq	GeneWorks
PIAS3SR1	CTC GAG CCA GCC ATG CAT CAT CAC CAC CAT CAC GTG ATG AGT TTC CGA GTG TCT	54	21	92	52	desalt	Sigma
PIAS3SR2	CTC GAG TCA CTT GTC ATC GTC GTC CTT GTA GTC GTC CAA GGA AAT GAC GTC TGA	54	21	71	52	desalt	Sigma
PIAS3SR3	AGC TGT CAC GGC AGT TCC	18	18	64	64.2	desalt	Sigma
PIAS3SR4	GAT CTT CAT CTG GAC GCT	18	18	58	57.7	desalt	Sigma
PIAS3SR5	CTC GAG AAC CAG CCA TGC ATC ATC ACC ACC ATC ACG TGA TGA GTT TCC GAG TGT CT	56	21	74	52	PCR/Seq	GeneWorks

Name	Sequence	Length	Gene Specific Length	Tm	Gene Specific Tm	Purity	Manufacturer
YY1							
YY1SR1	GAA TTC CCA GCC ATG GCC TCG GGC GAC ACC CTC TAC	36	21	72	62	PCR/Seq	GeneWorks
YY1SR2	GAA TTC TCA CTT GTC ATC GTC GTC CTT GTA GTC CTG GTT GTT TTT GGC TTT AGC	54	21	71	50	PCR/Seq	GeneWorks
YY1SR1	GAA TTC CCA GCC ATG CAT CAT CAC CAC CAT CAC GCC TCG GGC GAC ACC CTC TAC	54	21	94	62	desalt	Sigma
YY1SR2	GAA TTC TCA CTT GTC ATC GTC GTC CTT GTA GTC CTG GTT GTT TTT GGC TTT AGC	54	21	84	50	desalt	Sigma
YY1SR3	CGG CTG CAG CGC GAT CAT	18	18	73	73.4	desalt	Sigma
YY1SR4	CGC ACA CAT GTG GGA ATC	18	18	64	63.9	desalt	Sigma
YY1SR5	ATG GCC TCG GGC GAC ACC CTC TAC	24	24	64	64	PCR/Seq	GeneWorks
YY1SR6	TCA CTG GTT GTT TTT GGC TTT AGC	24	24	54	54	PCR/Seq	GeneWorks
HDAC1							
HDAC1SR1	ATA GAA TTC CCA GCC ATG GCG CAG ACT CAG GGC	33	18	83	55	desalt	Sigma
HDAC1SR2	ATA GAA TTC TCA GGC CAA CTT GAC CTC TTC	30	21	70	54	desalt	Sigma
HDAC1SR3	GAA TTC CCA GCC ATG CAT CAT CAC CAC CAT CAC GCG CAG ACT CAG GGC ACC AAG	54	21	76	60	PCR/Seq	GeneWorks
HDAC1SR4	GAA TTC TCA CTT GTC ATC GTC GTC CTT GTA GTC GGC CAA CTT GAC CTC TTC TTT GAC	57	24	72	57	PCR/Seq	GeneWorks
HDAC1SR5	CAT CTC CTC AGC ATT GGC	18	18	50	50	PCR/Seq	GeneWorks
RING1							
RING1SR1	GAA TTC CCA GCC ATG GAT GGT ACA GAG ATT GCG GTT	36	21	67	52	PCR/Seq	GeneWorks
RING1SR2	GAA TTC TCA CTT GTC ATC GTC GTC CTT GTA GTC TTT TGG GTC CTT CGT GGG GGC	54	21	74	58	PCR/Seq	GeneWorks
RING1SR1	GAA TTC CCA GCC ATG CAT CAT CAC CAC CAT CAC GAT GGT ACA GAG ATT GCG GTT	54	21	90	52	desalt	Sigma
RING1SR2	GAA TTC TCA CTT GTC ATC GTC GTC CTT GTA GTC TTT TGG GTC CTT CGT GGG GGC	54	21	88	58	desalt	Sigma
RING1SR3	GGT GAC GAT GCA GTC CGA	18	18	66	66.4	desalt	Sigma
RING1SR4	AGC CTG GAA GGT GTC AGC	18	18	63	63.1	desalt	Sigma
Ubc9							
UBC9SR1	TAT GAA TTC CCA GCC ATG TCG GGG ATC GCC CTC	33	18	84	55	desalt	Sigma
UBC9SR2	TTA GAA TTC TCA CTT GTC ATC GTC GTC CTT GTA GTC TGA GGG GGC AAA CTT CTT CGC	57	21	87	56	desalt	Sigma
UBC9SR3	GAA TTC CCA GCC ATG CAT CAT CAC CAC CAT CAC TCG GGG ATC GCC CTC AGC CGC	54	21	96	64	desalt	Sigma
FKBP4							
FKBP4SR1	GAA TTC CCA GCC ATG CAT CAT CAC CAC CAT CAC ACC GCC GAG GAG ATG AAG GCG	54	21	95	60	desalt	Sigma
FKBP4SR2	GAA TTC TCA CTT GTC ATC GTC GTC CTT GTA GTC CGC TTC TGT CTC CAC CCG AGA	54	21	88	58	desalt	Sigma
FKBP4SR3	GGC TGT GTG CCA GCA GCG	18	18	72	72.1	desalt	Sigma
FKBP4SR4	CTT TGT GCC ATC TAG CAG	18	18	57	56.9	desalt	Sigma

Name	*Sequence*	*Length*	*Gene Specific Length*	*Tm*	*Gene Specific Tm*	*Purity*	*Manufacturer*
General							
LacZSR1	CAA GGC GAT TAA GTT GGG TAA CG	23	18	55	55	desalt	GeneWorks
52-SR	AAG CAG GAA GAG CCG CAG CTA TC	23	23	59	59	desalt	GeneWorks
42-SR	AGG TTC GAG TGG GTG CTG CTT AA	23	23	57	57	desalt	GeneWorks
31-SR	GAG AGC ACA TCC GGT TTT AGC AG	23	23	57	57	desalt	GeneWorks
LUC-SR1	TAT GCA GTT GCT CTC CAG CGG	21	21	56	56	PCR/Seq	GeneWorks
SRGal4-AD	CAC TAC AAT GGA TGA TG	17	17	49	49	desalt	Sigma
GAL4SR2	GAG ATG GTG CAC GAT GCA	18	18	65	64.9	desalt	Sigma
actinA	ATGGATGACGATATCGCTG	19	19	49	49	desalt	Sigma
actinB	ATGAGGTAGTCTGTCAGGT	19	19	49	49	desalt	Sigma
Gel Shift Probes							
CP2 Binding A	TCG AGC AAG CAC AAA CCA GCC AAC	24		75		desalt	Sigma
CP2 Binding B	TCG AGT TGG CTG GTT TGT GCT TGC	24		75		desalt	Sigma
CP2 Non-Binding A	TCG AGA AAT CAC AAA ACA TCC AAC	24		66		desalt	Sigma
CP2 Non-Binding B	TCG AGT TGG ATG TTT TGT GAT TTC	24		66		desalt	Sigma
SV40 LP1	GAT CAT GGG CGG AAC TGG GCG GAG	24		80		desalt	Sigma
SV40 LP2	GAT CCT CCG CCC AGT TCC GCC CAT	24		80		desalt	Sigma
CP2RE-SR1	AGC TTG AGC AAG CAC AAA CCA GCC AAC TCG AGA GCA AGC ACA AAC CAG CCA AG	53		74		PCR/Seq	GeneWorks
CP2RE-SR2	TCG ACT TGG CTG GTT TGT GCT TGC TCT CGA GTT GGC TGG TTT GTG CTT GCT CA	53		74		PCR/Seq	GeneWorks
CP2RE-SR3	TCG ACG AGC AAG CAC AAA CCA GCC AAC TCG AGA GCA AGC ACA AAC CAG CCA AG	53		75		PCR/Seq	GeneWorks
CP2RE-SR4	GAT CCT TGG CTG GTT TGT GCT TGC TCT CGA GTT GGC TGG TTT GTG CTT GCT CG	53		75		PCR/Seq	GeneWorks
CP2RE-SR5	AGC TTG AGA AAT CAC AAA ACA TCC AAC TCG AGA GAA ATC ACA AAA CAT CCA AG	53		68		PCR/Seq	GeneWorks
CP2RE-SR6	TCG ACT TGG ATG TTT TGT GAT TTC TCT CGA GTT GGA TGT TTT GTG ATT TCT CA	53		68		PCR/Seq	GeneWorks
CP2RE-SR7	TCG ACG AGA AAT CAC AAA ACA TCC AAC TCG AGA GAA ATC ACA AAA CAT CCA AG	53		68		PCR/Seq	GeneWorks
CP2RE-SR8	GAT CCT TGG ATG TTT TGT GAT TTC TCT CGA GTT GGA TGT TTT GTG ATT TCT CG	53		68		PCR/Seq	GeneWorks

2.2.7.1 General sequencing primers

T7:	TAATACGACTCACTATAGGGAGA
T3:	ATTAACCCTCACTAAAGGGA
USP:	GTTTTCCCAGTCACGAC
RSP:	CAGGAAACAGCTATGAC

2.2.7.2 Gene specific primers

Gene specific primer details are provided in Table 2.1

2.2.8 Antibodies

Antibodies, dilutions for use and suppliers were as follows:

Anti-FLAG monoclonal antibody (1/500):	Kodak IBI
Anti-DIG F_{ab} - alkaline phosphatase conjugate (1/2000):	Roche
Goat anti-rabbit IgG (whole molecule) - HRP conjugate (1/2000):	DAKO
Goat anti-mouse IgG (whole molecule) - HRP conjugate (1/2000):	DAKO
Anti Gal4-DBD rabbit polyclonal antibody (1/500)	SantaCruz
Anti CRTR-1 rabbit polyclonal antibody (1/500)	Appendix 2
Anti-HA 12CA5 monoclonal antibody (1/750)	David Lawrence (IMVS)
Anti-Myc 9E10 monoclonal antibody (1/750)	David Lawrence (IMVS)
Goat anti-rabbit IgG (whole molecule) - FITC conjugate (1/50):	Sigma
Rabbit anti-mouse IgG (whole molecule) - FITC conjugate (1/50):	Dako
Goat anti-mouse IgG (whole molecule) - TRITC conjugate (1/50):	Sigma

2.2.9 Bacterial Strains

DH5α strain *E. coli* were used for chemical heat shock and electroporation transformations constituting routine subcloning. Genomic library screening was performed with LE392 strain *E. coli*. cDNA Library screening was carried out in *E. coli* strain BB4 (Clontech). Lambda zapping was carried out in *E. coli*. strain XL1-Blue. Protein expression from recombinant plasmids in bacteria were carried out in *E coli*. strain BL21.

E. coli strain genotypes were as follows:

DH5α:: *supE*44 Δ*lac* U169 (phi80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1*gyrA*96 *thi*-1*relA*1

LE392: F^- e14$^-$(McrA$^-$) *hsdR514* ($r_K^- m_K^+$) *supe44 supf58 lacY1 or Δ(lacIZY)6 galK2 galT22 metB1 trpR55*

BB4: *supF*58 *supE*44 *hsdR*514 *galK*2 *galT*22 *trpR*55 *metB*1 *tonA* Δ*lac*U169 F'[*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*(*tet*^{r})]

XL1-Blue: *supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac*$^-$ F'[*proAB*$^+$ *lacI*^{q} *lacZ* ΔM15 Tn*10*(tet^r)]

BL21: *hsdS gal* (λc*Its857 ind*1 *S*am7 *nin*5 *lac*UV5-T7 gene *1*)

Strain stocks were stored at -80°C in 50% glycerol.

2.2.10 Bacterial Growth Media

Luria broth:	1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract 1% (w/v) NaCl, adjusted to pH 7.0 with NaOH.
LMM broth:	1% (w/v) Bacto-tryptone, 0.5% (w/v) NaCl, 0.4 % maltose, 0.2% $MgSO_4$.
Psi broth:	2% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, 0.5% $MgSO_4$, adjusted to pH 7.6 with KOH.
SOC Medium:	2% Bacto-tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose.
Solid Media:	Agar plates were prepared by supplementing the above media with 1.5% Bacto-agar.
LMM agarose:	Prepared by dissolving 0.7 g agarose per 100 ml LMM broth.

Growth media were prepared in MQ water and sterilised by autoclaving. Ampicillin (100 μg/ml) or Kanamycin (25 μg/ml) was added after the medium cooled to 55°C to maintain selective pressure for recombinant plasmids in transformed bacteria.

2.2.11 Yeast Strains

AH109: MATa, trp-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2: :$GAL1_{UAS}$-$Gal1_{TATA}$-HIS3, $GAL2_{UAS}$-$GAL2_{TATA}$-ADE2, URA3: :$MEL1_{UAS}$-$MEL1_{TATA}$-lacZ.

2.2.12 Yeast Media

YPD Media: 20 g/L Difco peptone, 10 gL yeast extract, 2% (w/v) glucose and 20 g/L Agar (for plates only)

YPDA Media: YPD media + 100 μg/ml adenine.

SD Media: 6.7 g/L yeast nitrogen base without amino acids, 2% (w/v) glucose, 100 mls of the appropriate amino acids lacking selection amino acids and 20 g/L agar for plates only.

Amino acids: All supplied by Sigma

L-Isoleucine	300 mg/L
L-Adenine	1500 mg/L
L-Arginine	200 mg/L
L-Histidine	200 mg/L
L-Leucine	1000 mg/L
L-Lysine	300 mg/L
L-Methionine	200 mg/L
L-Phenylalanine	500 mg/L
L-Threonine	2000 mg/L
L-Tryptophane	200 mg/L
L-Tyrosine	300 mg/L
L-Uracil	200 mg/L

Growth media were prepared in MQ water and sterilised by autoclaving. Glucose and appropriate amino acids were added after the medium cooled to 55°C.

2.2.13 DNA Markers

*Hpa*II digested pUC19 markers were purchased from Geneworks.

Band sizes (bp): 501, 489, 404, 331, 242, 190, 147, 111, 110, 67, 34, 26.

*Eco*RI/*Hin*dIII λ DNA markers were prepared by digestion of λ DNA (NEB).

Band sizes (kb): 21.2, 5.14, 4.97, 4.26, 3.53, 2.02, 1.90, 1.58, 1.37, 0.97, 0.83, 0.56, 0.12.

*Eco*RI digested SPP-1 bacteriophage DNA markers were purchased from Geneworks.

Band sizes (kb): 8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, 0.48, 0.36.

DNA fragment sizes and approximate concentrations were determined by loading agarose gels with 500 ng of marker DNA.

2.2.14 Protein Markers

Benchmark^{TM} prestained protein ladder was purchased from Invitrogen.

Band sizes (Mr. in kDa) varied between batches and are indicated on figures.

2.2.15 Miscellaneous Materials

3mm chromatography paper:	Whatman Ltd.
X-ray film:	Fuji or Konica
Hybond-N^{+} membrane:	Amersham
Nytran nylon:	Schleicher and Schuell
Protran nylon	Schleicher and Schuell
Tissue culture grade plates and flasks:	Falcon
Freezing vials:	Nunc Inc
100 ASA Day roll slide film:	Sensia
100 ASA slide film:	Kodak

2.3 MOLECULAR METHODS

2.3.1 DNA Methods

2.3.1.1 Restriction endonuclease digestion of DNA

Plasmid DNA was digested with 4 units of enzyme per 1 µg of DNA and incubated at the appropriate temperature for 1-6 hours. All restriction digestions were carried out in SD buffer (33 mM Tris-HAc pH 7.8, 62.5 mM KAc, 10 mM MgAc, 4 mM Spermidine, 0.5 mM DTE).

2.3.1.2 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out using horizontal mini-gels by pouring 10 ml of gel solution (1% w/v agarose in 1 x TAE or 1 x TBE) onto a 7.5 cm x 5.0 cm glass microscope slide. Agarose mini-gels were flooded in 1x TAE or 1x TBE and samples containing 1 x GLB (5% glycerol, 0.01% SDS, 50 µg/µl bromophenol blue, 50 µg/µl xylene cyanol) were electrophoresed at 100 mA for up to one hour. DNA was stained in EtBr, visualised by exposure to medium wavelength UV light and photographed using a tracktel thermal imager.

2.3.1.3 Purification of linear DNA fragments

Linear DNA fragments were run on appropriate percentage TAE agarose gels and visualised under long wavelength UV light. Bands were dissected using sterile scalpel blades and purified using the Gel purification kit (GeneWorks) according to the manufacturer's instructions.

2.3.1.4 Blunting of DNA fragments with overhanging 5′ and 3′ ends.

Precipitated restriction digestion reactions were washed with 70% ethanol, air dried, and resuspended in 23 µl of MQ H_2O. 5′ overhanging DNA fragments were blunted by addition of 3 µl of 2 mM dNTPs (2 mM of each deoxynucleotide triphosphate), 3 µl of 10 x Klenow buffer (500 mM Tris-HCl pH 7.6, 100 mM $MgCl_2$) and 1 µl of DNA polymerase I Klenow fragment (6 units/µl) and incubation at 37°C for 30 minutes. DNA fragments with overhanging 3′ termini were blunted by the addition of 2 µl of 10 x Klenow buffer and 6 units of DNA polymerase I Klenow fragment and incubated at 37°C for 5 minutes. 6 µl of 2 mM dNTPs was subsequently added and incubated for a further 15

minutes at 37°C. Blunt ended DNA fragments were purified by electrophoresis on TAE agarose gels (2.3.1.3).

2.3.1.5 Removal of 5′ phosphate groups from vector DNA fragments

Restriction digested or blunted vector DNA was precipitated in the presence of glycogen, washed in 70% ethanol, dried and resuspended in 40 µl of MQ H_2O. 5 µl of 10 x CIP buffer (500 mM Tris-HCl pH 8.5, 1 mM EDTA) and 1 µl (1 unit/µl) of Calf Intestinal Phosphatase was then added and incubated for 30 minutes at 37°C prior to purification on 1% TAE agarose gels (2.3.1.3).

2.3.1.6 Ligation reactions

Complementary end ligation reactions were carried out with 25 ng purified vector, 50 - 100 ng DNA insert in the presence of ligation buffer (50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 10 mM DTT, 1 mM rATP) and 2 units T4 DNA ligase. Reactions were incubated at room temperature for 1 hour. Blunt end ligations were performed in ligase buffer containing PEG 6000 (50 mM Tris-HCl pH 7.5, 5% PEG 6000, 10 mM $MgCl_2$, 1 mM rATP, 1 mM DTT).

2.3.1.7 Southern analysis

Digested DNA samples were run on 1% TAE agarose gels prior to staining with EtBr and photographed adjacent to a ruler for a scaling reference. A level platform was constructed in a shallow tray which was subsequently covered with 3mm chromatography paper and moistened with 0.4 M NaOH. Three sheets of 3mm paper were positioned on top of the covered platform, moistened, and rolled flat with a pipette. The gel was placed on the 3mm paper and lengths of parafilm were placed over each edge of the gel. A section of Hybond-N^+ membrane was cut to the exact size of the gel and moistened for 5-10 minutes in MQ H_2O and then in 2 x SSC. The membrane was carefully placed on top of the gel and bubbles removed by rolling with a pipette. Incisions were made in the membrane to mark the position of the wells. Two pieces of 3mm paper soaked in 2 x SSC were placed on the membrane followed by a 10 cm stack of paper towels cut marginally smaller than the size of the gel. A glass plate was placed on top of the towels and a 500 g weight positioned on the plate. Transfers were allowed to proceed for at least 18 hours after which time the apparatus was dismantled and the blot placed DNA side up on 3mm paper soaked in 6 x

SSC. The blot was then UV crosslinked in a Stratalinker (Stratagene) prior to prehybridisation.

2.3.1.8 Synthesis of radioactive DNA probes

DNA probes were prepared using the Gigaprime labelling kit (Geneworks) or Megaprime labelling kit (Amersham) according to the manufacturer's instructions. 20-50 ng of purified fragment DNA was oligo-labelled for 20 minutes at 37°C in a reaction containing 50 µCi of [α-^{32}P] dATP. The reaction was stopped by the addition of 10 µl of stop buffer (50% glycerol, 100 mM EDTA pH 7.0, 0.02% bromophenol blue, 0.02% SDS) and addition of MQ H_2O up to a volume of 100 µl prior to incubation at 65°C for 10 minutes. Excess unincorporated label was removed from probe reactions by centrifugation at 1800 rpm for 3 minutes through a Sepharose CL-6B spin column.

2.3.1.9 Hybridisation of ^{32}P-labelled probes to Southern blots

Nylon mesh, moistened with pre-hybridisation solution (5.85g NaCl, 40% (v/v) deionised formamide, 50 mM Tris HCl pH 7.4, 16.5% (v/v) PEG, 1% (v/v) SDS, 5% (v/v) Denhardts solution (2.2.5), 100 µg/ml ssDNA) was laid flat in a shallow tray. The Southern filter was placed on the mesh DNA side up, rolled up with the mesh, and placed in a Hybaid cylinder. 10-15 ml of warm pre-hybridisation solution was added to the cylinder and incubated at 42°C for 1-4 hours with rotation in a Hybaid oven.

^{32}P-labelled DNA probes (2.3.1.8) were denatured at 100°C for 5 minutes and snap cooled on ice prior to replacement of the pre-hybridisation and addition of the denatured probe. The cylinder was returned to the Hybaid oven and rotated for 12-18 hours at 42°C.

2.3.1.10 Washing Southern filters

Hybridisation solution was replaced with 50 ml of 2 x SSC/0.1% SDS, placed in a Hybaid oven and incubated at 42°C for 15 minutes. The filter was removed from the cylinder, placed in 500 ml of prewarmed 2 x SSC/0.1% SDS and incubated at 42°C for 15 minutes. Wash solution was replaced with 500 ml of prewarmed 0.2 x SSC/0.1% SDS and incubated at 42°C for a further 15 minutes. More stringent washes were performed in 0.2 x SSC/0.1% SDS at 65°C as required.

2.3.1.11 cDNA synthesis

Reverse transcription was carried out with Superscript^{tm} II reverse transcriptase first strand synthesis system (Invitrogen) following the manufacturer's instructions. Briefly, 5 µg of total RNA (section 2.3.5.1) and 500 ng of Oligo $(dT)_{12-18}$ were heated to 65°C for 5 minutes in a volume of 10 µl. The mixture was snap cooled on ice and incubated at 42°C for 50 minutes in a 20 µl reaction containing 500 nM dNTPs, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 200 U Superscript^{tm} II. The reaction was diluted to 100 µl with H_2O and stored at -20°C.

2.3.1.12 PCR with Taq polymerase

Platinum Taq PCR supermix (Invitrogen) was used, following the manufacturers instructions, for PCR amplification from 1 µL of cDNA (2.3.1.11) or 10 ng of plasmid DNA in a 35 µL reaction volume with 100 ng of each primer in a PTC-100 hot bonnet thermal cycler.

2.3.1.13 PCR with pfu polymerase

PCR reactions carried out using Pfu Turbo (Stratagene) were done so according to the manufacturer's instructions. Briefly, 100 µl volume PCR reactions containing 1 µl of cDNA (2.3.1.11) or 10 ng of plasmid DNA, 250 ng of each primer, 200 µM dNTPs, 20 mM Tris-HCl pH 8.8, 2 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 100 ng/ml BSA, 0.1% Triton X-100, and 2.5 U of Pfu Turbo polymerase were overlaid with mineral oil, heated to 94°C for 3 minutes, and cycled in a PTC-100 Thermal cycler.

2.3.1.14 Automated sequencing of plasmid DNA

Plasmid DNA was prepared from 3 ml cultures of LB containing the appropriate antibiotic selection inoculated with a single bacterial colony and grown overnight at 37°C using the Miniprep Kit according to the manufacturer's instructions (GeneWorks). 1 µg of miniprep DNA was combined with 100 ng of sequencing primer and Big Dye terminator mix (PE Biosystems) in a total volume of 20 µl. The reaction was cycled through the following steps 30 times.

Step 1: 96°C for 30 seconds

Step 2: 50°C for 15 seconds

Step 2: 60°C for 4 minutes

80 µl of 75% isopropanol was added to reactions, mixed and allowed to precipitate for 15 minutes- 4 hours at room temperature. DNA was pelleted for 20 minutes at 14,000 rpm, washed in 250 µl of 75% isopropanol, centrifuged again for 5 minutes and air dried. Reactions were analysed at the Institute for Medical and Veterinary Science Sequencing Centre, Adelaide, Australia and viewed on the Editview program (PE Biosystems).

2.3.1.15 5' RACE PCR

5' RACE PCR was carried out using the SMART 5' RACE kit (Clontech) as described by the manufacturers instructions with 4 µg of adult mouse kidney RNA (2.3.5.2).

2.3.1.16 Sequencing software and database searches

Contiguous sequence alignments and Kyte and Doolittle hydrophobicity analysis were carried out using the MacDNASIS program. Homology searches and alignments were carried out using the BLAST program of Altschul *et al.* (1990). cDNA sequences were compared to the non-redundant nucleotide database, expressed sequence tag database and the MGSCV3 mouse genomic database, or translated in six reading frames and compared to the non-redundant nucleotide database translated in all reading frames using the National Centre for Biotechnology Information web site (www.ncbi.nlm.nih.gov/blast). Alignment of cDNA sequences were carried out using the ALIGN program (Genestream network IGH France; www2.igh.cnrs/bin/align-guess.cgi) and the BLAST 2 program of Tatusova and Madden (1999) (www.ncbi.nlm.nih.gov/blast). Multiple sequence alignments were carried out using the CLUSTALW program of Thompson *et al.* (1994) (dot.imgen.bcm.tmc.edu:9331/multi-align/Options/clustalw.html) and identities and similarities shaded using the Boxshade program (www.ch.embnet.org/software/BOXform.html). The predicted CRTR-1 protein sequence was examined for motifs using the PROSITE database (www.expasy.ch/prosite). Protein sequences were also analysed using the protein structure prediction server of Fischer and Eisenberg (1996) (www.doe-mbi.ucla.edu/people/frsvr/frsvr.html). BLAST searches of the GSF gene trap data base were done at the GSF home page (genetrap.gsf.de).

2.3.1.17 EMSA probe production

Dried oligonucleotides (2.2.7.2) were resuspended to a final concentration of 1 µg/µL. 25 µg of each oligonucleotide was added to FLB (2.2.5) and separated out by 16%

PAGE (2.3.1.19) containing 8 M urea. PAGE gels were pre-electrophoresed for 30 minutes at 180 V for 2.5 hours and then stained with EtBr. Gels were then placed onto thin layer chromatography plates and exposed to a hand held long wavelength UV lamp. Oligonucleotide bands were excised, dissected into small fragments, placed into 1.5 mL eppendorf tubes with 400 µL of TE of GEB (2.2.5) and left to elute overnight at room temperature. The eluate was transferred to a clean tube and precipitated with 2.5 volumes of ethanol, 0.1 volume of 3 M NaAc pH 5.2 in the presence of glycogen. The concentration of purified oligonucleotides was determined by spectrotometer absorbance at 260nm.

2.5 µg of complimentary oligonucleotides were combind in a single eppendorf in a total volume of 8 µL made up with MQ H_2O and 2 µL of 5x annealing buffer (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$ and 250 mM NaCl). Tubes were heated to 100°C for 2 minutes then transferred to a 65°C heating block for 2 minutes which was then removed from the heater and allowed to reach room temperature over 1-2 hours.

2 µL of annealed oligo was used in an endfill reaction in a 15 µL reaction volume containing 1.5 µL of 10x Klenow buffer (2.2.5), 2.5 mM of each dATP, dGTP and dTTP with 50 mCi of μ^{32}P-dCTP and 2 units of Klenow enzyme. Reactions were left at room temparature for 20 minutes with 2.5 mM dCTP being added and left at room temparature for a further 10 minuntes. Finally, reactions were made upto 200 µL with MQ H_2O and precipitated with 100% ethanol in the presence of NaAc and glycogen and washed with 70% ethanol. Pelleted DNA was resuspended in 100 µL of MQ H_2O and 1 µL was used for counting specific activity in a liquid scintillation counter while 5 µL was loaded on a non-denaturing 16% PAGE for analysis (2.3.1.19). Probes were then diluted to 100,000 cpm/µL and stored at –20°C.

2.3.1.18 Electrophoretic mobility shift assay

Electrophoretic mobility shift assays were carried out as described (Shirra and Hansen, 1998). Briefly, 2 µL of ^{35}S-methionine labelled *in vitro* transcribed translated protein (2.3.7.7) was added to the EMSA binding buffer (10mM Tris-HCl (pH 8.0), 10% glycerol, 2% polyvinyl alcohol, 0.1mM EDTA, 100mM KCl, 1mM DTT and 1µg poly-dI/dC) in a 20 µL reaction volume. Reactions were incubated for 15 minutes at room temperature prior to the addition of 100,000 cpm (equivalent to 0.2 ng) of labelled probe and incubated at room temperature for 20 minutes prior to electrophoresis on a 0.5x TBE buffered 5.5% PAGE gel that had been pre-electrophoresed for at least 30 minutes prior to

loading of reactions. Gels were run for 1.5-2 hours at 200V, transferred to 3mm chromatography paper, dried down and and exposed for 5-48 hours to autoradiography film in an autoradiography cassette at room temperature or at –80°C.

2.3.1.19 Non-denaturing PAGE

Oligonucleotides for use in EMSA (2.2.7.2) were separated by 16% PAGE either denaturing (containing 8 M urea, for the purification of synthesized oligonucleotides) or non-denaturing (lacking urea, for the analysis of labeled probes) buffered with 0.5x TBE.

Gels were pre-electrophoresed at 180 V for 30 minutes prior to loading samples and were electrophoresed for 1.5-2 hours once loaded. Denaturing gels stained with EtBr and viewed under long wavelength UV light while non-denaturing were dried down and exposed to autoradiography film overnight in an aoutoradiographic cassette.

2.3.1.20 Kinase treatment of oligonucleotides

2 µL of complementary loigonucleotides that had been annealed (2.3.1.17) were included in a kinase reaction with T4 PNK buffer (Promega), 10 mM ATP and 1 µL of PNK enzyme (Promega) in a total reaction volume of 10 µL. Reactions were left at 37°C for 30 minutes and then heat inactivated at 70°C for 10 minutes. 2 µL of annealed and phosphorylated oligos were then used in a ligation reaction (2.3.1.6).

2.3.2 Bacteria

2.3.2.1 Preparation of $RbCl_2$ competent cells

5 mls of Psi Broth was inoculated with a single colony of DH5α strain bacteria and grown overnight at 37°C with shaking. 500 µl of overnight culture was used to inoculate 15 mls of Psi broth. The culture was grown at 37°C to an OD_{600} of 0.6. 5 mls of bacteria were subcultured in 95 mls of Psi broth and grown to an OD_{600} of 0.6 at 37°C with shaking. Cells were poured into 40 ml Oakridge tubes and chilled on ice for 5 minutes prior to centrifugation at 6000 rpm for 5 minutes at 4°C. The supernatant was aspirated and the cell pellet was resuspended in 40 mls of TFB1 (30 mM KAc, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol, pH 5.8), left on ice for 5 minutes and centrifuged at 6000 rpm for 5 minutes at 4°C. The supernatant was aspirated and the pellet was resuspended in 4 mls of TFB2 (10 mM MOPS, 75 mM $CaCl_2$, 10 mM $RbCl_2$, 15% glycerol, pH 6.5). After

15 minutes on ice 100 µl aliquots were snap frozen in a dry ice/ethanol bath and stored at -80°C.

2.3.2.2 Bacterial heat shock transformation

$RbCl_2$ competent DH5α cells were thawed on ice for 5 minutes. 50 µl aliquots were mixed with DNA (approximately 10 ng of plasmid DNA; half of a ligation reaction) and left on ice for 30 minutes. The cell/DNA mixture was heat shocked for 2 minutes at 42°C and mixed with 1 ml of LB. Cells were allowed to recover by incubation at 37°C for 45 minutes and were pelleted by brief centrifugation in a microfuge at maximum speed. The majority of the LB was removed, leaving around 100 µl, and cells were resuspended and plated on LB plates containing 100 µg/ml ampicillin. 20 µl of 50 mg/ml BCIG (dissolved in dimethyl formamide) and 50 µl of 50 mg/ml IPTG were spread onto plates for colour selection of bacteria containing recombinant plasmids, prior to plating bacteria.

2.3.2.3 Preparation of electrocompetent cells

An isolated DH5α strain colony was picked into 10 mls of YENB medium in a 50 ml flask and incubated overnight at 37°C with shaking. Two baffled 2 litre flasks each with 500 ml of YENB, were inoculated with 5 mls of the overnight culture. The cells were allowed to grow to an OD_{600} of 0.8 before centrifugation at 2,600 x g for 10 minutes at 4°C in a pre-cooled rotor. The supernatant was carefully removed and the cells were resuspended in 100 ml of ice-cold 10% glycerol. Following centrifugation at 2,600 x g the supernatant was removed and the pellet resuspended in 100 ml cold 10% glycerol and centrifuged again. The supernatant was removed and the cells were resuspended 2 mls of 10% glycerol. 120 µl aliquots were snap frozen in a dry ice/ethanol bath and stored at -80°C.

2.3.2.4 Transformation of bacteria by electroporation

Completed ligation reactions were prepared for electroporation by extraction in a 50 µl volume with an equal volume of phenol/chloroform (1:1), then chloroform alone prior to precipitation in the presence of glycogen. Precipitated DNA was washed three times in 70% ethanol, air dried and resuspended in 20 µl of MQ H_2O. Electro-competent cells were thawed on ice for 10 minutes and 40 µl aliquots were mixed with 1-2 µl of DNA (10-100 pg of supercoiled DNA; $1/10^{th}$ of ligation). The cell/DNA mix was transferred to a chilled

0.2 cm gap electroporation cuvette (BIO-RAD) and electroporated at 25 μF, 2,500 V, and 200 ohms in a BIORAD Gene Pulser. Immediately after electric shock cells were suspended in 1 ml of SOC medium and incubated at 37°C for 1 hour. The bacteria were plated onto LB plates containing 100 μg/ml ampicillin and grown overnight at 37°C.

2.3.2.5 Mini-preparation of plasmid DNA

3 ml of LB containing the appropriate antibiotic was inoculated with a single bacterial colony and grown overnight at 37°C in a rotating drum. Each culture was poured into a 1.5 ml Eppendorf tube and centrifuged at maximum speed for 15 seconds. The majority of the medium was removed leaving approximately 100 μl. Bacterial pellets were resuspended by vortexing and lysed by the addition of 300 μl of Megadeath solution (0.1 M NaOH, 0.5% SDS, 10 mM Tris-HCl pH 8.0, 1 mM EDTA). Cell debris was precipitated by mixing 160 μl of NaAc pH 5.2 with the mixture and centrifugation at maximum speed for 4 minutes. Nucleic acids were precipitated by mixing 1 ml of 100% ethanol with the supernatant. The sample was briefly vortexed and centrifuged at maximum speed for 4 minutes prior to removal of the supernatant. The nucleic acid pellet was washed by the addition of 400 μl of 70% ethanol followed by vortexing and a brief centrifugation. The remaining liquid was removed and the pellet was dried for 5 - 10 minutes at 37°C. Mini-prep DNA was resuspended in 20 μl of MQ H_2O containing 10 μg/ml RNase A.

2.3.2.6 Midi-preparation of plasmid DNA

50 ml of LB containing 100 μg/ml ampicillin in a 250 ml flask was inoculated with a single bacterial colony and grown overnight at 37°C with shaking. The culture was transferred to a 40 ml oakridge tube and centrifuged in a RC-5 Sorvall centrifuge and SS-34 rotor (Dupont) at 6,000 rpm for 10 minutes. The supernatant was removed and the bacterial pellet was resuspended in 3 ml of Solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA). The suspension was gently mixed with 6 ml of fresh Solution II (0.2 M NaOH, 1% SDS) to lyse the cells. Following 5 minutes incubation on ice, cell debris was precipitated by the addition of 4.5 ml of Solution III (3 M KAc, 2 M HAc). The solution was gently mixed by inversion, left on ice for 5 minutes, then briefly mixed vigorously, left on ice for a further 15 minutes, and centrifuged at 14,000 rpm for 15 minutes at 4°C. The supernatant was mixed with 8 ml of isopropanol in a clean oakridge tube, and nucleic acids were precipitated by centrifugation at 12,000 rpm for 5 minutes at

4°C. The supernatant was aspirated and the pellet was dissolved in 400 µl of MQ H_2O. RNA was removed by incubation at 37°C for 30 minutes with 2 µl of RNase A (10 mg/ml). 8 µl of 10% SDS and 2 µl of Proteinase K (20 mg/ml) were added to the solution and incubated for a further 15 minutes at 37°C. The sample was extracted 2-3 times with an equal volume of phenol/chloroform and then with once with an equal volume of chloroform alone. The aqueous phase was precipitated by addition of 100 µl of 7 M NH_4Ac and 1 ml of 100% ethanol. DNA was precipitated for 20 minutes at -20°C then pelleted at 14,000 rpm in a bench-top centrifuge for 15 minutes. The DNA was washed in 400 µl of 70% ethanol, dried, and resuspended in 200 µl of MQ H_2O.

2.3.2.7 Large-scale plasmid preparation

500 ml of LB containing 100 µg/ml ampicillin was inoculated either with a single bacterial colony or 5 ml from an overnight culture, and incubated overnight at 37°C in an orbital shaker. The cells were harvested by centrifugation at 6000 rpm for 5 minutes at 4°C, and the bacterial pellets drained. Bacteria were resuspended in 6.5 mls of GTE (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA) before the addition of 13 ml of fresh lysis solution (0.2 M NaOH, 1% SDS). The mixture was thoroughly mixed by inversion approximately 20 times and placed on ice for 5 minutes. 6.5 ml of 3 M NaAc pH 4.6 was added, gently mixed by inversion and incubated on ice for 5 minutes. The mixture was mixed more vigorously and placed back on ice for a further 15 minutes. Cell debris was pelleted by centrifugation at 14,000 rpm for 15 minutes at 4°C in a SS-34 rotor and RC-5 Sorvall centrifuge (Dupont). The supernatant was transferred to a clean Oakridge tube and nucleic acid was precipitated with the addition of 15 ml of isopropanol and centrifugation at 8000 rpm for 5 minutes at 4°C. The pellet was resuspended in 7 ml of TE. 7g CsCl and 700 µl EtBr (10 mg/ml) was added and mixed immediately. EtBr/protein aggregates were removed by centrifugation at 3,500 rpm for 5 minutes at 4°C. The supernatant was transferred to a 10 ml Nalgene polycarbonate Oakridge tube and balanced with paraffin oil. A CsCl density gradient was formed by centrifugation at 45,000 rpm for 20-22 hours at 20°C. Plasmid DNA was visualised under long wavelength UV light and recovered using a 1 ml syringe and a 1 1/2 inch, 22 gauge needle. EtBr was removed by 5 - 10 extractions with NaCl/TE saturated isopropanol. The DNA solution was diluted 1 in 4 with MQ water in a 30 ml Corex tube before precipitation with 2.5 volumes of ethanol. Plasmid DNA was recovered by centrifugation at 9500 rpm for 20 minutes at 4°C and the pellet was resuspended in 400 µl MQ water. The DNA solution was transferred to an Eppendorf tube

and precipitated again with 20 µl of NaAc pH 5.2 and 1 ml of 100% ethanol before final resuspension in MQ water. Yield and quality of plasmid DNA was determined from the absorbance of a 1 in 500 dilution at wavelengths between 210-320 nm and by electrophoresis on 1% TBE agarose gels.

2.3.3 Bacteriophage

2.3.3.1 Library screening

20 ml LMM broth was inoculated with a single LE392 bacterial colony and grown overnight at 37°C in an orbital shaker. Bacterial cultures were centrifuged at 5,000 rpm for 5 minutes at 4°C and resuspended in 10 ml of 10 mM $MgSO_4$. Approximately 5 x 10^4 recombinant phage were added to 200 µl of plating bacteria and incubated at 37°C for 15-20 minutes. 10 ml of molten LMM agarose was transferred to the phage/bacteria mixture, briefly mixed and overlayed onto 15 cm LB plates. Plates were incubated at 37°C for 8 hours until the plaques were almost in contact and were stored overnight at 4°C. Colony/Plaque Screen hybridisation transfer membranes were spotted asymmetrically with a permanent marker and placed onto library plates for 1 minute. Spots were duplicated on the plate, the lift removed and transferred, phage side up, to 3mm paper. A second lift was placed on the plates and removed after 3 minutes. Lifts were allowed to air dry for 5 minutes. Filters were then baked at 100°C for 2 minutes, moistened with 6 x SSC, and UV crosslinked twice in a Stratalinker (Stratagene).

Lifts were pre-hybridised with 15-25 ml of pre-hybridisation solution (40% deionised formamide, 50 mM Tris-HCl pH 7.4, 1% SDS, 16.5% PEG 6000, 5 x Denhardt's reagent, 1 M NaCl, 100 µg/ml herring sperm DNA) in a large petri dish with a piece of nylon on top of the last filter at 42°C for at least 4 hours. ^{32}P-labelled DNA probes (2.3.1.8) were denatured at 100°C for 5 minutes and snap cooled on ice. The lifts were removed from the petri dish, pre-hybridisation solution replaced, ^{32}P-labelled DNA probe added, and the lifts placed back in the dish, one by one. Nylon mesh was placed on top of the lifts to prevent drying of the top lift. The petri dish was wrapped in cling wrap and placed in a 42°C Hybaid oven overnight.

Lifts were removed from the dish and placed in 500 ml of preheated 2 x SSC/0.1% SDS and incubated at 42°C for 15 minutes. This procedure was repeated and individual lifts were checked for counts with a hand held Geiger counter. Lifts were sufficiently washed if lifts had counts of between 5 and 10 cpm. More stringent washes were performed in 2 x SSC/0.1% SDS at 65°C for 15 minutes and finally in 0.2 x SSC/0.1% SDS at 65°C

for 15 minutes. Excess moisture was removed by blotting lifts on 3mm chromatography paper. Lifts were positioned between two sheets of clear plastic in an autoradiograph cassette and exposed to fast X-ray film overnight at -80°C. Orientation marks were copied onto autoradiographs and double positives were picked with the wide end of a Pasteur pipette into 1 ml of PSB and eluted overnight at 4°C. The eluate was placed into a clean tube with 1 drop of chloroform to eliminate bacterial contaminants.

Second round screening was performed as detailed above with the following changes. Dilutions of phage eluted from the first round positive plugs were used to infect 100 µl of plating bacteria and plated onto 10 cm LB plates in 5 mls of LMM agarose. Duplicate nitrocellulose lifts were taken from plates with around 100-300 plaques and phage were lysed by placing the lifts onto 3mm paper soaked in denaturing solution (1.5 M NaCl, 500 mM NaOH) for 5 minutes. Lifts were then placed on 3mm paper soaked in neutralisation solution (1.5 M NaCl, 500 mM Tris-HCl pH 8.0) for 5 minutes and finally onto 3mm paper soaked in 6 x SSC. DNA was crosslinked to the lifts in a Stratalinker (Stratagene). Pre-hybridisation and hybridisation was carried out in a 60 mm diameter petri dish. Isolated duplicate positive plaques were picked with the narrow end of a Pasteur pipette and phage were eluted overnight at 4°C in 400 µl of PSB.

2.3.3.2 High titre stock production

Isolated second round positive plaques were eluted in 400 µl of PSB overnight at 4°C. 50 µl of the eluate was incubated with 100 µl of LE392 *E. coli* before plating onto 10 cm LB plates. Plates observed to have confluent lysis were soaked in 1-2 mls of PSB for 3 hours with shaking. The PSB was transferred to Eppendorf tubes and a drop of chloroform was added to kill bacteria.

2.3.3.3 Phage DNA preparations

10^5 phage (50 µl of high titre stock) were mixed with 500 µl of an LE392 strain overnight culture. The mixture was incubated for 15 minutes at room temperature and added to 50 ml of LMM broth in a 250 ml flask and incubated at 37°C overnight. A few drops of chloroform were added the next day to kill bacteria. The solution was poured into 40 ml oakridge tubes and centrifuged at 5000 rpm for 5 minutes. The supernatant was poured into a new oakridge tube and treated with 10 µl RNase A (10 mg/ml) and 10 µl DNase I (100 U/µl) for 30 minutes at 37°C. 7.125mls of 4 M NaCl and 3.75 ml of 50%

PEG 6000 were thoroughly mixed into the solution and the tubes chilled to 4°C for at least 2 hours. Phage particles were pelleted by centrifugation at 10,000 rpm for 10 minutes. The supernatant was drained away and the phage pellet was resuspended in 500 µl of PSB. Phage particles were lysed by the addition of 20 µl of 500 mM EDTA, 10 µl of 10% SDS, and 2.5 µl of Proteinase K (20 mg/ml). Phage proteins were digested for 60 minutes at 37°C and the protease was inactivated at 65°C for 15 minutes. The sample was phenol/chloroform extracted twice, chloroform extracted once. Phage DNA was recovered by precipitation by the addition of 1 ml of 100% ethanol and centrifuged, 70% ethanol washed, and resuspended in 100 µl of MQ H_2O.

2.3.3.4 Lambda zapping

cDNA clones were isolated from second positive plaques using the Lambda ZAP excision process (Clontech). The Lambda ZAP excision process relies on the life cycle of m13/F1 coliphage to excise pBluescript out of the Lambda arms. XL1-blue E.coli were infected with a positive Lambda and helper bacteriophage. The cells were incubated for 4.0 hours at 37°C then at 68°C for 20 minutes. Tubes were then centrifuged for 5 minutes at 3000rpm. Supernatant from the culture (containing the phagemid) was added to XL1-blue cells and platted on 100µg/ml ampicillin plates and incubated at 37°C overnight. Successful excision was determined by the presence of bacterial colonies being present on the experimental plates.

2.3.4 Yeast

2.3.4.1 Amplification of the pACT2-mouse testis cDNA library

The pACT2-mouse testis cDNA library (Clontech) was obtained and amplified as described by the manufacturers instructions.

2.3.4.2 Large scale yeast transformation for library screening

Simultaneous cotransformation:

2-3 large colonies of AH109 strain yeast that had been streaked out onto a YPD yeast plate (2.2.12) were used to inoculate 150 ml of YPDA media (2.2.12) in a flask and incubated at 30°C overnight OVERNIGHT with shaking. The OD_{600} was measured and determined to be greater than 1.5. 50 ml of this was then subcultured into 2x 500 ml of YPDA media (2.2.12) and grown to an OD_{600} of 0.5 ± 0.1 at 30°C. The culture was then divided between sterile 50 ml tubes and pelleted by centrifugation at 1000x g for 5 minutes

at room temperature. The supernatant was discarded and the pellet was resuspended in several mls of sterile MQ H_2O and combined into one 50 ml tube were the yeast were re-pelleted. The supernatant was discarded and the yeast were resuspended in 8 ml of 1x TE/LiAc solution (2.2.5). In a sterile 50 ml tube 500 ng of the yeast-2-hybrid library and 1 mg of pGB-CRTR-1(47-481) were combined with 20 mg of herring testis carrier DNA and 8 ml of competent yeast. This was mixed and equally divided between two 50 ml tubes. To each tube 30 ml of fresh made PEG/LiAc (2.2.5) was added and mixed. Tubes were incubated at 30°C for 30 minutes with shaking and then had 3.5 ml of DMSO added to each tube prior to being equally divided between yellow cap tubes and heat shocked at 42°C for 15 minutes. Yeast were allowed to recover on ice for 1-2 minutes prior to being pelletted by pulse spin in an Eppendorf centrifuge and being resuspended in a total volume of 10 ml of 1x TE. 200 µL of transformed yeast were then spread onto 15 cm -leu/-trp/-his plates incubated at 30°C until transformants appeared (4-5 days). Colonies were then picked and streaked onto 15 cm -leu/-trp/-his/-ade plates and incubated at 30°C until transformants appeared (4-5 days).

Sequential transformation:

Yeast were transformed with pGB-CRTR-1(47-481) as described (2.3.4.2). Transformed colonies were then picked and used to set up over night cultures for transformation with the yeast-2-hybrid cDNA library as described above with the exception that pGB-CRTR-1(47-481) DNA was not included in the transformation protocol.

2.3.4.3 Small scale yeast transformation

2-3 large colonies of AH109 strain yeast that had been streaked out onto a YPD yeast plate (2.2.12) were used to inoculate 50 ml of YPDA media (2.2.12) in a flask and incubated at 30°C overnight OVERNIGHT with shaking. The OD_{600} was measured and determined to be greater than 1.5. 15 ml of this was then subcultured into 300 ml of YPDA media (2.2.12) and grown to an OD_{600} of 0.5 ± 0.1 at 30°C. The culture was then divided between sterile 50 ml tubes and pelleted by centrifugation at 1000x g for 5 minutes at room temperature. The supernatant was discarded and the pellet was resuspended in several mls of sterile MQ H_2O and combined into one 50 ml tube were the yeast were re-pelleted. The supernatant was discarded and the yeast were resuspended in 1.5 ml of 1x TE/LiAc solution (2.2.5). To a 1.5 ml Eppendorf tube 100 ng of each the bait and prey plasmids were added together with 100 µg of herring testis carrier DNA and 100 µL of competent

yeast with 600 µL of fresh made PEG/LiAc solution (2.2.5). Tubes were mixed and incubated at 30°C for 30 minutes with shaking prior to the addition of 70 µL of DMSO and heat shock treatment at 42°C for 15 minutes. Yeast were allowed to recover on ice for 1-2 minutes prior to being pelletted by pulse spin in an eppendorf centrifuge and being resuspended in 100 µl of 1x TE and being spread onto appropriate selective media plates and incubated at 30°C until transformants appeared (4-5 days).

2.3.4.4 Plasmid preparations from transformed yeast

Single transformed yeast colonies were picked and used to inoculate 4 ml YPD (2.2.5) cultures in yellow cap tubes and incubated with rotation over night at 30°C. Yeast were then pelleted by centrifugation and the supernatant removed. Yeast were then resusdpended in 500 mL of 1 M sorbitol, 0.1 M EDTA pH 7.5 and transferred to a 1.5 ml Eppendorf tube. 5 µL of 10 mg/ml lyticase (Sigma) was then added and mixed. The tubes were then incubated at 37°C for 60 minutes with mixing. Tubes were then spun at low speed for 1 minute, the supernatant was removed, and the pellet resuspended in 500 µL of 50 mM Tris pH 7.4 and 20 mM EDTA. Spaeroplasts were then lysed by the addition of 50 µL SDS (10%), mixing and incubation at 65°C for 30 minutes. 200 µL of 5 M Kac was added and incubated on ice with mixing prior to spinning the tubes at full speed for 5 minutes in an eppendorf centrifuge. Supernatants were transferred to fresh eppendorf tubes and 750 µL of isopropanol was added and tubes mixed. After 5 minutes at room temperature, tubes were spun at full speed for 10 seconds, the supernatant was removed and pellets were air dried for several minutes. Pellets were resuspended in 300 µL 1x TE and RNA was removed by the addition of 2 µL of Rnase A at 10 mg/ml and incubated at 37°C for 30 minutes. DNA was precipitated by the addition of 30 µL of 3 M NaAc and 200 µL of isopropanol and left at room temperature for 10 minutes. Tubes were spun at full speed for 5 minutes and the supernatant was removed. Pellets were resuspended in 100 µL of MQ H_2O and 10 µL of each DNA preperation was used for electroporation into DH5α *E.coli* (2.3.2.4). Electroporated bacteria were then plated onto the appropriate antibiotic selection plates and incubated over night at 37°C. Colonies were then selected and used for DNA mini preparations (2.3.2.5) for BDT automated sequence (2.3.1.14) and PCR (2.3.1.12) analysis.

2.3.5 RNA

2.3.5.1. RNA isolation using RNAzol^{TM} B

Total RNA from cultured cells and mouse tissues was isolated using RNAzol^{TM} B (Tel-test) following the manufacturers instructions.

2.3.5.2 Isolation of RNA from mouse tissues using guanidium thiocyanate

RNA from tissue samples was isolated using the guanidium thiocyanate method (Chomczynski and Sacchi, 1987). Tissues were harvested and immediately frozen at -80°C until required. Tissues were thawed on ice in 1 ml of solution D (2.2.5) and homogenised using a glass teflon homogeniser. The solution was transferred to a 10 ml polypropylene tube and 0.1 ml 2 M NaAc pH 4, 1 ml of phenol and 0.2 ml chloroform:isoamylalcohol (49:1) added sequentially and mixed by gentle inversion, before vigorous mixing once all reagents were added. The homogenate was incubated on ice for 15 min., before centrifugation at 3800 r.p.m. (Jouan, C412) and the aqueous layer transferred to a Corex tube. RNA was precipitated by the addition of 1 ml of isopropanol and incubation at -20°C for a minimum of an hour. RNA was recovered following centrifugation at 9000 r.p.m. for 30 min. at 4°C (SS-34 rotor, Sorvall RC-5) and the pellet resuspended in 400 µl of solution D. Following transfer to an eppendorf tube, RNA was re-precipitated, washed with 75% (v/v) ethanol, dried and resuspended in an appropriate volume of MQ H_2O. RNA concentration was determined by spectrophotometric analysis at 260 nm.

2.3.5.3 Selection for polyadenylated RNA

Poly $(A)^+$ RNA selection was carried out essentially as in (Celano *et al.*, 1993). Oligo-dT cellulose (Type 7, Pharmacia) was hydrated by three washes in Na-TES (500 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1 % SDS). 1 mg of cytoplasmic RNA (2.3.26) was brought up to a final volume of 600 µl in TES (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1 % SDS) and denatured at 65°C for 5 minutes and snap cooled on ice. 60 µl of 5 M NaCl was mixed with the RNA and added to 600 µl (60 mg) of hydrated oligo-dT cellulose. The tube was mixed well and incubated at 37°C for 10 minutes. Oligo-dT cellulose was pelleted by centrifugation in a benchtop centrifuge at maximum speed for 15 seconds. The supernatant was removed and the pellet was resuspended in 1 ml of Na-TES and the tube was centrifuged again. The supernatant was removed and the pellet resuspended in 1 ml of ice-cold MQ H_2O and centrifuged once more. Poly $(A)^+$ RNA was

eluted by resuspending the oligo-dT cellulose in 400 µl of MQ H_2O and incubation at 55°C for 5 minutes. Following centrifugation the supernatant was placed into a siliconised Eppendorf tube. The elution was repeated twice more and eluates were again selected on oligo-dT cellulose as described above. Following the second round of selection eluted poly $(A)^+$ RNA was precipitated by the addition of 1/10th volume of 3 M NaAc pH 5.2 and 2.5 volumes of 100% ethanol. Precipitates from each tube were resuspended and pooled in 10-15 µl and the quantity and quality of the poly $(A)^+$ RNA was ascertained by spectrophotometric analysis and agarose gel electrophoresis, respectively.

2.3.5.4 Ribonuclease protection analysis

2.3.5.4.1 Riboprobe templates and probe synthesis

Linearised riboprobe templates were prepared by digesting 20 µg of DNA with the appropriate enzyme (2.3.1.1), followed by phenol/chloroform extraction, ethanol precipitation and resuspension in 20 µl of MQ H_2O. CRTR-1 riboprobes for RNAse protection were generated from clone 1.2 (Figure 3.1.) digested with *Hinc*II and transcribed with T3 polymerase yielding a 460 bp transcript. CP2 riboprobes for RNAse protection were generated from paltCP2-RP (3.7.3) digested with *Nco*I and transcribed with SP6 polymerase yielding a 490 bp transcript. NF2d9 riboprobes for RNAse protection were generated by *Sal*I digestion of paltNF2d9-RP and transcription with T7 polymerase yielding a 540 bp transcript. *mGap* riboprobes for RNAse protection were generated from the 300 bp *mGap* cDNA clone (2.2.6.2). The antisense template was generated by *Bam*HI digestion and transcribed with SP6 RNA polymerase.

Transcription reactions (in 15 µl) contained 125 µCi of [α-^{32}P]-rUTP for *CRTR-1* probes and 25 µCi [α-^{32}P]-rUTP for *mGap* riboprobes, transcription buffer (2.2.5), 2.25 µl of 3.3 mM rATP/rCTP/rGTP mix, 0.5 µl of 1 mM rUTP, 1 µg DNA template, 10 U RNAsin and 10 U of the appropriate RNA polymerase. Reactions were incubated at 37°C for 75 min., before the addition of 20 U of DNAse 1 and incubation at 37°C for a further 15 min. Unincorporated label was removed using a sephadex G-50 column and spinning at 3000 r.p.m. for 5 min. (Jouan, C412).

2.3.5.4.2 RNAse protection assay

RNAse protection analysis was carried out using the method of (Krieg and Melton, 1987). 150,000 c.p.m. of single stranded antisense *CRTR-1* riboprobe and 37,000 c.p.m. of single stranded antisense *mGap* riboprobe were added to each 10 µg RNA sample.

Probe/RNA samples were precipitated, and the recovered pellet resuspended in 6 µl 5x Hyb (2.2.5) and 24 µl deionised formamide, before denaturation at 85°C for 3 min., and hybridisation OVERNIGHT at 45°C. The following day, samples were digested at 37°C for 30 min. in RNAse digestion buffer (2.2.5), incubated for a further 15 min. with 2 µl proteinase K and 10 µl of 10% (w/v) SDS, and then phenol/chloroform extracted. RNA digestion products were re-precipitated and resuspended in 1 µl of MQ H2O and 4 µl of FLB (2.2.5), heated to 85°C for 3 min., snap cooled and analysed by electrophoresis at 60 V on a 6% (v/v) denaturing polyacrylamide gel prepared from 40% sequagel 6. Following electrophoresis, the gel was transferred to 3MM chromatography paper (Whatman), and dried at 70°C under vacuum. Radioactivity was detected by exposure to phosphorimager screens (Molecular Dynamics).

2.3.5.5. Northern blot analysis

1% agarose gels for northern blot analysis were prepared by dissolving 2.5 g agarose in 210 ml MQ H_20. Once the gel solution had cooled to 60°C, 25 ml 10x MOPS (2.2.5) and 15 ml 20% (w/v) formaldehyde (freshly prepared by dissolving 4 g paraformaldehyde in 20 ml MQ H_2O) were added before pouring the gel.

Poly-$(A)^+$ RNA samples (4 µg), or total RNA samples (25 µg) were prepared for electrophoresis as follows:

11.25 µl	RNA + MQ H_2O
5 µl	10x MOPS
8.75 µl	formaldehyde (37%, pH 4.5)
25 µl	deionised formamide

RNA samples were denatured by heating at 65°C for 15 min., snap cooled on ice, before the addition of 5 µl of RNAse free 10x GLB (2.2.5). 5 µg *Eco*RI digested SPP-1 phage markers (2.2.10) were also loaded. Northern gels were run at 80 V in 1x MOPS (2.2.5) until the bromophenol blue dye had run to within 3/4 of the bottom of the gel. The DNA marker containing lane was removed from the gel, stained in EtBr for 45 min. and destained in water OVERNIGHT before photographing under medium wavelength UV light. The remainder of the gel was blotted onto Hybond-N^+ membrane (Amersham) using capillary transfer as described in 2.3.6, with the exception of the transfer being carried out in 20 X SSC. RNA was cross-linked to the filter using a UV Stratalinker™ (Stratagene). The Northern filter was then placed on nylon mesh RNA side up, rolled up, and prehybridised in a Hybaid cylinder at 42°C for 4-16 hours in 10 ml of pre-hyb solution (2.2.5). Filters were then probed, washed and analysed as described in 2.3.1.9 and 2.3.1.10.

2.3.5.6 in situ hybridisation

2.3.5.6.1 Digoxygenin labelled RNA probes templates preparation

Templates for DIG riboprobe synthesis were prepared as described in section 2.3.21.1, by digesting plasmids described in section 2.2.6.2 with the appropriate restriction enzymes to linearise the plasmids. Specific DIG labelled riboprobes were generated using the following templates and RNA polymerases:

CRTR-1: The sense template was generated by *Bam*HI digestion, and riboprobes transcribed using T7 RNA polymerase. Antisense templates were generated by *Xho*I digestion, and riboprobes produced with T3 RNA polymerase.

K7: The sense template was generated by *Bam*HI digestion, and riboprobes transcribed using T3 RNA polymerase. Antisense templates were generated by *Hind*III digestion, and riboprobes produced with T7 RNA polymerase.

Psc1: The sense template was generated by *Hind*III digestion, and riboprobes transcribed using T7 RNA polymerase. Antisense templates were generated by *Bam*HI digestion, and riboprobes produced with T3 RNA polymerase.

Oct4: The sense template was generated by *Xho*I digestion, and transcripts polymerised with T7 RNA polymerase. The antisense template was generated by *Eco*RI digestion, and transcripts produced with T3 RNA polymerase.

Rex1: Sense and antisense riboprobes were produced using *Bam*HI or *Xba*I linearised templates and T7 and Sp6 RNA polymerases respectively.

Fgf5: Sense and anti-sense *Fgf5* probes were generated by linearising the plasmid with *Eco*RI or *Bam*HI, and transcribing with T7 and T3 RNA polymerases respectively.

2.3.5.6.2 DIG labelled riboprobe synthesis

Riboprobes were synthesised in transcription reactions containing 1 µg linearised plasmid, transcription buffer (2.2.5), 1x DIG labelling mix (10 mM each of rATP/rCTP/rGTP, 6.5 mM UTP, 3.5 mM DIG-UTP), 20 U of RNAsin, and 20 U of the appropriate RNA polymerase. Transcription reactions were incubated at 37°C for 2 hours before the template was removed by addition of 40 U RNAse free DNAse 1 and incubation at 37°C for 15 min. Reactions were precipitated at -20°C (1 hour-overnight) after addition of 60 µl MQ H_2O, 20 µl 100 mM EDTA, 10 µl 3M NaAc pH 5.2, and 250 µl ethanol. After

centrifugation for 15 min., DIG probes were resuspended in 100 µl of RNAse free MQ H_2O containing 40 U of RNAsin. A 5 µl sample of resuspended probe was used to assess riboprobe yield and quality by agarose gel electrophoresis (2.3.5).

2.3.5.6.3 Embryonic in situ hybridisation

In situ hybridisation to pre-implantation and post-implantation mouse embryos was carried out using the whole mount method of (Rosen and Beddington, 1993) with the following modifications. Embryos were treated with 2 ml solution volumes in 24 well trays using processing baskets (2.4.2) to minimise embryo loss. Dehydrated embryos in 100% methanol were rehydrated on ice with successive 5 min. washes in 75% (v/v) methanol/PBT, 50% (v/v) methanol/PBT, and 25% (v/v) methanol/PBT before rinsing twice with PBT for 5 min. at RT. All further washes were carried out at RT unless specified. Embryos were bleached with 6% (v/v) H_2O_2/PBT for 1 hour and rinsed 3 times with PBT for 5 min. Embryos were washed with RIPA buffer (2.2.5) 3 times for 20 min., rinsed twice in PBT for 5 min., and postfixed in 4% (w/v) PFA / 0.2% (v/v) gluteraldehyde in PBT for 20 min. Embryos were then washed 3 times for 5 min. in PBT, for 5 min. in 1:1 *in situ* Hyb:PBT, and for a further 5 min. in *in situ* Hyb (2.2.5) before being prehybridised in fresh *in situ* Hyb for 1-4 hours at 65°C. Prehybridisation and hybridisation steps were carried out in a sealed humidified box containing paper towels soaked in 50% (v/v) formamide. DIG-labelled riboprobes were diluted 1/200, denatured at 80°C for 10 min., snap cooled on ice, added to fresh *in situ* Hyb and hybridised to embryos OVERNIGHT at 65°C. The following day, embryos were washed at 65°C for 5 min. sequentially in *in situ* buffer 1 (2.2.5), 70% *in situ* buffer 1:30% 2x SSC, and 30% *in situ* buffer 1:70% 2x SSC. Embryos were then washed twice in 2x SSC/0.1% CHAPS at 65°C for 30 min. and twice in 0.2x SSC/0.1% CHAPS at 65°C for 30 min. Embryos were washed twice in TBST (2.2.5) for 10 min., followed by incubation in 10% FCS/TBST for at least one hour. Preblocked anti-digoxygenin Fab fragments-AP conjugate was prepared as described in section 2.4.8, and incubated with the embryos for 6-24 hours at 4°C with gentle rocking. Embryos were rinsed for 5 min. with TBST before being washed overnight in TBST with gentle rocking. The following day, embryos were washed for 3 x 10 min. in AP buffer (2.2.5). The embryos were gently flushed from the processing baskets into siliconised dishes containing AP buffer. Embryos were subsequently identified and transferred to siliconised welled microscope slides (Crown Scientific) for staining. *In situ* hybridisations were developed in humid chambers by addition of 50-100 µl of *in situ* substrate mix (2.2.5) and incubation in

the dark until purple staining appeared (1-6 hours). The staining reaction was terminated by transferring embryos through several rinses of PBT/1 mM EDTA. The stain was fixed by incubation in 4% (w/v) PFA / 0.2% (v/v) gluteraldehyde in PBT for 20 min. and embryos were stored in 0.4% (w/v) PFA/PBT. Embryos were viewed on a Nikon Eclipse TE300 inverted microscope using Hoffmann modulation contrast system optics, and photographed with Ektachrome 100 ASA slide film (Kodak).

2.3.5.6.4 Embryonic tissue whole mount *in situ* hybridisation

16.5 d.p.c embryos were dissected from BALB/c female mice. Embryonic tissues were removed and rinsed in PBS and then 4% PFA/PBS prior to fixation with 4% PFA/PBS for 16 hours at 4°C. Tissues were rinsed in PBT (0.1% Tween 20/PBS) solution and dehydrated on ice in sequential washes of ice cold 25%, 50% and 75% methanol/PBS for 5 minutes each and finally in 100% methanol prior to storage at -20°C. Whole mount *in situ* hybridization was carried out as described in 2.3.6.3.

2.3.5.6.5 Embedding and Sectioning of Tissues From Whole Mount *in situ* Hybridisation

Fixed tissues that had been used for whole mount *in situ* hybridisation were dehydrated with 1 mL volumes in 100% in sterile glass tubes for 10 minutes. Methanol was then removed and the tissue was rinsed in isopropanol and then left in fresh isopropanol for 15 minutes. This process was repeated twice using histoclear. Tissues were then placed into sterile tissue baskets and incubated with fresh melted wax for 15 minutes. This process was repeated three times with the tissues being put under vacuum for the last wax incubation. Tissues were then placed into heat sterilized and RNase free moulds and cast with fresh melted wax. Blocks were allowed to set at room temparature and stored at –20°C until required.

Embedded tissues were then cut into 7 μm thick sections using the Leica microtome. Sections were then floated on water at 45°C in order to be placed onto silanised microscope slides. Slides were dried at 37°C and stored at room temperature. Slides were then incubated two times for 5 minutes in histoclear and then re-hydrated through a 100%, 70% methanol series for two by 5 minutes washes each. Tissues were counterstained with methyl green for 1 minute and then washed in H_2O several times before being dehydrated with 2 by 5 minute incubations in 70% and 100% methanol then two by 5 minute incubations with histoclear and mounted with DePex and a glass cover slip.

2.3.5.6.6 Whole mount *in situ* hybridization of cultured embryonic mouse kidneys

Metanephric kidney rudiments were isolated from 12.5 d.p.c. embryos of out bred CD1 mice. Explants were cultured for 3 days at 37°C in 5 % CO_2 on 1 µm polycarbonate trans well filters (Corning) in MEM supplemented with 10% FCS. Whole mount *in situ* hybridization was then carried out as described in (Piper *et al.*, 2000) using DIG-labelled sense and antisense CRTR-1 specific riboprobes generated as described in 2.3.6.1.

2.3.5.6.7 Riboprobe transcription for radiolabelled *in situ* hybridisation

RNA probes for *in situ* hybridisation were generated by *in vitro* transcription of linearised plasmid, clone 1.2.8, containing the CRTR-1 cDNA encompassing the 460 bp probe used for RNAse protection analysis. Clone 1.2.8 linearised with BamHI was transcribed with T3 RNA polymerase generating the sense transcript while clone 1.2.8 digested with *Xho*I was transcribed with T7 polymerase generating the antisense transcript. Transcription reactions were set up using approximately 1 µg of linearised template incubated with 0.6 µl of 10 mM UTP, 1 µl 10 x transcription buffer, 2 µl rNTPs (2.5 mM each ATP, CTP, GTP), 0.5 µl RNasin, and 100µCi (10µl) dried ^{33}P-UTP, and 1 µl of the appropriate RNA polymerase, with sterile MQ H_2O to 10 µl at 37°C for 1 hour. An additional 1 µl of enzyme was then added before incubation for a further hour. 1µl of DNase 1 (Rnase free) was added then incubated for a further 15 minutes at 37°C. 40 µl of sterile MQ H_2O was added then the probe spun through a Sephadex G 50 column for 5 minutes at 3000 rpm. 2-5µl of the collected probe was run on a 6 % acrylamide gel to check that the transcripts were full length with 15 µl formamide load buffer after denaturing for 5 minutes at 95°C then cooling on ice. 1µl of probe was counted in a liquid scintillation counter in two ml of Optiphase scintillation fluid. Remaining probe was stored at -20°C with addition of 2.5 µl of ribonucleoside vanadyl complex and 1 µl of 0.5 M ßME.

2.3.5.6.8 Embedding and Sectioning of Tissues For Radiolabelled *in situ* hybridisation

Tissues were dissected from BALB/c female mice and directly placed into PBS at 4°C. The tissues were then fixed in 4% paraformaldehyde in PBS at 4°C for 20 hours. Tissues were subsequently washed in PBS at 4°C prior to transfer to 70% ethanol. Tissues were either stored at 4°C in 70% ethanol or used directly for embedding.

Prior to embedding, tissues were dehydrated by 70% ethanol for 1 hour, 80% ethanol for 30 minutes, 2x 95% ethanol for 45 minutes, 2x 30 minutes and 1x 45 minutes in crude ethanol, 1x 30 minutes and 2x 45 minutes in safesolvent. Dehydrated tissues were then washed in molten wax 2x for 45 minutes at 60°C followed by a 15-20 minute molten wax wash at 60°C with a vacuum of 15-20 inches mercury. Finally tissues were embedded into pouring moulds and placed at 4°C.

Embedded tissues were then cut into 7µm thick sections using the Leica microtome. Sections were then floated on water at 45°C in order to be placed onto silanised microscope slides. Slides were dried at 37°C and stored at room temperature until use.

2.3.5.6.9 Silane treatment of microscope slides

Single frosted microscope slides were placed in a stainless steel rack. The slides were briefly cleaned in 0.1% hypochlorite. The slides were rinsed in MQ water briefly, soaked in 10% nitric acid for 1 hour, dipped in acetone and the left in 2% TESPA (3 amino propyl triethoxy silane, Sigma, in acetone) for about 10 minutes. The slides were dipped in acetone and MQ water prior to drying of the slides. Slides were made RNase free by baking at 180°C for 6 hours.

2.3.5.6.10 Radiolabelled *in situ* hybridisation on paraffin embedded tissue sections.

Tissues fixed in 4 % paraformaldehyde in PBS were embedded in paraffin and cut as 7 µm thick sections and placed onto silane treated slides, dried at 42°C overnight the stored at room temperature until use. For use in an assay, slides were heated to 55°C for 30 minutes to attach tissue sections to the slides. Slides were cooled to room temperature then dewaxed in two changes of Safesolvent (Histoclear) for 5 minutes each, then hydrated through two minute changes of 100%, 80% and 70% ethanol, then one minute in 2x SSPE. Sections were then acetylated in 0.1 M triethanolamine pH 8 supplemented with 1: 400 acetic anhydride for 10 minutes and washed twice in 2x SSPE for 2 minutes then 10 minutes. Slides were air dried and regions on the slide were separated from each other using nail polish. Sections were prehybridised at 52°C for 1 hour with 36 µl of prehybridisation solution under parafilm coverslips in humid chambers. Slides were washed in 2x SSPE twice for two minutes each, then air dried and brought to 52°C, before incubation with 36µl of hybridisation solution under glass coverslips in humid chambers overnight at 52°C.

Slides were washed in 50% formamide, 2x SSPE, 0.1% SDS, 10mM ßME at 52°C for 5 minutes then in the same wash buffer excluding SDS for 5 minutes at 52°C then 10 minutes at 60°C. Slides were then washed in two changes of 2x SSPE for five minutes each at room temperature before being air dried and warmed to 37°C. Sections were then RNase treated to remove non-specifically hybridised probe for 30 minutes at 37°C, with 0.1 mg/ml RNase A, 0.05 U/µl RNase T1 in 2x SSPE under parafilm coverslips in humid chambers. RNase treated sections were washed twice in 2x SSPE for 5 minutes each at 52°C, the once in 0.1x SSPE at 60°C for 30 minutes. Sections were dehydrated for two minutes each in 70 %, 80%, and 100% ethanol, then air dried at room temperature before dipping in a solution of 50% Ilford L4 emulsion, 49% MQ H_2O, 1% glycerol, and air drying for two hours at room temperature. Slides were then packed into air tight, light tight dry boxes and exposed at 4°C.

2.3.5.6.11 Animal manipulations

All procedures involving animals were carried out with the approval of the University of Adelaide and Institute of Medical and Veterinary Science animal ethics committees.

2.3.6 Protein Methods

2.3.6.1 Polyclonal antibody generation

2.3.6.1.1 Generation of GST-CRTR-1 peptide constructs

In order to generate anti-CRTR-1 polyclonal antibodies the 510bp *Eco*RI fragment of clone 8.2.1 (encoding sequence at the 5′ end of *Crtr-1* excluding the most N terminal 7 amino acid) was excised by *Eco*RI digestion, blunt ended, gel purified and ligated into the *Eco*RI digested and blunt ended pGEX-2T such that *Crtr-1* is cloned in frame with the GST (figure 3.13). generating the plasmid pGEX-2T-CRTR-1(8-177) (figure 3.14). The correct orientation was determined by restriction digest with *Bam*HI and *Pst*I. BDT automated sequencing using the RACE1 primer was used to sequence across the multiple cloning site confirming that the cloned insert was in-frame confirm.

2.3.6.1.2Preparation of competent BL21 cells

50 ml of LB (2.2.9) was inoculated with a single BL21 *E. coli* colony and cultured to an OD_{600} of 0.6. The cells were harvested by centrifugation at 4000 r.p.m. for 10 minutes

at 4°C (SS-34 rotor, Sorvall RC-5). Cell pellets were resuspended in 25 ml of cold 0.1 M $MgCl_2$ and pelleted by centrifugation at 4000 r.p.m. for 10 min. at 4°C. Cells were resuspended in 12.5 ml of cold 0.1 $CaCl_2$ and incubated on ice for 20 min. Cells were harvested by centrifugation at 4000 r.p.m. for 10 min. at 4°C and resuspended in 1.6 ml of cold 0.1 M $CaCl_2$ and 15% (v/v) glycerol. 200 µl aliquots were placed into eppendorf tubes, snap frozen in a dry ice/ethanol bath and stored at -80°C.

2.3.6.1.3 Determination of protein concentration by bradford assay

Samples and standards were performed in triplicate and averages were used in further calculations. 2 µl of BSA standards (1-10 mg/ml) or samples were mixed 200 µl of 1 in 4 diluted Bradford Reagent (BIO-RAD) in a 96-well tray. Absorbance at 505 nm wavelength was measured in a Emax plate reader (Molecular Dynamics). Protein concentrations of samples were determined by calculation from the line of best fit of the standard curve.

2.3.6.1.4 Small scale GST-fusion protein induction

Following transformation of competent BL21 with the appropriate plasmid DNA (2.3.7.1.1), a single transformed colony was picked and grown in 2 ml of LB plus ampicillin (100 µg/ml) overnight. The overnight culture was then sub-cultured 1/100 into 10 ml of fresh LB and ampicillin (100 µg/ml) and grown at 37°C with agitation to an OD_{600} of approximately 0.6. Fusion protein expression was induced by the addition of 0.2 mM IPTG following the removal of a 1 ml pre-induction sample. Incubation continued for 4 hours at 30°C. Cells were harvested by centrifugation and resuspended in 100 µl bacterial lysis buffer (2.2.5). For ease of manipulation, the cell lysates were heated at 100°C for several minutes before transferring 50 µl of bacterial cell lysate into 50 µl of 2x SDS load buffer (2.2.5). This solution was again heated at 100°C for 3 min. before loading 20 µl onto a 12.5% SDS-polyacrylamide gel for electrophoresis (2.3.29).

2.3.6.1.5 Large scale GST fusion protein induction

200 ml of LB (2.2.9) plus ampicillin (100 µg/ml) was inoculated with diluted overnight culture, grown to log phase (OD_{600} = 0.6) and fusion protein expression induced as described in section (2.3.7.1.4). The cells were harvested by centrifugation at 5000 r.p.m. for 10 min. (GSA rotor, Sorvall RC-5) and the cell pellet resuspended in 20 ml of

TBST containing 0.25 mM PMSF. Cells were lysed by 3 rounds of sonication for 30 sec., adding 0.25 mM PMSF between sonications. Cell debris was pelleted by centrifugation at 10000 r.p.m. for 15 min. (SS-34 rotor, Sorvall RC-5) and the supernatant removed. Protein solubility was determined by resuspending the pellet in 1.5 ml of TBS and analysing 10 µl of both resuspended pellet and supernatant in 10 µl of 2x SDS load buffer by SDS-PAGE (2.3.7.4).

2.3.6.1.6 Preparation of GST-fusion protein for immunisation

Insoluble fusion proteins were prepared for immunisation into rabbits using the large scale induction protocol outlined above (2.5.4). Insoluble GST-fusion protein concentration was estimated by analysing several dilutions of the resuspended pellet by SDS-PAGE (2.3.29), along with BSA standards of known concentration (1-10 mg/ml). Approximately 100 µg of fusion protein was then loaded onto 10% SDS-polyacrylamide preparative gels, along with prestained molecular weight standards (2.2.12), and a band excised which corresponded to the correct molecular weight of the fusion protein of interest. Gel bands were then processed serially through 18 to 25 gauge needles in readiness for immunisation.

2.3.6.1.7 Immunisation regime

Preimmune samples (5 ml) were taken from each rabbit prior to immunisation. Approximately 100 µg GST-fusion protein was injected subcutaneously into two Semi lop male rabbits. Heat killed *mycobacteria* and muramyl dipeptide, which is a component of the bacterial cell wall known to activate macrophages, was added at 100µg per rabbit in the primary immunisation as an adjuvant. Boosting injections of approximately 100 µg of protein were carried out on 2 subsequent occasions, 3 weeks apart. Serum samples were taken 14 days following the final booster injection, cleared by centrifugation and stored at 4°C with 0.1% sodium azide. The presence of anti-CRTR-1 antibodies was assayed in COS-1 cells transiently transfected (2.6.7) with CRTR-1 containing expression vectors by immunofluorescence (2.3.31) and western analysis (2.3.30) of COS-1 cell extracts (2.3.27). Once bleed, the final sera collection was cleared and stored at –80°C after being smap frozen.

2.3.6.2 COS-1 cell lysis

24 hours post transfection (2.4.5) $3x10^5$ COS-1 cells, grown in 6 cm petri dishes, were wash in PBS and then harvested using TEN buffer (2.2.5). Cells were transferred to a 1.5 ml eppendorf tube, and pelleted for 30 seconds at 1200 rpm and lysed in 50 µl of Western lysis buffer (2.2.5) at 4°C with rotation for 30 min. Cell debris was removed following centrifugation for 10 min. at 14000 r.p.m. in an eppendorf centrifuge. Supernatants were collected and added to 50 µL 2x SDS-load buffer (2.2.5) and analysed for protein expression by SDS-PAGE (2.3.7.4) and western analysis (2.3.7.5).

2.3.6.3 Co-purification of expressed proteins

$3x10^5$ COS-1 cells, grown in 6 cm petri dishes, were transfected (2.4.5) and cultured for 24 hours prior to being washed in PBS and lysed for Ni-affinity purification by the addition of 1 ml of EDTA-free cell lysis buffer (2.2.5) and incubation on ice for 30 min. Cells were harvested by pipetting up and down across the dish with lysis buffer and transferred into a 1.5 ml eppendorf tube. Cell debris was removed by centrifugation for 10 min. at 14000 r.p.m. in an eppendorf centrifuge, and the supernatant transferred to a new tube. 40 µL of Talon metal affinity beads (20 µL actual beads volume with 20 µL of buffer) pre-equilibrated by washing and storage of beads in EDTA-free cell lysis buffer (2.2.5) was added to each sample and incubated over night with rotation at 4°C. Beads from each sample were pelletted and washed three times in 1 ml volumes of EDTA-free cell lysis buffer without protease inhibitors. Beads were pelleted and resuspended in 30 µl of 2x SDS-load buffer, boiled for 10 min. and analysed by SDS-PAGE (2.3.7.4).

2.3.6.4 SDS-PAGE analysis

SDS-polyacrylamide gels, containing 1x Tris-SDS buffer (2.2.5), 0.1% (w/v) APS and 0.1% (v/v) TEMED, were poured using 0.75-1 mm spacers and allowed to polymerise for approximately 20 min. under a distilled water overlay. After polymerisation, the water was removed and a 4% stacker gel, containing 1x Tris-SDS buffer, 0.1% APS (w/v) and 0.1% (v/v) TEMED was applied. 10 well combs were inserted and the gel left to polymerise. Gels were electrophoresed using a PAGE minigel apparatus (Biorad) in SDS-PAGE buffer (2.2.5) at 100-150 V. Gels were either stained in Coomassie stain (2.2.5) over night, and destained (2.2.5) overnight to visualise the protein bands, or transferred to nitrocellulose by western blot (2.3.6.6).

2.3.6.5 Visualisation of proteins via Coomassie staining

SDS- PAGE gels were stained in Coomassie stain (2.2.5) for 4hrs-overnight at room temperature or for 1hr at 37°C . The gels were then destained in Coomassie destain (2.2.5) solution for several hours until protein bands could be detected.

2.3.6.6 Western analysis

Proteins were transferred from SDS-polyacrylamide gels to nitrocellulose (Protran, Schneider and Schell) in western transfer buffer (2.2.5), using a mini trans-blot electrophoretic transfer cell (Biorad). Filters were blocked by incubation in 5% (w/v) milk powder in PBT overnight at 4°C. An appropriate dilution of primary antibody (2.2.8) in PBT was added to the filter and incubated for 1 hour at room temperature. Filters were washed using 4 x 15 min. washes in PBT before incubation with the appropriately diluted HRP-conjugated secondary antibody (2.2.8) for 1 hour. Following a further 6 x 10 min. washes in PBT, the blot was developed by bathing in enhanced chemiluminescence reagents for 5 min. (SuperSignal™ Substrates, Pearce), drained and exposed on autoradiographic film (Kodak/Fuji) for an appropriate time (1 sec-5 min.). Antibodies were blocked in milk powder (w/v) in PBT as follows:

Goat anti-mouse IgG (whole molecule) - HRP conjugate (1/2000):	1%
Anti Gal4-DBD rabbit polyclonal antibody (1/500)	1.5%
Anti CRTR-1 rabbit polyclonal antibody (1/500)	2%

2.3.6.7 In vitro transcription translation

^{35}S-methionine labeling and In vitro transcription translation of proteins from plasmid DNA was carried out using the TNT Quick coupled transcription/translation system (Promega) following the manufacturers instructions in 20 µL reaction volumes. 1-3 µL samples of reactions were mixed with 2x SDS-load buffer and analysed by SDS-PAGE (2.3.7.4) dried down and visualized by autoradiography.

2.3.6.8 GST-pull down

Glutathione beads were prerpared by washing in water and then two times in MQ H_2O. Beads were resuspended and stored in NTEN buffer (2.2.5). Prior to use beads were pre-blocked against non-specific binding by incubation of beads with 0.1% BSA in NETN buffer for 1 hour at room temperature. 25 µL of the glutathione slurry was incubated with 1

mL of the protein lysate for 30 minutes at room temparature. Beads were then washed three times with NETN. Beads were resuspended in 1 ml of binding buffer and mixed with 2 µL of ^{35}S-methionine labelled IvTT CRTR-1 (3.3.2 and Figure 3.8) for 1hr at room temperature. 20 µL samples of the binding reactions containing bound and unbound IvTT CRTR-1, referred to as the soup samples, were added to 20 µL of 2xSDS-gel loading buffer and retained for PAGE analysis. Beads were extensively washed with NETN buffer (2.2.5, 2.3.6.8) to remove unbound proteins and resuspended in 2xSDS-gel loading buffer (2.2.5). Proteins bound to the beads were separated by 10% SDS-PAGE (2.3.6.4) together with soup samples.

2.3.6.9 Immunohistochemistry

$3x10^5$ COS-1 cells were seeded onto coverslips (Crown Scientific) in 6 well dishes and transiently transfected (2.4.5) with 1 µg of the appropriate mammalian expression plasmid. Cells were washed twice with PBS, and fixed by immersing in methanol for 2 min. at -20°C and re-hydrating for 15 min. in PBS. Cells were incubated for 2 min. in 0.1% Triton X-100 in 80 mM PIPES, 5 mM EDTA and 1 mM $MgCl_2$ at RT, prior to methanol fixation for 3 min.. Primary antibodies were diluted appropriately (2.2.8) in 3% (w/v) BSA/PBT and incubated with the cells for 60 min. at RT. Unbound primary antibody was removed following 3 x 10 min. washes in PBT. Cells were incubated with the appropriate secondary antibodies (2.2.8), diluted 1/1200 in 3% (w/v) BSA/PBT, at RT for 60 min. in the dark, before being washed 3 times in PBT for 5 min. Nuclei were visualised by staining with 0.5 µg/ml Hoechst 33258 Trihydrochloride (BisBenzamide) for 1 min., or 2 µg/ml of propidium iodide for 1 min. before washing twice for 5 min. with PBT. Coverslips were viewed using either a Zeiss Axioplan microscope equipped for 3 channel fluorescence (Zeiss filter sets II, IX, and XV) and photographed with a Zeiss MC 100 camera attachment using 35 mm ektachrome 160T film (Kodak) or a Nikon Eclipse TE300 inverted microscope equipped with a TE-FM Epi-Fluorescence attachment, and photographed with a Nikon F70/F70D camera attachment using ektachrome 100 ASA slide film (Kodak). Images were overlayed using Photoshop (Adobe).

2.4 TISSUE CULTURE METHODS

2.4.1 Cell Lines

Cell lines used during the course of this work were obtained from:

Dr Lindsay Williams, Ludwig Institute, Melbourne, Australia

E14TG2a ES cells	Dr Austin Smith, CGR Edinburgh, UK.
COS-1 cells	ATCC
293T	ATCC
TBV-2 ES cells (Clone WO64C03)	German Gene trap consortium

2.4.2 Solutions

PBS:	136 mM NaCl, 2.6 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4 pH 7.4. Sterilised by autoclaving (20 psi for 25 minutes at 140°C).
PBS/gelatin:	0.2% (w/v) gelatin in PBS
Trypan blue:	0.4 g trypan blue, 0.06 g KH2PO4, in 100 ml MQ H2O
Trypsin:	0.1% trypsin (Difco) and 1 x EDTA Versene buffer solution (CSL). Sterilised by filtration through a 0.2 µM filter (Whatman).
0.1M βME:	100 mM β-mercaptoethanol (Sigma) in 14.1 ml PBS. β-mercaptoethanol/PBS solutions were not kept longer than two weeks.
L-glutamine:	100 mM L-glutamine in PBS.
LIF:	COS cell conditioned medium containing LIF prepared as described by Smith (1991) except that transfections were performed by electroporation.

2.4.3 Media

Incomplete ES cell medium:	85% DMEM medium, 15% FCS (Gibco BRL), 1% L-glutamine, 0.1 mM β-mercaptoethanol/PBS and 1000 units/ml penicillin and streptomycin.
Complete ES cell medium:	Incomplete ES cell medium with 0.1% LIF.
EPL cell medium:	50% ES cell media (with or without LIF) and 50% HepG2 conditioned medium (medium was isolated from HepG2 cells cultured in COS medium for 4-5 days and

supplemented with 0.1 mM β-mercaptoethanol before use).

COS/NIH3T3 cell medium: 90% DMEM medium, 10% FCS, 25 mM HEPES pH 7.5

2.4.4 Maintenance of Cells

ES Cells:

ES cells were maintained on gelatinised 10 cm petri dishes (Corning or Falcon) in complete ES medium at 37°C in 10% CO_2. Cells were harvested by washing in PBS and incubation with trypsin (1 ml) at 37°C for 1 minute prior to being transferred to 9 ml of complete ES cell medium. The cells were centrifuged at 1,200 rpm for 4 minutes, medium aspirated, resuspended in 10 ml complete ES medium, and re-seeded at clonal density (10^6-10^5 cells per plate). Medium was replaced with fresh medium on the second day of the passage. ES cells were re-seeded every 3 - 4 days.

EPL Cells:

ES cells were differentiated into EPL (early primitive ectoderm-like) by the addition of EPL cell media (2.4.3), in the presence or absence of LIF. EPL cells were grown at 37°C in 10% CO_2 and seeded at clonal density (10^6 per plate) in EPL cell medium (with or without LIF) every 2 days as described above for ES cells.

COS-1 and 293T Cells:

Cells were maintained in standard culture media (2.6.5), grown at 37°C in 5% CO_2 and passaged every 3-4 days when cultures had reached near confluence. Cell passaging involved two washes with PBS and then trypsinisation for 5 min. at 37°C. Cells were dislodged from flask, transferred into standard culture media, and spun at 1200 r.p.m. for 2 min. Cell pellets were resuspended in 10 ml of media and re-seeded using 5-20 fold dilutions.

2.4.5 Transient Transfection Of Cells

Effective transfection efficiency was achieved FuGENE™ 6 transfection reagent according to the manufacturer's instructions (Roche).

2.4.6 Freezing And Thawing Of Es Cells

10 cm plates of ES cells were trypsinised (2.4.4) and centrifuged at 1,200 rpm for 4 minutes. The supernatant was aspirated and the cells resuspended in 4 ml of freezing mix (90% FCS, 10% DMSO). 500 µL was placed in each freezing vial (Nunc) and stored overnight at -80°C. Vials were placed in liquid nitrogen for long term storage.

Freezing vials were thawed in a 37°C water bath and the cells were seeded onto 60 mm plates containing 4 ml of ES cell complete medium. The next day the cells were washed in PBS and the medium replaced.

2.4.7 Cell Counting

Cells were trypsinised as in section 2.4.4. 100 µl of cell suspension was mixed with 900 µl of Trypan Blue. 50 µl of this mixture placed on a haemocytometer and unstained cells were scored under light field microscopy at 20 x magnification. The number of cells per ml were determined by multiplying the number cells counted by a factor of 10^4.

2.4.8 Histochemical Staining For β-Galactosidase Activity

β-galactosidase activity was detected as described in (Winegar and Lutze, 1990). Briefly, cell culture medium was aspirated and the cells were washed three times in PBS before fixing for 5 minutes in 2% gluteraldehyde/PBS. The cells were again washed three times in PBS before the addition of staining solution (0.45 mM $K_3Fe(CN)_6$, 0.45 mM $K_4Fe(CN)_6$, 1mM $MgCl_2$, 400 µg/ml BCIG). Plates were incubated for 24 hours at 37°C.

2.4.9 Luciferase Assay

Cell transfections were carried out with FuGENE™ 6 transfection reagent (2.4.5). COS-1, 293T and ES cells were plated out in 24 well trays (Falcon) at densities of 35,000, 50,000 and 100,000 cells/well respectively, and transfected the following day with 200ng/well Gal4-DBD plasmid + 200ng/well pTk-MH100x4-LUC (Kang *et al.*, 1993) + 50ng/well pRLTK (Promega), or 200ng/well pGalO.CRTR-1 + 200ng/well pHRE-Luc (37, 38) + 50ng/well pRLTK. Control transfections carried out with 200ng/well pTk-MH100x4-LUC or 200ng/well pHRE-Luc + 50ng/well pRLTK were made up to 450ng total/well with pBluescript-KS^+ carrier DNA. Luciferase activity was assayed on a TD-20/20 Luminometer (Turner Designs) using the Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's instructions. Firefly luciferase expression was normalised against Renilla luciferase. Experiments were repeated in triplicate.

2.4.10 Alkaline Phosphatase Assay

Alkaline phosphatase assays were carried out using an alkaline phosphatase assay kit (2.2.3, Sigma) following the manufacturers instructions.

2.4.11 Karyotyping Of Es Cells

ES cells were seeded at a density of approximately $4x10^6$ cells in a 10 cm tissue culture dish and cultured over night. The next day, culture media was removed and replaced with fresh media for 3 hours to induce rapid growth. This media was then replaced with media containing 10 µg/ml colchicine and cultured for 1 hour. This media was then removed and kept while the cells were harvested by trypsinisation and placed back into the colchicine containing media. Cells were pelleted by centrifugation at 1200 rpm for 2 minutes. The pellet was resuspended in 9 ml of 0.075 M KCl for 30 minutes at room temperature. 1 ml of fixative (methanol : acetic acid, 3:1). Cells were re-pelletted by centrifugation and washed 3 times in 10 ml of fixative. On the last wash, approximately 0.5 ml of fixative was retained to resuspend the cell pellet and stored at $-20^{\circ}C$.

Glass slides were washed in 100% methanol and air dried. Several drops of cells were dropped onto glass slides held at a 45° angle 40-50 cm away from the pipette tip. Slides were allowed to air-dry standing up. Slides were then stained with giemsa stain (Gibco) diluted 1/20 with MQ H_2O for 5 minutes, rinsed with MQ H_2O, air-dried and mounted with DePex and a cover slide.

2.5 PHOSPHORIMAGING, AUTORADIOGRAPHY AND DATA MANIPULATION

Filters and gels were exposed to storage Phosphorimager screens (Molecular Dynamics) for 1 - 7 days and analysed using a Phosphorimager (Molecular Dynamics) and the ImageQuant software package. Filters and gels were also exposed to BioMax X-ray film for 1 - 3 days. Autoradiographs and gel photographs were scanned using a scanjet 7400c scanner (Hewlett packard), slide scanner and Photoshop (Adobe). Images were manipulated in Canvas 6 (Deneba) and printed using a HP LaserJet 5 MP or Epson Stylus Photo 870 printer. Gels and filters exposed to phosphorimager screens (Molecular Dynamics) were processed using a Molecular Dynamics phosphorimager, running the ImageQuant software package. Quantitation by volume integration was carried out using ImageQuant. Phosphorimager files were manipulated using the Adobe Photoshop™ and

PowerPoint programs. Autoradiographic film and photographic slides were scanned and manipulated using Adobe Photoshop™.

2.6 CONTAINMENT FACILITIES

Manipulations that involved organisms containing recombinant DNA were carried out in accordance with the regulations and approval of the Australian Academy of Science Committee on Recombinant DNA and the University Council of the University of Adelaide.

CHAPTER 3

Isolation of new CP2 family members: Identification of the full-length *CRTR-1* cDNA and novel *CP2* and *NF2d9* transcripts.

3.1 INTRODUCTION

CRTR-1 was identified as a gene differentially expressed between ES and EPL cells *in vitro* and demonstrated to be expressed in the pluripotent cells of the early mouse embryo with expression down regulated upon differentiation of these cells (Pelton *et al.*, 2002). Further analysis has demonstrated *CRTR-1* expression to be spatially and temporally regulated with specific *CRTR-1* expression detected in the epithelial cells lining the embryonic and adult kidney DCTs (Rodda *et al.*, 2001). Alignment of the available CRTR-1 amino acid sequence has identified CRTR-1 as a likely member of the CP2 family of transcription factors which are generally described as ubiquitously expressed (Jane *et al.*, 1995; Murata *et al.*, 1998; Sueyoshi *et al.*, 1995; Swendeman *et al.*, 1994). Regulated expression therefore distinguishes CRTR-1 from CP2 family members and defines it as a protein warranting further investigation. Characterisation of CRTR-1 at the molecular level with regards to confirmation of CRTR-1 as a member of the CP2 family and the ability of CRTR-1 to function as a transcription factor requires isolation of the full CRTR-1 ORF and isolation of ORFs encoding mouse CP2 family members CP2 and NF2d9.

3.2 ISOLATION OF *CRTR-1* cDNAs.

Alignment of the predicted CRTR-1 amino acid sequence and CP2 suggested that the CRTR-1 amino acid sequence was incomplete at the 5' end and in particular did not contain an initiating methionine, a 5' untranslated region, a consensus Kozak sequence or the amino terminus of the CRTR-1 protein. To obtain the full-length cDNA sequence for *CRTR-1*, the Lambda Zap II ES D3 cDNA library (Clontech) used previously (Rodda, 1998) was re-screened for *CRTR-1* specific cDNAs from the 5' end of the transcript. This library was constructed by RT-PCR on RNA prepared from undifferentiated D3 ES cells using both poly(dT) and random primers (Clontech).

3.2.1 Design and Production of *CRTR-1* Specific Probes.

A 500 bp *Eco*R1 fragment (E2) from the clone 8.2.1 (Figure 3.1a), representing the most 5' sequence of *CRTR-1* obtained from the previous library screen, was used to probe 1st and 2nd round phage lifts from the fifth cDNA library screen (2.3.3.1). In order to restrict phage clones to those most likely to contain new 5′ sequences, a 92bp *Eco*RI/ *Pst*I fragment (ED2) (Figure 3.1a) from the most 5′ region of the E2 probe was used to screen positive clones at the Southern analysis stage (2.3.1.7).

3.2.2 First and Second Round Isolation of *CRTR-1* Positive Plaques.

450,000 first round library phage plaques were screened at a density of approximately 45,000 plaques per 150 mm diameter petri dish (2.3.3.1). Duplicate lifts were probed with α^{32}P-dATP oligo-labelled E2 probe (2.3.1.8) resulting in the identification of 13 duplicate positive plaques. All duplicate positive plaques were picked for second round amplification and re-selection, and were plated out at approximately 200 plaques per 90 mm diameter dish (1/100 dilution of the first round plaque eluate) (2.3.3.1). Second round duplicate lifts were taken and probed with α^{32}P-dATP oligo-labelled E2 probe (2.3.3.1, 2.3.1.8). Of the original 13 double positive plaques, seven plates produced multiple duplicate positive plaques. A third round screen was not necessary as the phage density of second round plaques enabled single plaques to be isolated. 10 individual double positive plaques from 7 independent first round plaques were picked and high titre stocks were generated (2.3.3.2).

Bacterial colonies carrying the pBluescript SK plasmid with putative 5' *CRTR-1* cDNA fragments were obtained by the λ ZAP excision process (2.3.3.4). Colonies from each of the 10 Zapped clones were grown and plasmids were purified (2.3.2.5) and digested (2.3.1.1) with *Eco*RI to release the cloned cDNA fragments. Digests were separated on a 1% TAE gel (2.3.1.2) (Figure 3.2a), transferred to a nytran filter and probed with a α^{32}P-

FIGURE 3.1

Schematic diagram showing *CRTR-1* transcript, cDNA library clones and probes.

A) Schematic representation of the relative positions and sizes of four *CRTR-1* cDNA clones, termed q1, 1.2, 6A and 8.2.1, isolated previously from the λ ZAPII D3 ES cell cDNA library. Probes used for the isolation of clones are indicated together with the restriction sites used for their excision. Also indicated are the location, size and restriction sites for excision of the E2 and ED2 probes.

B) Schematic representation of the relative size and position of clones 2a and 8b isolated from the fifth λ ZAPII D3 ES cell cDNA library screen. Size and restriction sites are indicated for clone 8b sub-clones. Arrowheads depict the direction of sequencing reactions.

RI; *Eco*RI

A; *Acc*I

N; *Nco*I

P; *Pst*I

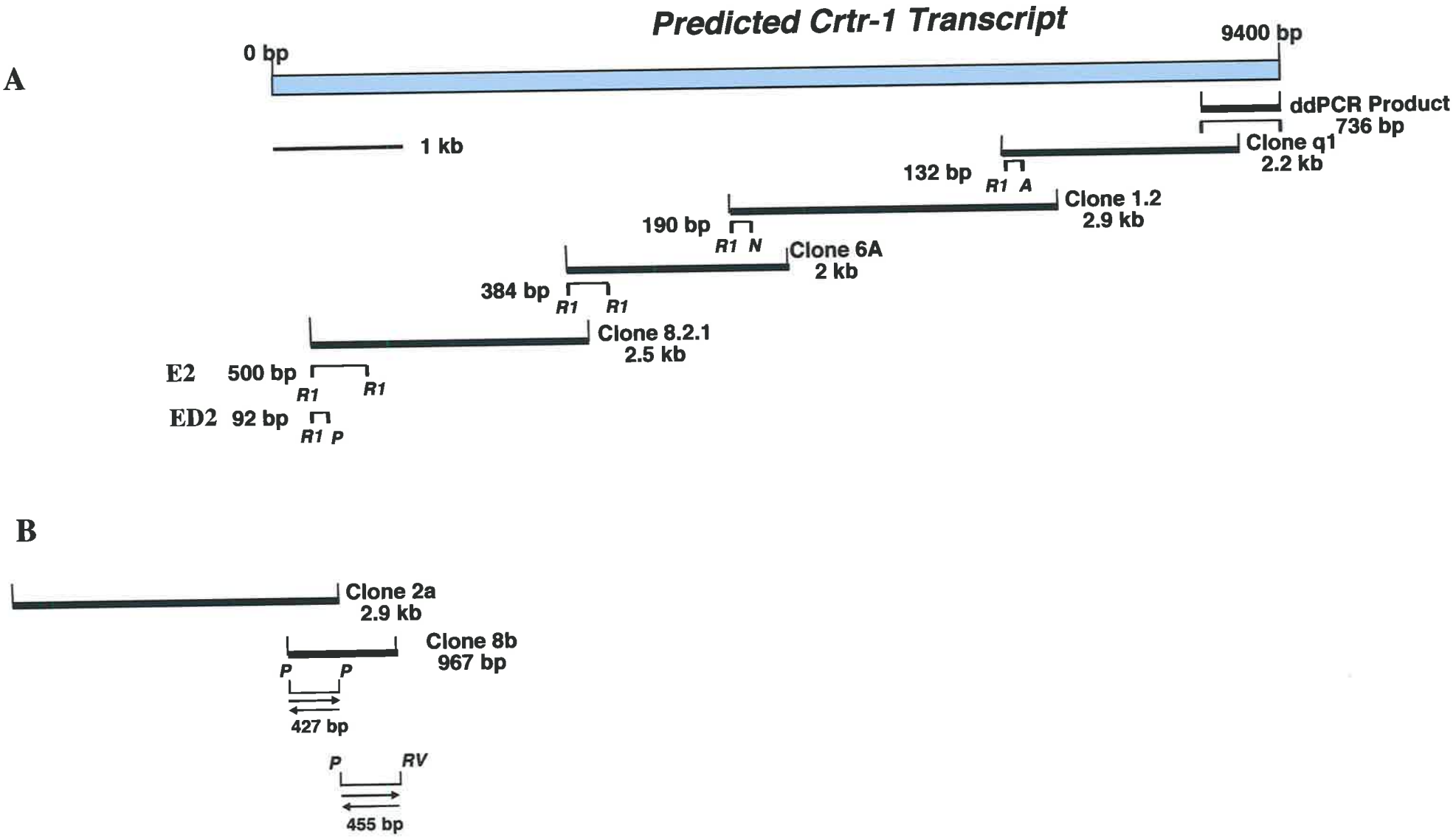

A
Predicted Crtr-1 Transcript
0 bp
9400 bp
ddPCR Product
736 bp
1 kb
Clone q1
2.2 kb
132 bp
R1 A
Clone 1.2
2.9 kb
190 bp
R1 N
Clone 6A
2 kb
384 bp
R1 R1
Clone 8.2.1
2.5 kb
E2 500 bp
R1 R1
ED2 92 bp
R1 P
B
Clone 2a
2.9 kb
Clone 8b
967 bp
P P
427 bp
P RV
455 bp

FIGURE 3.2

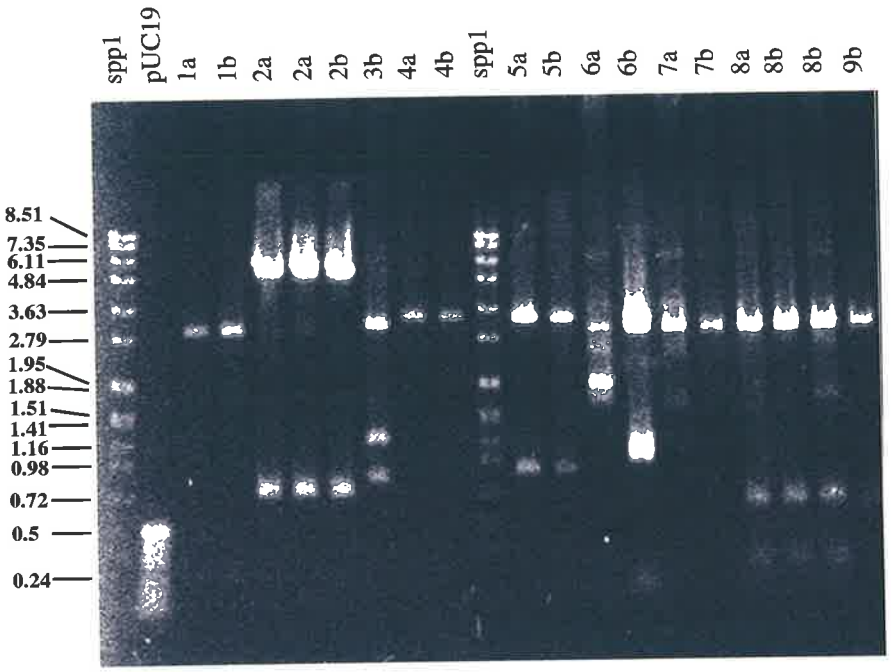
Southern analysis of cDNA clones isolated in the fifth library screen.

A) Plasmid pBluescript SK containing cDNA inserts were isolated from high titre λ phage stocks using the Zap method (2.3.3.4). DNA was digested with *Eco*RI to release the cDNA insert and run on a 1% TAE agarose and visualised by exposure to UV light after staining with ethidium bromide (2.3.1.2). 1-9, independent zapped cDNA clones; a-d, independent isolates of each zapped clone. Markers; 500 ng of *Eco*RI digested Spp1 phage DNA and 500 ng of *Hpa*II digested pUC19 DNA markers (2.2.13).

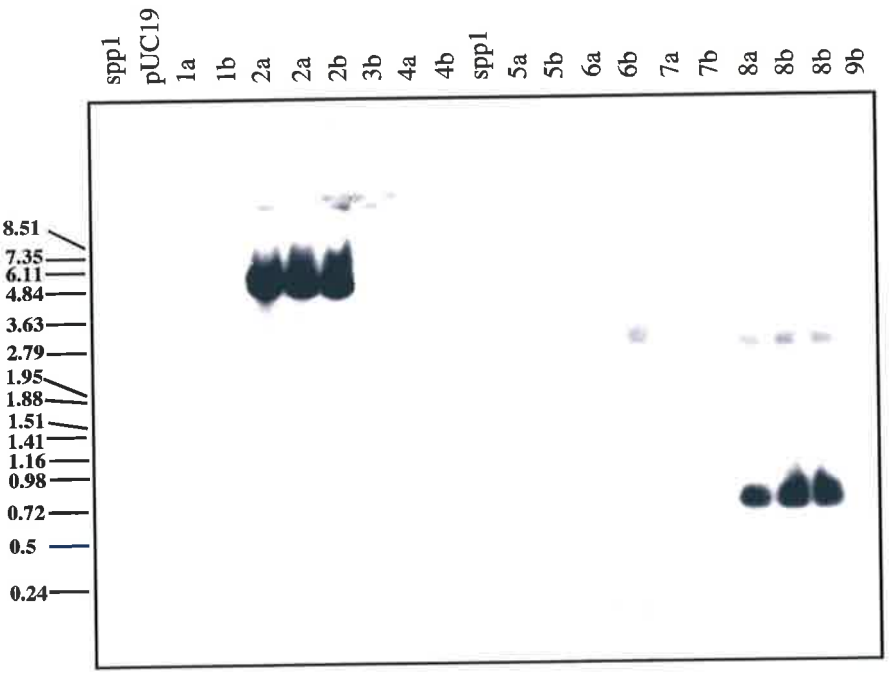
B) The agarose gel **(A)** was transferred to a nytran filter for Southern analysis (2.3.1.7) using an α^{32}P-dATP-oligo labelled 92bp ED2 probe.

Work done in association with Michaela Scherer.

A


spp1
pUC19
1a
1b
2a
2a
2b
3b
4a
4b
spp1
5a
5b
6a
6b
7a
7b
8a
8b
8b
9b
8.51
7.35
6.11
4.84
3.63
2.79
1.95
1.88
1.51
1.41
1.16
0.98
0.72
0.5
0.24

B


spp1
pUC19
1a
1b
2a
2a
2b
3b
4a
4b
spp1
5a
5b
6a
6b
7a
7b
8a
8b
8b
9b
8.51
7.35
6.11
4.84
3.63
2.79
1.95
1.88
1.51
1.41
1.16
0.98
0.72
0.5
0.24

dATP oligo-labelled ED2 probe (2.3.1.7-9). Two clones, 2a and 8b, hybridised with the 92 bp most 5′ region of the known *CRTR-1* cDNA sequence (Figure 3.2b).

Clone 8b contained a cDNA insert that was excised from pBluescript SK by *Eco*RI digestion as fragments of 630 bp, hybridising to the ED2 probe, and 240 bp (figure 3.2a and b). The cDNA insert in clone 2a was not excised by *Eco*RI digestion, suggesting that this clone had lost an *Eco*RI site during library construction, but was estimated to be approximately 2.9 kb. Hybridisation to the ED2 probe confirmed that clones 8b and 2a contain 5' *CRTR-1* sequence.

3.2.3 Sequence Analysis of 5′ *CRTR-1* Clones.

Sequencing of clone 2a and clone 8b was carried out using big dye terminator (BDT) automated sequencing (2.3.1.14) with primers USP and RSP (2.2.7.2) and confirmed that both clones contained the ED2 probe region.

Clone 2a contained an overlapping 380 bp of known *CRTR-1* sequence (figure 3.1b). However, sequence alignment was lost before the 5′ end of known sequence indicating that the additional 5' sequence was artefactual, resulting from ligation of independent cDNA transcripts to the 5′ region of *CRTR-1* during library construction. This clone was discarded. Clone 8b contained 772 bp of known *CRTR-1* sequence extending 110bp further 5′ than the known 5′ boundary (figure 3.1b). *Pst*I/*Eco*RV digestion of clone 8b produced a *Pst*I fragment of 427 bp and a 455 bp *Pst*I/*Eco*RV fragment (figure 3.1b) that were sub-cloned into pBluescript KS and sequenced in both directions.

Clone 8b sequence contained an ATG in frame with the deduced *CRTR-1* open reading frame (ORF). No further in frame ATG codons or stop codons were present in the additional 5' sequence. The ATG codon was preceded by a consensus Kozak sequence (figure 3.5) (Kozak 1987) and was located in a position consistent with other CP2 family

members. This ATG codon was therefore considered to represent the start codon, defining the CRTR-1 ORF as 1446 bp in length.

3.3 CONFIRMATION OF *CRTR-1* cDNAs.

It has been shown previously (Dr. Shiwani Sharma, pers comm), and in this thesis (3.2.3), that the ES D3 Zap II cDNA library used for the isolation of *CRTR-1* cDNAs contains clones comprising more than one ligated cDNA. It was therefore necessary to confirm the additional 110 bp 5′ sequence of the *CRTR-1* transcript. This was achieved by RT PCR, *in vitro* transcription translation (IvTT) and RACE-PCR.

3.3.1 RT-PCR.

Platinum Taq PCR (2.3.1.12) using primers SR1 and SR2 (2.2.7.2) was carried out on poly-T primed cDNA (2.3.1.11) generated from total ES cell RNA (2.3.5.1). The PCR reaction yielded a 1475 bp PCR product consistent with that predicted for the *CRTR-1* cDNA sequence (Figure 3.3b). This confirmed that the additional sequence isolated from clone 8b was contiguous with the *CRTR-1* cDNA and presentation of the 5' end of the *CRTR-1* transcript. This product was gel purified (2.3.1.3) and cloned into pGEM-T easy (Promega), generating pGEM-T-*CRTR-1*. pGEM-T-*CRTR-1* was sequenced with USP, RSP (2.2.7.1) and *CRTR-1* specific primers (2.2.7.2) in both directions using BDT automated sequencing (2.3.1.14), confirming the sequence of the *CRTR-1* ORF obtained from clones 8b and 8.2.1.

3.3.2 *In Vitro* Transcription Translation.

Expression of a full-length CRTR-1 protein of the expected molecular weight was confirmed by IvTT (2.3.6.7) using 1µg of pGEM-T-*CRTR-1*. 1 µl and 3 µL samples of IvTT CRTR-1 were separated by 10% SDS-PAGE (2.3.6.4), revealing a single band at approximately 64 kDa (Figure 3.3c, lanes 1 and 2). The discrepancy in the size of the

FIGURE 3.3

Isolation of full length *CRTR-1* ORF by RT-PCR and confirmation by IvTT.

A) Schematic figure representing the position of PCR primers SR1 and SR2 on the *CRTR-1* ORF sequence.

B) Platinum Taq PCR (2.3.1.12) using the primers SR1 with SR2 (2.2.7.2) was carried out on poly-T primed cDNA generated from ES cell total RNA (2.3.5.1). PCR reactions were separated on a 1% TAE agarose gel and visualised by exposure to UV light after ethidium staining (2.3.1.2). SR1 and SR2, PCR using individual primers with cDNA; cDNA, PCR with cDNA alone; actin, PCR using actin specific primers, actinA and actinB (2.2.7.2) with cDNA. Markers; together with 500 ng of *Eco*RI digested Spp1 phage DNA (2.2.13).

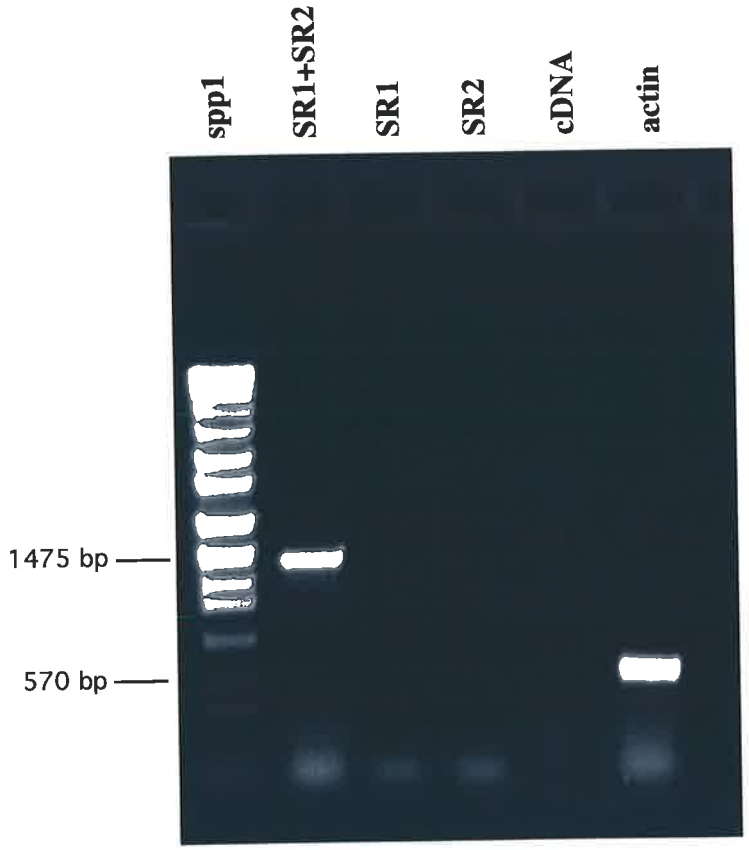
PCR Parameters:
Step 1: 94°C for 3 minutes
Step 2: 94°C for 30 seconds
Step 3: 50°C for 30 seconds
Step 4: 72°C for 2 minutes
Step 5: cycle back to step 2, 24 times.
Step 6: 72°C for 4 minutes
Step 7: 4°C
Step 8: End

C) CRTR-1 protein was synthesised and ^{35}S-methionine labelled by IvTT 9 (2.3.6.7) using 1 µg of pGEMT-CRTR-1. 1 µl and 3 µL samples of IvTT CRTR-1 were separated by 10% SDS-PAGE (2.3.6.4), dried down and visualised by autoradiography. –ve, 3 µL of a negative IvTT control carried out with 1 µg of pGEM-T easy. Markers; 10 µL of Benchmark pre-stained protein ladder (2.2.14).

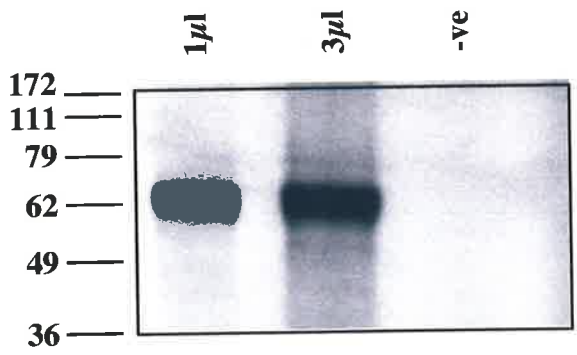
A


ATG
TGA
Crtr-1 open reading frame, 1446bp
5' UTR
3' UTR
Primer SR1
Primer SR2

B


spp1
SR1+SR2
SR1
SR2
cDNA
actin
1475 bp
570 bp

C


1µl
3µl
-ve
172
111
79
62
49
36

observed CRTR-1 band versus the expected band size is consistent with the reported behaviour of CP2, predicted to be approximately 58 kDa, which runs as two bands of 64-66 kDa when analysed by SDS-PAGE (Lim *et al* 1992). This result confirmed that pGEM-T-*CRTR-1* contained the *CRTR-1* ORF encoding a full-length protein of the appropriate size.

3.3.3 RACE PCR

5' RACE PCR (Clontech, 2.3.1.15) was used to isolate the 5'UTR of *CRTR-1*. 5' RACE PCR was carried out on RACE ready cDNA generated from total adult mouse kidney RNA (2.3.1.15) using the RACE 1 primer (Figure 3.4a) (2.2.7.2) and yielded two bands, 582 bp and 398 bp (Figure 3.4b). Both bands were excised (2.3.1.3), cloned into pGEM-T easy and sequenced by BDT automated sequencing (2.3.1.14) using primers USP and RSP (2.2.7.1). The 398 bp product contained *CRTR-1* sequence that extended 5' from the RACE primer and terminated at nucleotide 178 of the known sequence (Figure 3.4c). The function and importance of this sequence was unknown and not analysed further. The sequence of the 582 bp product aligned exactly to the 5' end of the nucleic acid sequence of clone 8b confirming the isolation of the complete 5' *CRTR-1* UTR. Determination of the complete 5' *CRTR-1* UTR by two independent methods, cDNA library screening and RACE-PCR, confirmed the successful isolation of a full length *CRTR-1* cDNA sequence.

3.4 FULL LENGTH *CRTR-1* cDNA AND AMINO ACID SEQUENCES

The full *CRTR-1* nucleic acid and amino acid sequence is presented in Figure 3.5. The 9405 bp *CRTR-1* transcript contained a 91 bp 5' UTR, a 1446 bp open reading frame extending from nucleotide 92 to nucleotide 1537 and a 3' untranslated region of 7868 bp. The ATG codon is preceded by a consensus Kozak sequence (Figure 3.5) (Kozak 1987). A poly(A) tail 25 bp downstream of a consensus polyadenylation signal (AATAAA) was identified and is consistent with the typical polyadenylation signal 10-30 bp upstream of

FIGURE 3.4

Confirmation of the *CRTR-1* 5'UTR by RACE-PCR.

A) Schematic representation of the position of RACE1 primer on the *CRTR-1* ORF sequence.

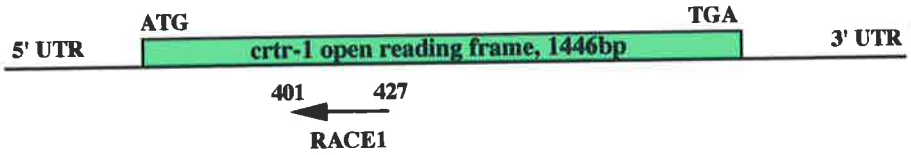
B) RACE PCR (2.3.1.15) with the RACE1 primer on RACE ready cDNA generated from adult mouse kidney total RNA (2.3.5.2). PCR reactions were separated on a 1% TAE agarose gel together with 500 ng of *Eco*RI digested Spp1 phage DNA (2.2.13) and visualised by exposure to UV light after ethidium staining.

PCR Parameters:

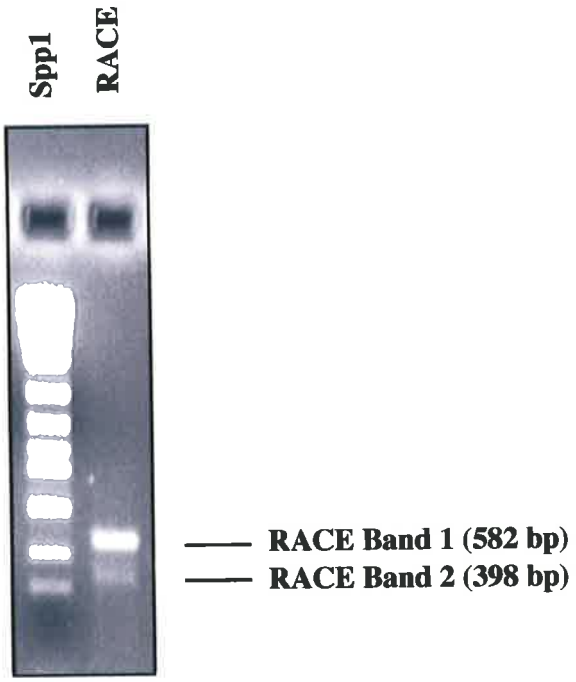
Step 1: 94°C for 3 minutes
Step 2: 94°C for 30 seconds
Step 3: 50°C for 30 seconds
Step 4: 72°C for 2 minutes
Step 5: cycle back to step 2, 24 times.
Step 6: 72°C for 4 minutes
Step 7: 4°C
Step 8: End

C) Nucleotide sequence alignment of cloned RACE Band 1 and RACE Band 2 **(B)**. Alignment carried out using ClustalW (2.3.1.16). The underlined region is the 5' ligated universal primer (2.2.7.1).

A


ATG
TGA
5' UTR
crtr-1 open reading frame, 1446bp
3' UTR
401
427
RACE1

B


Spp1
RACE
RACE Band 1 (582 bp)
RACE Band 2 (398 bp)

C

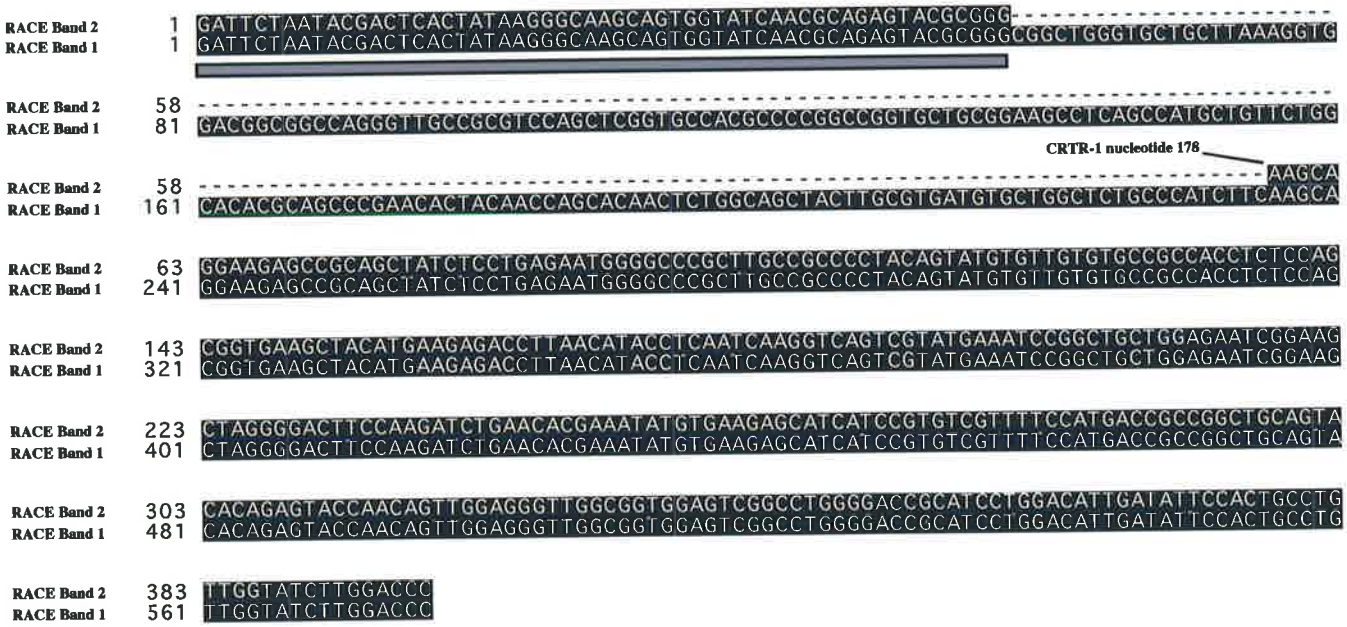
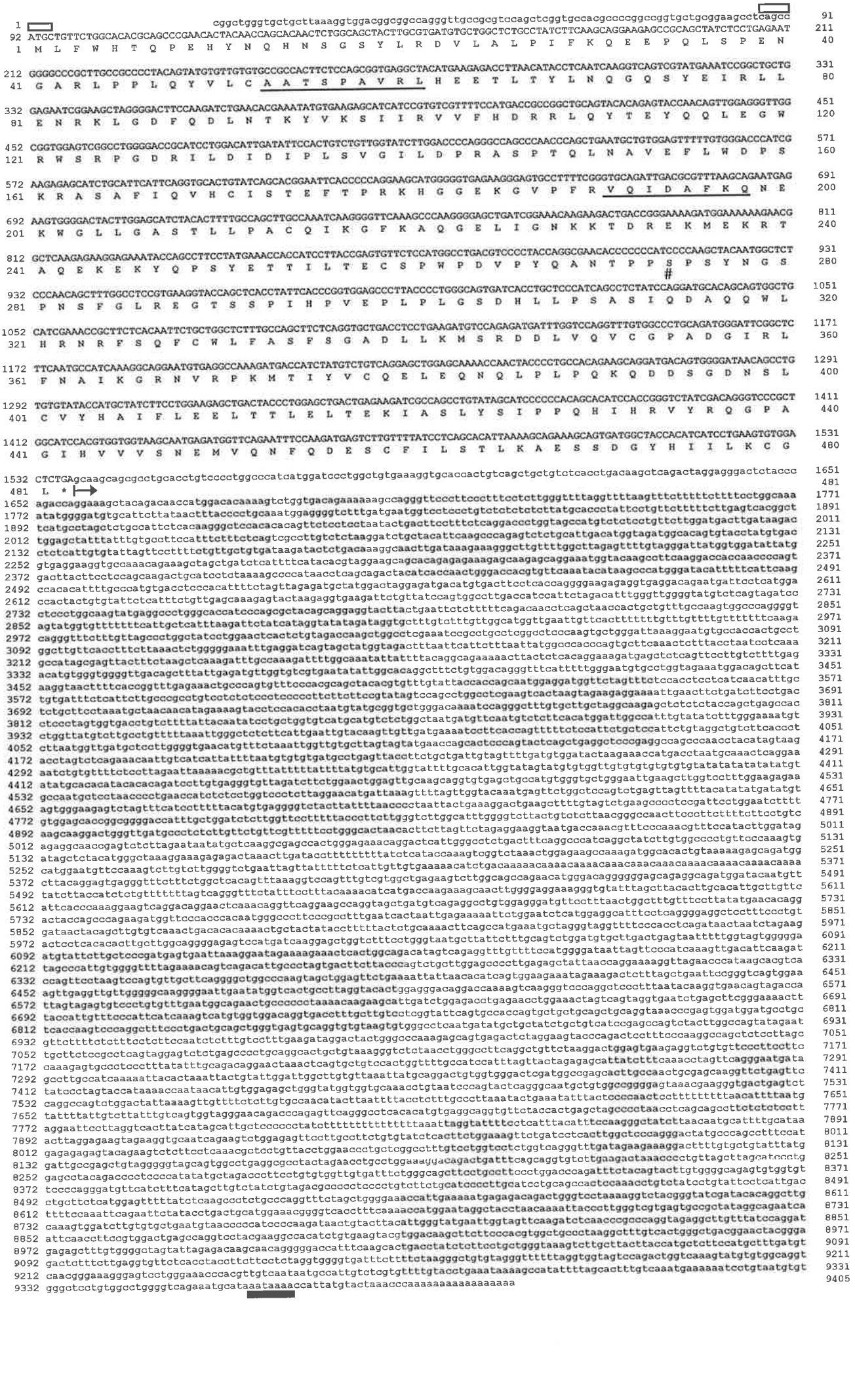

RACE Band 2 1 GATTCTAATACGACTCACTATAAGGGCAAGCAGTGGTATCAACGCAGAGTACGCGGG
RACE Band 1 1 GATTCTAATACGACTCACTATAAGGGCAAGCAGTGGTATCAACGCAGAGTACGCGGGCGGCTGGGTGCTGCTTAAAGGTG
RACE Band 2 58
RACE Band 1 81 GACGGCGGCCAGGGTTGCCGCGTCCAGCTCGGTGCCACGCCCGGCCGGTGCTGCGGAAGCCTCAGCCATGCTGTTCTGG
CRTR-1 nucleotide 178
RACE Band 2 58 AAGCA
RACE Band 1 161 CACACGCAGCCCGAACACTACAACCAGCACAACTCTGGCAGCTACTTGCGTGATGTGCTGGCTCTGCCCATCTTCAAGCA
RACE Band 2 63 GGAAGAGCCGCAGCTATCTCCTGAGAATGGGGCCCGCTTGCCGCCCCTACAGTATGTGTTGTGTGCCGCCACCTCTCCAG
RACE Band 1 241 GGAAGAGCCGCAGCTATCTCCTGAGAATGGGGCCCGCTTGCCGCCCCTACAGTATGTGTTGTGTGCCGCCACCTCTCCAG
RACE Band 2 143 CGGTGAAGCTACATGAAGAGACCTTAACATACCTCAATCAAGGTCAGTCGTATGAAATCCGGCTGCTGGAGAATCGGAAG
RACE Band 1 321 CGGTGAAGCTACATGAAGAGACCTTAACATACCTCAATCAAGGTCAGTCGTATGAAATCCGGCTGCTGGAGAATCGGAAG
RACE Band 2 223 CTAGGGGACTTCCAAGATCTGAACACGAAATATGTGAAGAGCATCATCCGTGTCGTTTTCCATGACCGCCGGCTGCAGTA
RACE Band 1 401 CTAGGGGACTTCCAAGATCTGAACACGAAATATGTGAAGAGCATCATCCGTGTCGTTTTCCATGACCGCCGGCTGCAGTA
RACE Band 2 303 CACAGAGTACCAACAGTTGGAGGGTTGGCGGTGGAGTCGGCCTGGGGACCGCATCCTGGACATTGATATTCCACTGCCTG
RACE Band 1 481 CACAGAGTACCAACAGTTGGAGGGTTGGCGGTGGAGTCGGCCTGGGGACCGCATCCTGGACATTGATATTCCACTGCCTG
RACE Band 2 383 TTGGTATCTTGGACCC
RACE Band 1 561 TTGGTATCTTGGACCC

FIGURE 3.5

Full-length *CRTR-1* cDNA and predicted amino acid sequence.

The complied *CRTR-1* sequence was generated from the ddPCR product and five overlapping cDNA library clones isolated from the l ZAP II D3 ES cell cDNA library. The predicted amino acid sequence is shown by capital single letter amino acid symbols below the ORF nucleotide sequence shown in upper case. The consensus Kozack sequence, CCPuCCATGG, is indicated by an open box. Thin underlined amino acids represent putative tyrosine kinase phosphorylation sites identified by a MOTIF search (2.5). The stop codon is indicated by (*) and the beginning of the 3'UTR by an arrow. The polyadenylation signal is shown by thick underline. #; conserved serine residue shown for LBP-1c to be phosphorylated.



the poly(A) tail (39). The predicted ORF encodes a protein of 481 amino acids. No other significant open reading frames were identified.

3.5 CRTR-1 SEQUENCE COMPARISONS.

Comparison of the predicted CRTR-1 amino acid sequence with entries in the BlastP protein sequence database (2.3.1.16) revealed considerable sequence similarity to proteins the CP2 family (Table 1). Figure 3.6 shows a multiple sequence alignment of reported CP2 family members with CRTR-1. Included in Table 1 and Figure 3.6 are the mouse family members CP2 and NF2d9, human family members LBP-1a, LBP-1b, LBP-1c, LBP-1d and LBP-9 and the DNA binding domain of *GRH*. Conservation between CRTR-1 and the other mammalian proteins was extensive and included 72% identity to the LBP-1c DNA binding domain (amino acids 45-366) (Figure 3.7a) and 67% identity to the LBP-1c oligomerisation domain (amino acids 248-403) (Figure 3.7b). Conservation of these regions is characteristic of CP2 family members, defining CRTR-1 as a novel CP2 family member, and suggests that CRTR-1 will be able to bind DNA and form homomeric and heteromeric protein complexes in a fashion analogous to other CP2 family members. Amino acids 1-47 and 381-401 of CRTR-1 were not homologous to other CP2 family members except LBP-9, which showed 97% identity across both regions (Figure 3.6 and 3.8). The 51 amino acid deletion at position 189 specific to LBP-1d and the 36 amino acid insertion (amino acids 274-312) specific to LBP-1b were not found in CRTR-1 (Figure 3.6).

The CP2 family member that showed the greatest identity to CRTR-1 was the human protein LBP-9. CRTR-1 showed extensive alignment across the full length of LBP-9, including the N-terminal amino acids not conserved in CP2 and other family members. This suggests that LBP-9 may be the human homologue of CRTR-1. Similarity between

TABLE 3.1: Percentage identity and similarity of CRTR-1 to other proteins from GenBank.

Name	Origin	% Identity	% Similarity	Reference
LBP-9	Human	88	91	9
CP2	Mouse	68	79	2
LBP-1c	Human	67	79	2
NF2d9	Mouse	62	78	4
LBP-1a	Human	61	76	8
LBP-1d	Human	60	71	8
LBP-1b	Human	57	71	8
grh	*Drosophila*	24*	40*	40
CG11867	*Drosophila*	44	59	–

* Percentage identity and similarity is over amino acids 632-865 of grh

FIGURE 3.6

Multiple amino acid sequence alignment of CRTR-1.

Reported members of the CP2 family of transcription factors and the DNA binding domain (amino acids 632-865) of GRH were aligned using ClustalW (2.3.1.16). Dark shading indicates conservation of identical amino acids while lighter shading indicates conservation of similar amino acids as defined by default parameters of Thompson *et al* (1994).

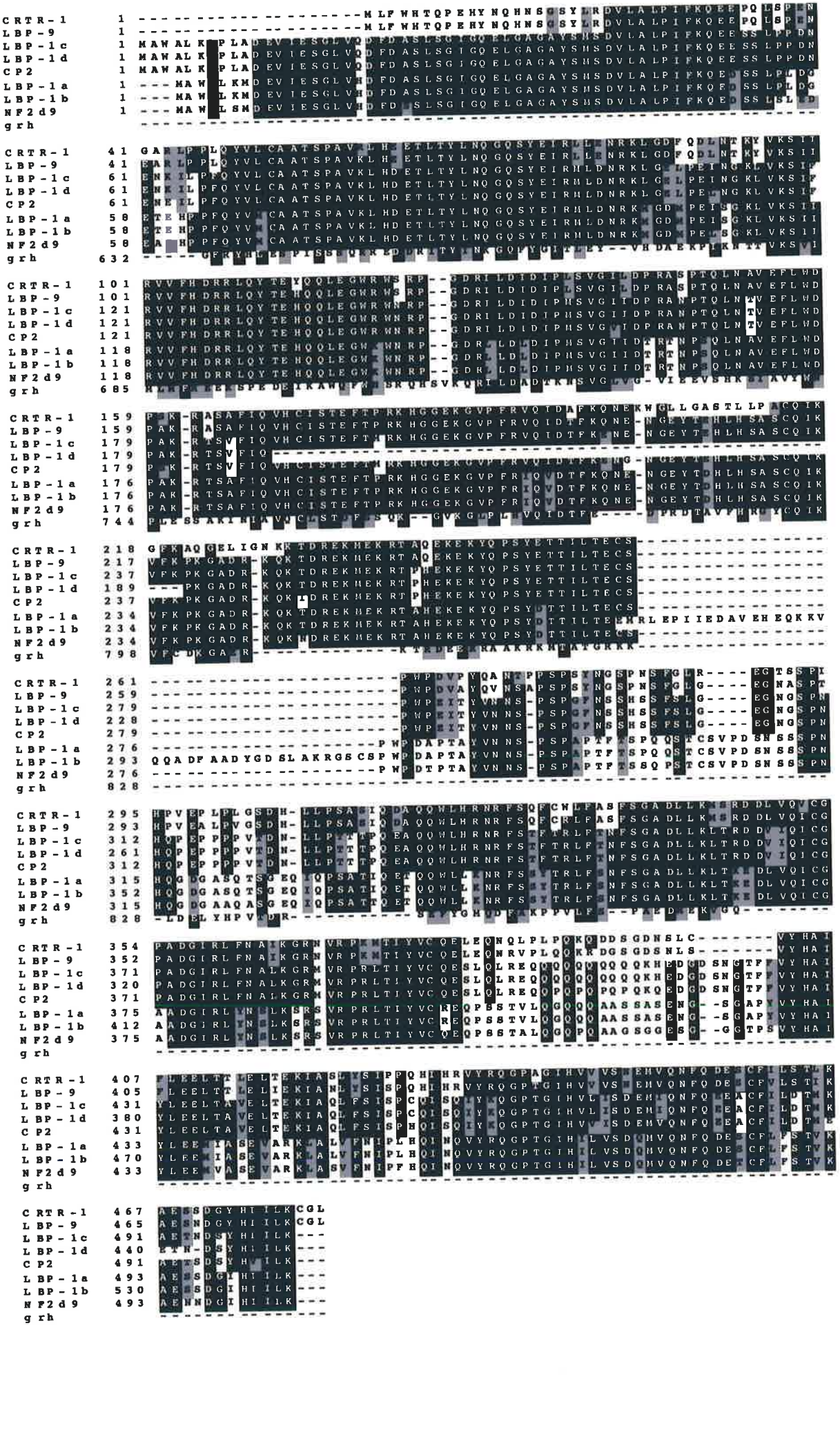

CRTR-1
LBP-9
LBP-1c
LBP-1d
CP2
LBP-1a
LBP-1b
NF2d9
grh

FIGURE 3.7

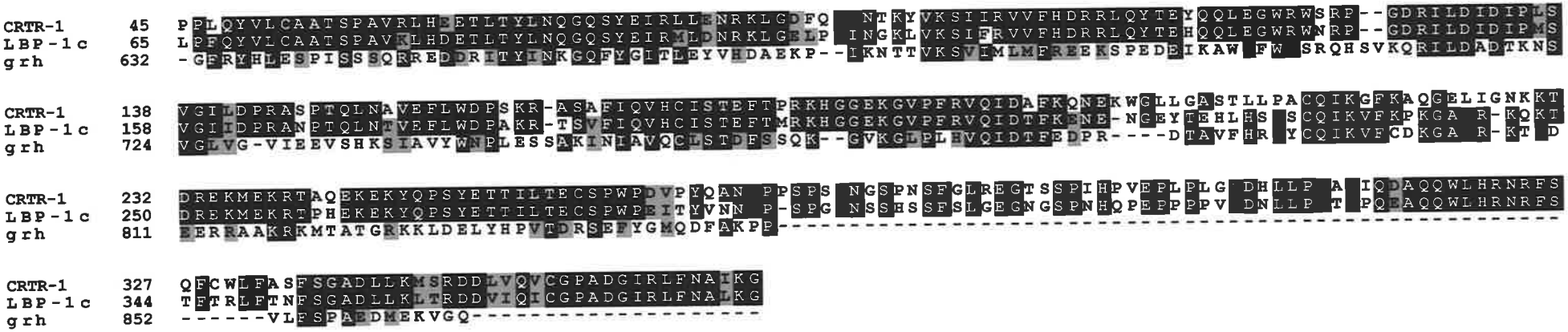
CRTR-1 contains conserved DNA binding and oligomerisation domains.

A) Alignment of CRTR-1 amino acids 45-366 with the analogous region of LBP-1c, amino acids 65-383, demonstrated to act as the minimal DNA binding region and the DNA binding domain of GRH, amino acids 632-865.

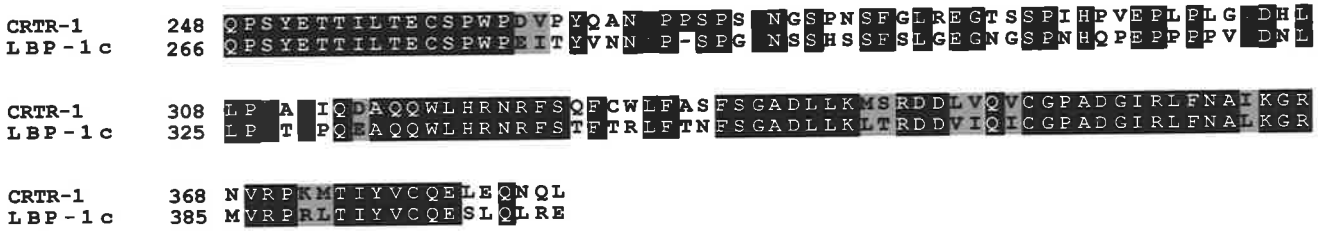
B) Alignment of CRTR-1 amino acids 248-386 with the analogous region of LBP-1c, amino acids 266-403, demonstrated to act as the minimal oligomerisation region.

Details as described in Figure 3.6.

A


CRTR-1 45
LBP-1c 65
grh 632
CRTR-1 138
LBP-1c 158
grh 724
CRTR-1 232
LBP-1c 250
grh 811
CRTR-1 327
LBP-1c 344
grh 852

B


CRTR-1 248
LBP-1c 266
CRTR-1 308
LBP-1c 325
CRTR-1 368
LBP-1c 385

CRTR-1 and LBP-9 at the nucleotide level did not extend into the reported 5' UTR and incomplete 3' UTR of LBP-9.

The predicted CRTR-1 amino acid sequence was entered into a MOTIF (2.3.1.1.6) search for common and well characterised protein motifs and domains at ExPasy using the PROSITE database (2.3.1.16). CRTR-1 contains two tyrosine kinase phosphorylation consensus sites positioned between amino acids 53-61 and amino acids 191-198 (Figure 3.5) which were both conserved in CP2 family members. Serine 291, the site of MAPK phosphorylation in CP2 (Volker *et al.*, 1997), which is conserved in all CP2 family members, was also present in CRTR-1 suggesting post-translational modification by phosphorylation of this serine residue (Figure 3.5). The serine, threonine and proline (S-T-P) rich region (Lim *et al.*, 1992) in CP2 was also conserved in CRTR-1 between amino acids 233-388. However, the predicted transactivation domain (Lim *et al.*, 1992) C-terminal to the SPXX motifs in CP2 and LBP-1c was not conserved in CRTR-1 or LBP-9 (summarised in Figure 3.8).

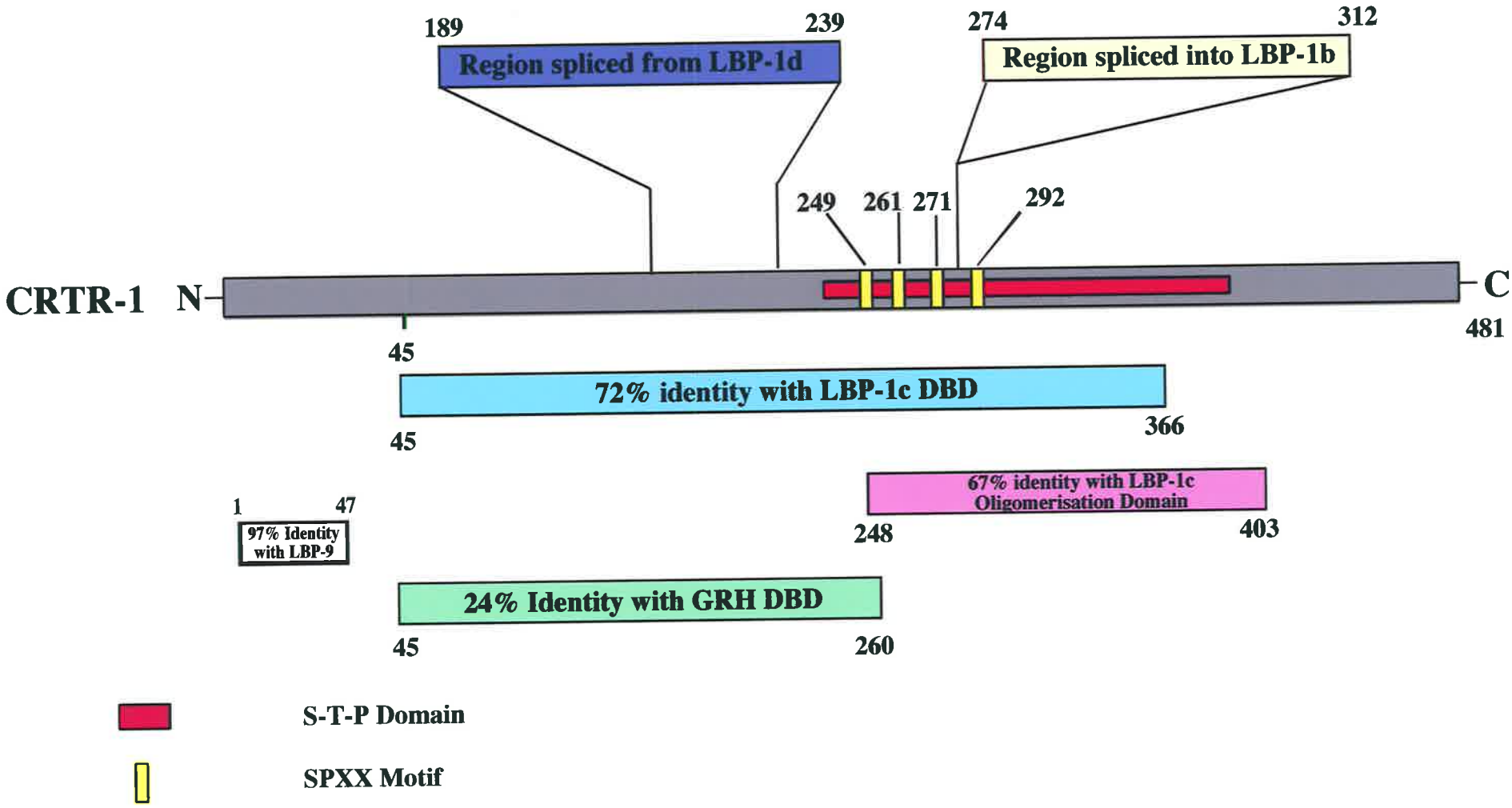
3.6 ISOLATION OF *CP2* AND *NF2d9* OPEN READING FRAMES AND IDENTIFICATION OF NOVEL TRANSCRIPTS.

Amino acid sequence conservation defines CRTR-1 as a novel member of the CP2 family and suggests that CRTR-1 will be able to bind a CP2 consensus DNA response element and form heteromeric protein complexes with other CP2 family members. Investigation of CRTR-1 molecular interactions at this level required isolation of cDNAs encoding the ORFs for CP2 and NF2d9, the other mouse members of the CP2 family. Regions encompassing amino acid 260 to the C-terminal end of LBP-1c and amino acid 247 to the C-terminal end of LBP-1a, referred to as the dimerisation domain (DD), have been used previously for the analysis of CP2 family protein/protein interactions (Tuckfield *et al.*, 2002b; Wilanowski *et al.*, 2002; Zhou *et al.*, 2000).

FIGURE 3.8

Schematic representation of the CRTR-1 ORF.

Schematic summary of conserved regions in CRTR-1 functionally important in LBP-1c (14) and conserved between members of the CP2 family of transcription factors. CRTR-1, amino acids 45-366, shares 72% identity with the LBP-1c DNA binding domain (DBD) and an overlapping region, amino acids 45-260, which is 24% identical to the GRH DBD. CRTR-1 also shares 67% identity with the LBP-1c oligomerisation domain and contains an S-T-P domain and SPXX motif conserved with other CP2 family members. The N-terminal 47 amino acids of CRTR-1 shared identity (97%) only with LBP-9. The 51 amino acid deletion at position 189, specific to LBP-1d, and the 37 amino acid insert at position 274, specific to LBP-1b, were not found in CRTR-1.


189
239
Region spliced from LBP-1d
274
312
Region spliced into LBP-1b
249
261
271
292
CRTR-1
N
C
481
45
72% identity with LBP-1c DBD
45
366
67% identity with LBP-1c Oligomerisation Domain
248
403
1
47
97% Identity with LBP-9
24% Identity with GRH DBD
45
260
S-T-P Domain
SPXX Motif

Platinum Taq PCR (2.3.1.12) was used to isolate cDNAs encoding full length CP2 and NF2d9 as well as CP2 amino acids 260-501 and NF2d9 amino acids 247-504, referred to as CP2-DD and NF2d9-DD respectively. PCR was carried out on a yeast-2-hybrid adult mouse testis cDNA library (Clontech, 2.3.4.1) using primer pairs CP2SR1/CP2SR2 and CP2SR3/CP2SR2 for isolation of CP2 and CP2-DD (2.2.7.2) (Figure 3.9a) and on poly-T primed cDNA (2.3.1.11) generated from adult mouse kidney total RNA (2.3.5.2) with primer pairs NF2d9SR1/NF2d9SR2 and NF2d9SR3/NF2d9SR2 (2.2.7.2) (Figure 3.9c) for isolation of NF2d9 and NF2d9-DD. In addition to the expected PCR products of approximately 1500 bp for full length CP2 and NF2d9 and approximately 760 bp and 780 bp for CP2-DD and NF2d9-DD additional lower abundance products of approximately 120 bp greater in size were observed (Figure 3.9b and d). All PCR products were excised (2.3.1.3), cloned into pGEM-T easy and sequenced using BDT automated sequencing (2.3.1.14) with primers USP and RSP (2.2.7.1).

In each case the smaller product showed 100% sequence alignment with the published CP2 and NF2d9 sequences (Lim *et al.*, 1992; Sueyoshi *et al.*, 1995). These clones are referred to as pGEM-T-CP2, pGEM-T-CP2-DD, pGEM-T-NF2d9 and pGEM-T-NF2d9-DD. The higher bands contained inserts of 132 nucleotides located between nucleotide 1152 and 1153 of the CP2 ORF sequence (Lim *et al.*, 1992) and 108 nucleotides located between nucleotide 821 and 822 of the NF2d9 ORF sequence (Sueyoshi *et al.*, 1995). These clones are referred to as pGEM-T-altCP2, pGEM-T-altCP2-DD, pGEM-T-altNF2d9 and pGEM-T-altNF2d9-DD.

3.7 VALIDATION AND SEQUENCE ANALYSIS OF *altNF2d9* AND *altCP2*.

The existence of altNF2d9 and altCP2 transcripts within cells was validated using techniques which enable simultaneous assessment of message identity and quantitative or semi-quantitative assessment of expression within various mouse tissues.

FIGURE 3.9

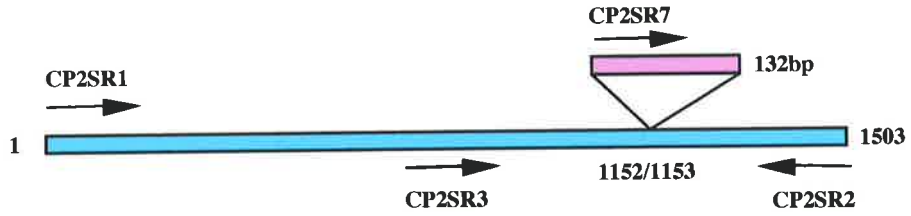
PCR analysis of *CP2* and *NF2d9* transcripts.

Schematic figure representing the position of PCR primers CP2SR1, CP2SR2, CP2SR3 and CP2SR7 on the *CP2* and *altCP2* cDNAs **(A)** and PCR primers NF2d9SR1, NF2d9SR2, NF2d9SR3 and NF2d9SR7 on the *NF2d9* and *altNF2d9* cDNAs **(C)**. Arrowheads indicate the primer direction 5'-3'.

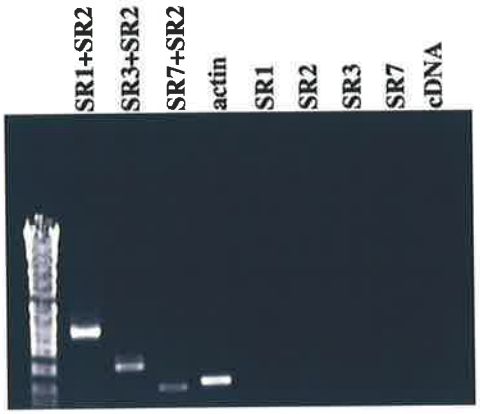
Platinum Taq PCR (2.3.1.12), using the primes indicated was carried out on a mouse testis yeast-2-hybrid cDNA library (Clontech) **(B)**, poly-T primed cDNA generated from mouse adult kidney **(D)**, and mouse ES cell total RNA **(E)**. PCR reactions were separated on a 1% TAE agarose gel and visualised by exposure to UV light after ethidium staining. cDNA, PCR with cDNA alone; actin, PCR using actin specific primers, actinA and actinB (2.2.7.2) with cDNA. Markers, 500 ng of *Eco*RI digested Spp1 phage DNA (2.2.13)

PCR Parameters:
Step 1: 94°C for 3 minutes
Step 2: 94°C for 30 seconds
Step 3: 50°C for 30 seconds
Step 4: 72°C for 2 minutes
Step 5: cycle back to step 2, 24 times.
Step 6: 72°C for 4 minutes
Step 7: 4°C
Step 8: End

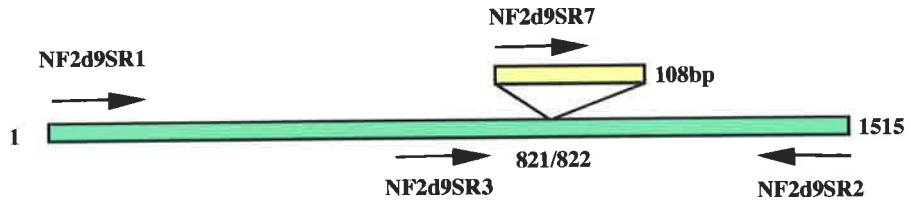
A


CP2SR7
132bp
CP2SR1
1
1503
1152/1153
CP2SR3
CP2SR2

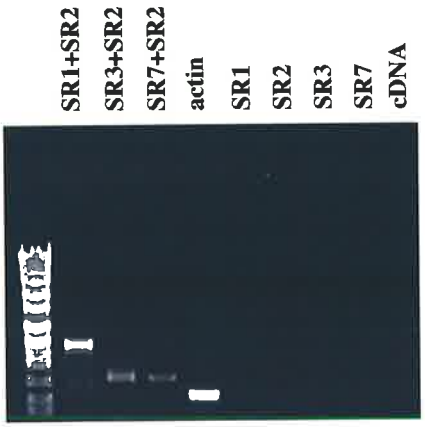
B


SR1+SR2
SR3+SR2
SR7+SR2
actin
SR1
SR2
SR3
SR7
cDNA

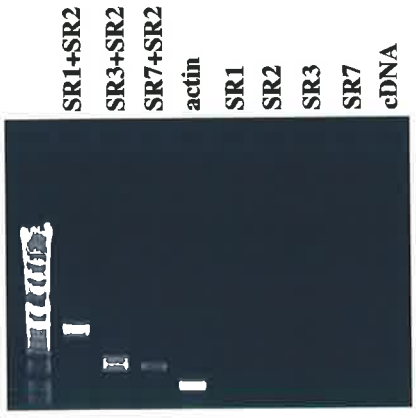
C


NF2d9SR7
NF2d9SR1
108bp
1
1515
821/822
NF2d9SR3
NF2d9SR2

D


SR1+SR2
SR3+SR2
SR7+SR2
actin
SR1
SR2
SR3
SR7
cDNA

E


SR1+SR2
SR3+SR2
SR7+SR2
actin
SR1
SR2
SR3
SR7
cDNA

3.7.1 Validation Of *altNF2d9* By RT-PCR and Ribonuclease Protection.

Platinum Taq PCR (2.3.1.12) was carried out on poly-T primed cDNA (2.3.1.11) generated from mouse adult kidney (2.3.5.2) using primers NF2d9SR7, specific for the 5' end of the additional nucleotide sequences, in conjunction with NF2d9SR2 (2.2.7.2) and poly-T primed cDNA (2.3.1.11) generated from ES cell total RNA (2.3.5.1) using primer pairs NF2d9SR1/NF2d9SR2, NF2d9SR3/NF2d9SR2 and NF2d9SR7/NF2d9SR2 (2.2.7.2). PCR reactions (Figure 3.9e) yielded doublet bands for primer sets NF2d9SR1/NF2d9SR2 and NF2d9SR3/NF2d9SR2 consistent with the size of bands predicted for NF2d9 and altNF2d9 (Figure 3.9d). PCR (2.3.1.12) using kidney (Figure 3.9d) and ES cell (Figure 3.9e) cDNA with primer set NF2d9SR7/NF2d9SR2 produced a single band of the expected size (790bp) for altNF2d9 in mouse kidney and ES cells. All observed bands were isolated and cloned into pGEM-T easy for subsequent sequencing (2.3.1.14) to confirm their identity. These results demonstrate that the isolated sequence is contiguous with the 3' end of the transcript and therefore confirms its existence in both cultured cells and mouse tissues.

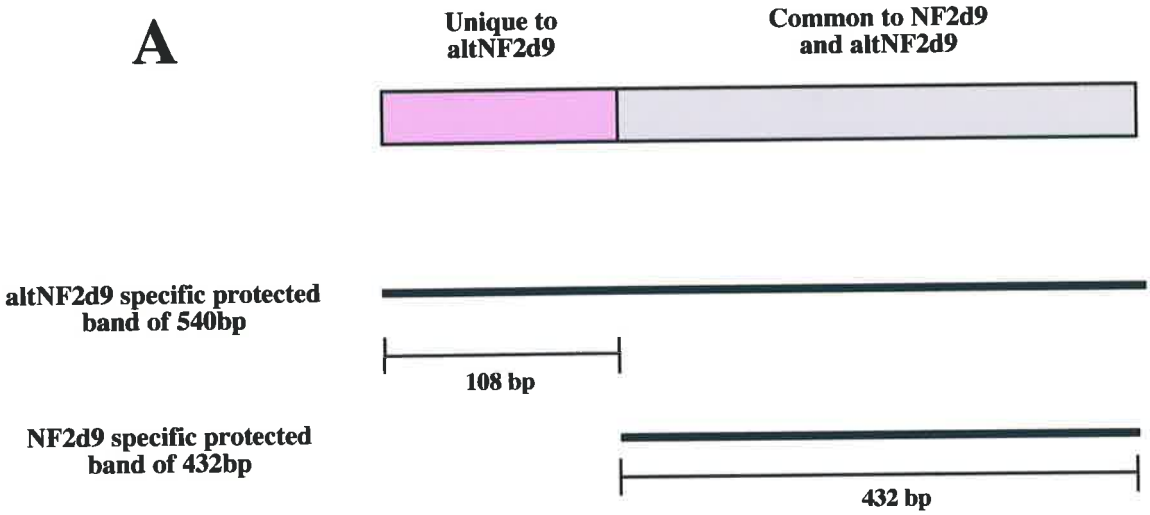
For ribonuclease protection, Platinum Taq PCR (2.3.1.12) was used to isolate a fragment of altNF2d9 that encompassed the inserted sequence and the region 3' to the insert (Figure 3.10a). pGEM-T-altNF2d9(247-504) was used as a PCR template with primers NF2d9SR7 and NF2d9SR8 (2.2.7.2). A single band at 540 bp was observed, cloned into pGEM-T easy, excised by *Not*I digestion (2.3.1.1) and cloned into *Not*I, CIP treated (2.3.1.5) pBluescript SK, generating the clone paltNF2d9-RP. paltNF2d9-RP was linearised with *Sal*I (2.3.1.1) and used for transcription of an α^{32}P-rUTP labelled anti-sense riboprobe using T7 RNA polymerase (2.3.5.4.1). Ribonuclease protection analysis (2.3.5.4.2) using 10 µg of total RNA isolated from mouse ES cells and adult mouse kidney (2.3.5.2) is presented in Figure 3.10b. Two bands were protected in both ES cell and kidney

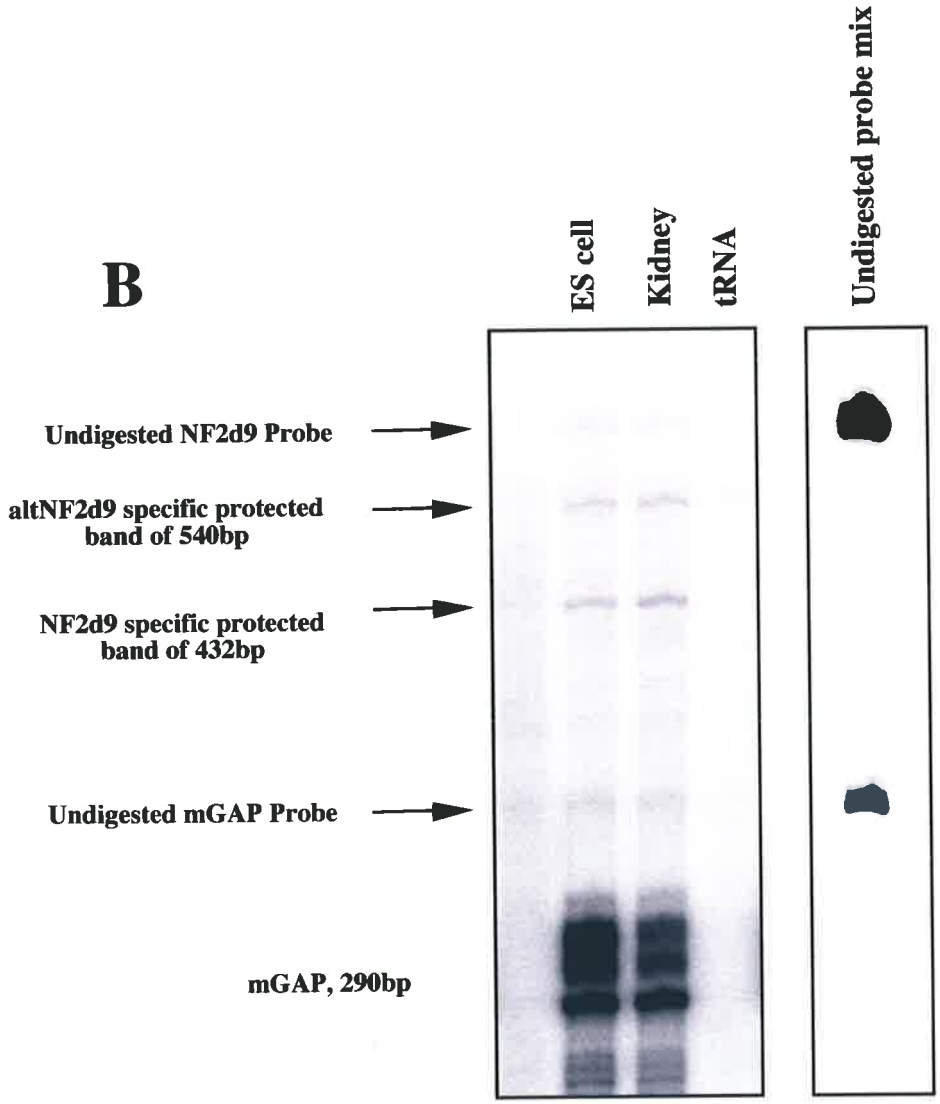
FIGURE 3.10

Validation of altNF2d9 by ribonuclease protection.

A) Schematic representation of the region of *altNF2d9* used to generate anti-sense riboprobes for use in ribonuclease protection assays. The size of protected fragments of *altNF2d9* and *NF2d9* is indicated.

B) Ribonuclease protection analysis (2.3.54) was carried out on 10 µg of total RNA isolated from mouse ES cells and mouse adult kidney using the 540bp antisense riboprobe generated from paltNF2d9-RP. *mGAP*; anti-sense riboprobe specific for mouse glyceraldehyde phosphate dehydrogenase (*mGAP*) was used as a loading control; tRNA, yeast tRNA was used as a negative control. Identity and size of protected bands are indicated.


A
Unique to altNF2d9
Common to NF2d9 and altNF2d9
altNF2d9 specific protected band of 540bp
108 bp
NF2d9 specific protected band of 432bp
432 bp


B
ES cell
Kidney
tRNA
Undigested probe mix
Undigested NF2d9 Probe
altNF2d9 specific protected band of 540bp
NF2d9 specific protected band of 432bp
Undigested mGAP Probe
mGAP, 290bp

RNA samples. The lower, more abundant, band was consistent with the expected size of a protected transcript corresponding to NF2d9. The higher, less abundant, band was consistent with the expected size of a protected altNF2d9 transcript confirming the existence of this transcript. The levels of expression of each species appeared equivalent in ES cells and kidney with NF2d9 being approximately 2 fold more abundant.

3.7.2 Sequence Analysis of *altNF2d9*.

The additional 108 bp insert in altNF2d9 contributes an additional 36 amino acids to the NF2d9 ORF (Figure 3.11a and b). Alignment of the predicted amino acid sequence of altNF2d9 with sequences from the BlastP database (2.3.1.16) revealed that altNF2d9 is the equivalent of LBP-1a, the alternatively spliced isoform of LBP-1b, the human homologue of NF2d9 (Yoon *et al.*, 1994) (Figure 3.12). Isolation of altNF2d9 demonstrates that the alternative splicing event described for LBP-1a to generate LBP-1b is conserved in mice, suggesting that altNF2d9 has conserved cellular functions that are distinct from NF2d9 and other CP2 family members. Mapping of LBP-1b expression has not been reported.

3.7.3 Validation Of *altCP2* By RT-PCR and Ribonuclease Protection.

Platinum Taq PCR (2.3.1.12) was carried out on the yeast-2-hybrid mouse testis cDNA library (2.3.4.1) and poly-T primed cDNA (2.3.1.11) generated from adult mouse testis total RNA (2.3.5.2) using primers CP2SR7, specific for the 5' end of the additional nucleotide sequences, in conjunction with CP2SR2 (2.2.7.2). A PCR product of expected size, approximately 490 bp, was observed, cloned into pGEMT-T easy and sequenced (2.3.1.14) and confirmed the existence of the altCP2 RNA species in mouse testis (Figure 3.9b and Figure 3.13a).

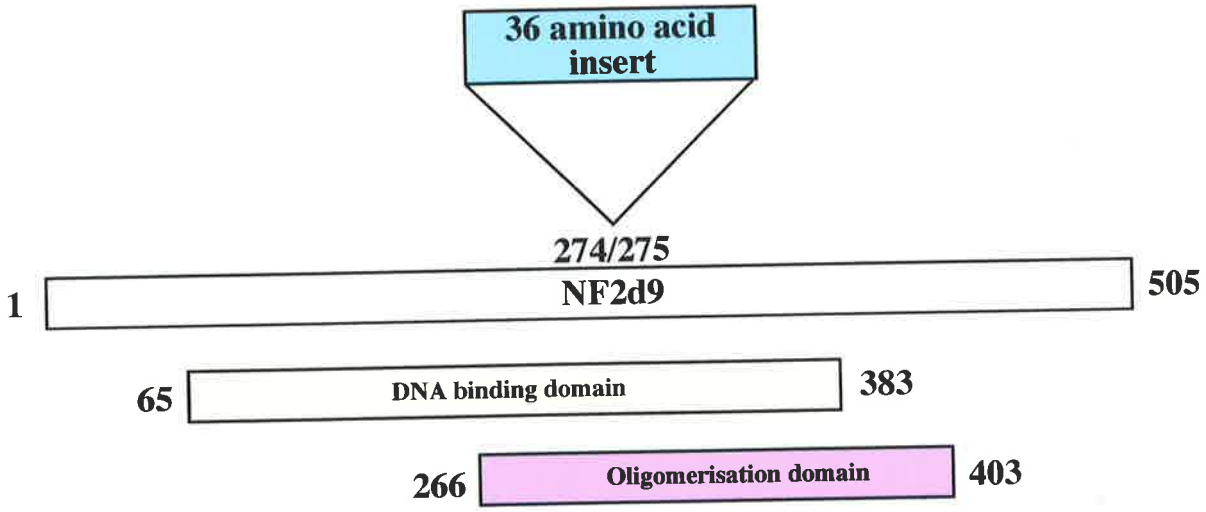
FIGURE 3.11

Amino acid sequence of altNF2d9 and alignment with NF2d9.

A) Schematic representation of the relative position of the additional 36 amino acid insert in altNF2d9 with the NF2d9 amino acid sequence and regions conserved in NF2d9 analogous to the DNA binding domain and dimerisation domain of LBP-1c.

B) Sequence alignment of the predicted NF2d9 and altNF2d9 amino acid sequences. Details as described for figure 3.6.

A



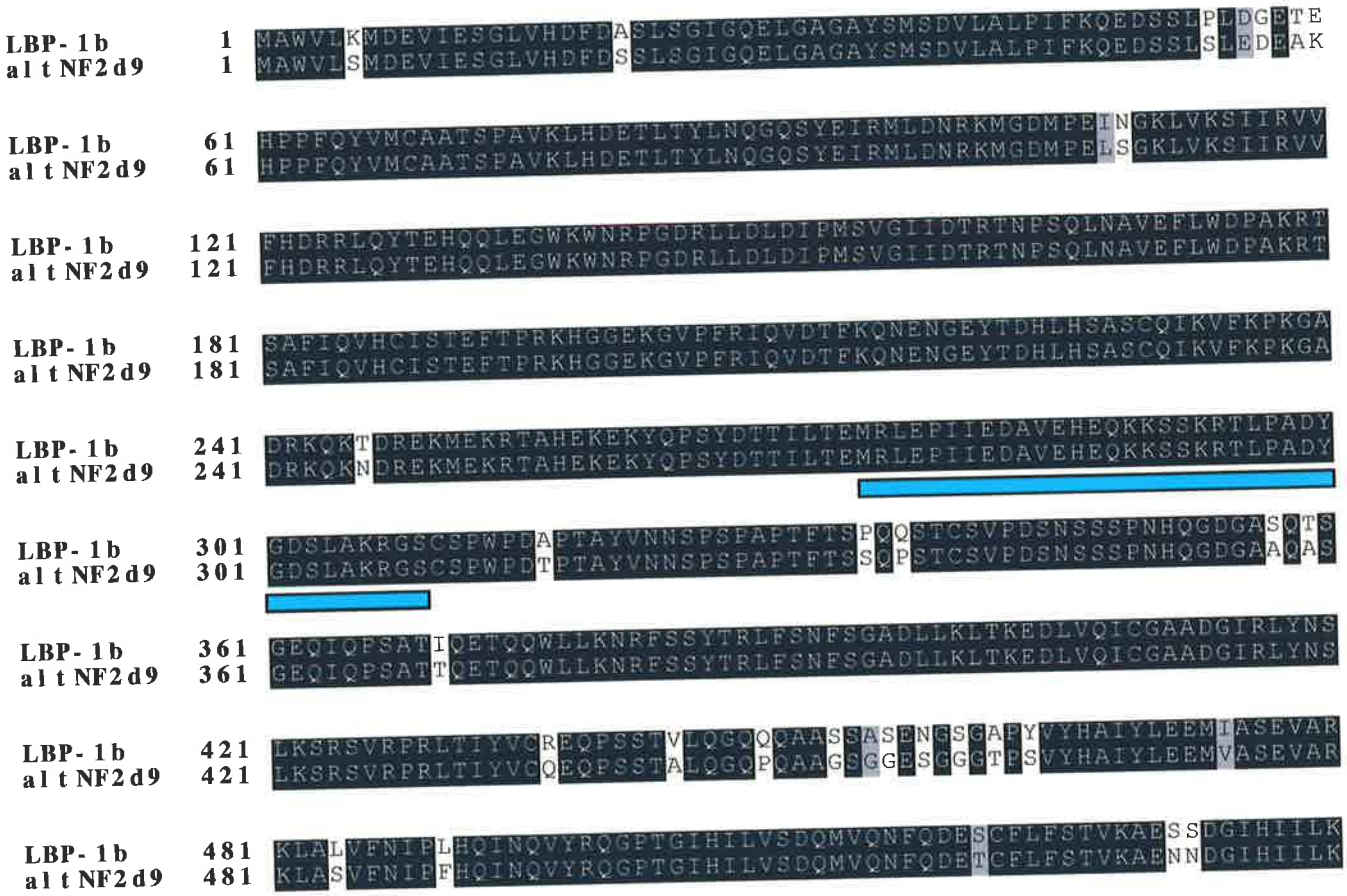
B

```
mNF2d9          1 MAWVLSMDEVIESGLVHDFDSSLSGIGQELGAGAYSMSDVLALPIFKQEDSSLSLEDEAK
alt-mNF2d9      1 MAWVLSMDEVIESGLVHDFDSSLSGIGQELGAGAYSMSDVLALPIFKQEDSSLSLEDEAK

mNF2d9         61 HPPFQYVMCAATSPAVKLHDETLTYLNQGQSYEIRMLDNRKMGDMPELSGKLVKSIIRVV
alt-mNF2d9     61 HPPFQYVMCAATSPAVKLHDETLTYLNQGQSYEIRMLDNRKMGDMPELSGKLVKSIIRVV

mNF2d9        121 FHDRRLQYTEHQQLEGWKWNRPGDRLLDLDIPMSVGIIDTRTNPSQLNAVEFLWDPAKRT
alt-mNF2d9    121 FHDRRLQYTEHQQLEGWKWNRPGDRLLDLDIPMSVGIIDTRTNPSQLNAVEFLWDPAKRT

mNF2d9        181 SAFIQVHCISTEFTPRKHGGEKGVPFRIQVDTFKQNENGEYTDHLHSASCQIKVFKPKGA
alt-mNF2d9    181 SAFIQVHCISTEFTPRKHGGEKGVPFRIQVDTFKQNENGEYTDHLHSASCQIKVFKPKGA

mNF2d9        241 DRKQKNDREKMEKRTAHEKEKYQPSYDTTILTEC----------------------------
alt-mNF2d9    241 DRKQKNDREKMEKRTAHEKEKYQPSYDTTILTEMRLEPIIEDAVEHEQKKSSKRTLPADY

mNF2d9        275 ----------SPWPDTPTAYVNNSPSPAPTFTSSQPSTCSVPDSNSSSPNHQGDGAAQAS
alt-mNF2d9    301 GDSLAKRGSCSPWPDTPTAYVNNSPSPAPTFTSSQPSTCSVPDSNSSSPNHQGDGAAQAS

mNF2d9        325 GEQIQPSATTQETQQWLLKNRFSSYTRLFSNFSGADLLKLTKEDLVQICGAADGIRLYNS
alt-mNF2d9    361 GEQIQPSATTQETQQWLLKNRFSSYTRLFSNFSGADLLKLTKEDLVQICGAADGIRLYNS

mNF2d9        385 LKSRSVRPRLTIYVCQEQPSSTALQGQPQAAGSGGESGGGTPSVYHAIYLEEMVASEVAR
alt-mNF2d9    421 LKSRSVRPRLTIYVCQEQPSSTALQGQPQAAGSGGESGGGTPSVYHAIYLEEMVASEVAR

mNF2d9        445 KLASVFNIPFHQINQVYRQGPTGIHILVSDQMVQNFQDETCFLFSTVKAENNDGIHIILK
alt-mNF2d9    481 KLASVFNIPFHQINQVYRQGPTGIHILVSDQMVQNFQDETCFLFSTVKAENNDGIHIILK
```

FIGURE 3.12

altNF2d9 is the homologue of LBP-1b.

Sequence alignment of the predicted altNF2d9 and LBP-1b amino acid sequences. Details as described for figure 3.6. The shaded box underlines the additional amino acids specific for and conserved between LBP-1b and NF2d9.


LBP-1b 1 MAWVLKMDEVIESGLVHDFDASLSGIGQELGAGAYSMSDVLALPIFKQEDSSLPLDGETE
alt NF2d9 1 MAWVLSMDEVIESGLVHDFDSSLSGIGQELGAGAYSMSDVLALPIFKQEDSSLSLEDEAK
LBP-1b 61 HPPFQYVMCAATSPAVKLHDETLTYLNQGQSYEIRMLDNRKMGDMPEINGKLVKSIIRVV
alt NF2d9 61 HPPFQYVMCAATSPAVKLHDETLTYLNQGQSYEIRMLDNRKMGDMPELSGKLVKSIIRVV
LBP-1b 121 FHDRRLQYTEHQQLEGWKWNRPGDRLLDLDIPMSVGIIDTRTNPSQLNAVEFLWDPAKRT
alt NF2d9 121 FHDRRLQYTEHQQLEGWKWNRPGDRLLDLDIPMSVGIIDTRTNPSQLNAVEFLWDPAKRT
LBP-1b 181 SAFIQVHCISTEFTPRKHGGEKGVPFRIQVDTFKQNENGEYTDHLHSASCQIKVFKPKGA
alt NF2d9 181 SAFIQVHCISTEFTPRKHGGEKGVPFRIQVDTFKQNENGEYTDHLHSASCQIKVFKPKGA
LBP-1b 241 DRKQKTDREKMEKRTAHEKEKYQPSYDTTILTEMRLEPIIEDAVEHEQKKSSKRTLPADY
alt NF2d9 241 DRKQKNDREKMEKRTAHEKEKYQPSYDTTILTEMRLEPIIEDAVEHEQKKSSKRTLPADY
LBP-1b 301 GDSLAKRGSCSPWPDAPTAYVNNSPSPAPTFTSPQQSTCSVPDSNSSSPNHQGDGASQTS
alt NF2d9 301 GDSLAKRGSCSPWPDTPTAYVNNSPSPAPTFTSSQPSTCSVPDSNSSSPNHQGDGAAQAS
LBP-1b 361 GEQIQPSATIQETQQWLLKNRFSSYTRLFSNFSGADLLKLTKEDLVQICGAADGIRLYNS
alt NF2d9 361 GEQIQPSATTQETQQWLLKNRFSSYTRLFSNFSGADLLKLTKEDLVQICGAADGIRLYNS
LBP-1b 421 LKSRSVRPRLTIYVCREQPSSTVLQGQQQAASSASENGSGAPYVYHAIYLEEMIASEVAR
alt NF2d9 421 LKSRSVRPRLTIYVCQEQPSSTALQGQPQAAGSGGESGGGTPSVYHAIYLEEMVASEVAR
LBP-1b 481 KLALVFNIPLHQINQVYRQGPTGIHILVSDQMVQNFQDESCFLFSTVKAESSDGIHIILK
alt NF2d9 481 KLASVFNIEFHQINQVYRQGPTGIHILVSDQMVQNFQDETCFLFSTVKAENNDGIHIILK

For ribonuclease protection, Platinum Taq PCR (2.3.1.12) was used to isolate a fragment of altCP2 that encompassed the additional insert and sequence 3' to the insert (Figure 3.13b). pGEM-T-altCP-DD was used as a template for PCR (2.3.1.12) using primers CP2SR7 and CP2SR2 (2.2.7.2). The PCR product of 490 bp was cloned into pGEM-T easy generating paltCP2-RP. paltCP2-RP was linearised (2.3.1.1) with *Nco*I and used for transcription of an α^{32}P-rUTP labelled anti-sense riboprobe using SP6 RNA polymerase (2.3.5.4.1). Ribonuclease protection analysis (2.3.5.4.2) using 10 µg of total RNA isolated from mouse ES cells, adult mouse heart and 16.5 d.p.c brain is presented in Figure 3.13c.

While a band corresponding to the CP2 transcript was present in each sample, a band of the expected size for altCP2 was detected at low levels only in the 16.5 d.p.c brain RNA sample (Figure 3.13c). Loading control levels for the 16.5 d.p.c. embryonic brain are significantly lower than for ES cells and heart samples suggesting that expression of altCP2 would be greater than observed in comparison to other RNAs. This result confirmed the existence of altCP2 RNA species and provides evidence that altCP2 may be differentially expressed between various tissues in contrast to the ubiquitous expression of CP2.

3.7.4 Sequence Analysis of *altCP2*.

The additional 132bp insert within the *altCP2* sequence contained an in frame TGA stop codon, resulting in C-terminal truncation of the CP2 protein sequence at amino acid 384 (Figure 3.14a and b). Alignment of the nucleotide sequence and predicted amino acid sequence of altCP2 with sequences from the BlastN and BlastP databases (2.3.1.16), respectively, revealed that the insert sequence in altCP2 is novel. altCP2 could be the product of alternative splicing which, for CP2, has not previously been described in mice.

FIGURE 3.13

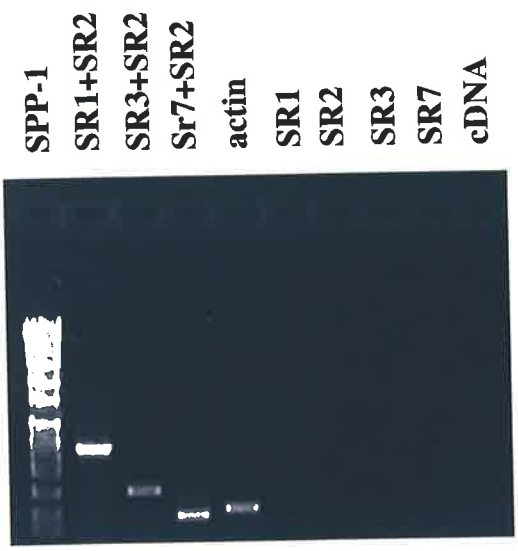
Validation of *altCP2* by RT-PCR and ribonuclease protection.

A) Platinum Taq PCR (2.3.1.12), using primers for *CP2* cDNAs SR1, SR2, SR3 and SR7 (2.2.7.2) was carried out on poly-T primed cDNA generated from adult mouse testis total RNA (2.3.5.1). PCR reactions were separated on a 1% TAE agarose gel and visualised by exposure to UV light after ethidium staining. cDNA, PCR with cDNA alone; actin, PCR using actin specific primers, actinA and actinB (2.2.7.2) with cDNA; Markers, 500 ng of *Eco*RI digested Spp1 phage DNA.

B) Schematic representation of the region of *altCP2* used to generate anti-sense riboprobes for use in ribonuclease protection assays. The size of protected fragments of *altCP2* and *CP2* indicated.

C) Ribonuclease protection analysis (2.3.5.4) was carried out on 10 μg of total RNA isolated from mouse ES cells, mouse adult heart and 16.5 d.p.c. brain using the 489 bp antisense riboprobe generated from paltCP2-RP. Details are as described for Figure 3.10b. Identity and size of protected bands are indicated.

A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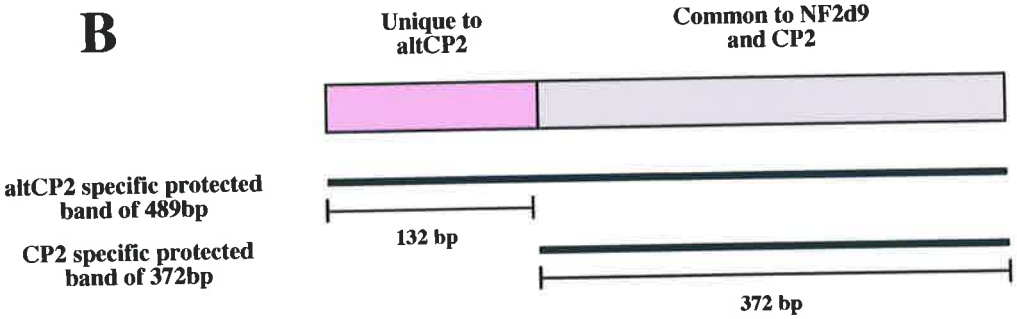
SR3+SR2
Sr7+SR2
actin
SR1
SR2
SR3
SR7
cDNA

B


Unique to altCP2
Common to NF2d9 and CP2
altCP2 specific protected band of 489bp
CP2 specific protected band of 372bp
132 bp
372 bp

C

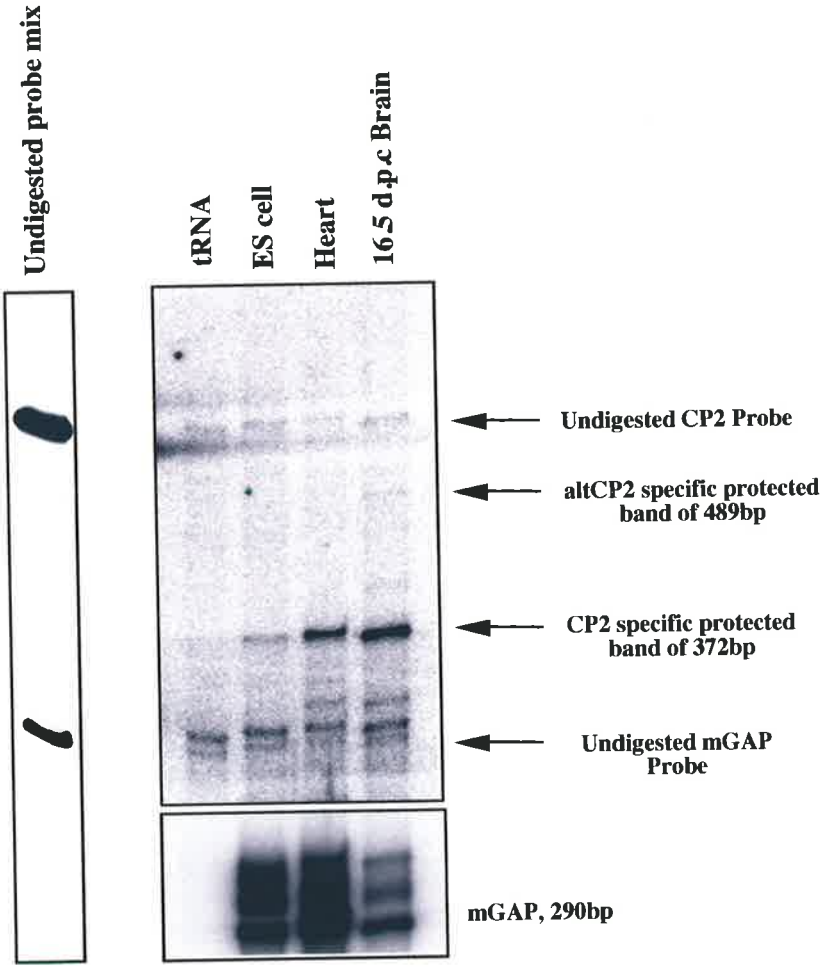

Undigested probe mix
tRNA
ES cell
Heart
16.5 d.p.c Brain
Undigested CP2 Probe
altCP2 specific protected band of 489bp
CP2 specific protected band of 372bp
Undigested mGAP Probe
mGAP, 290bp

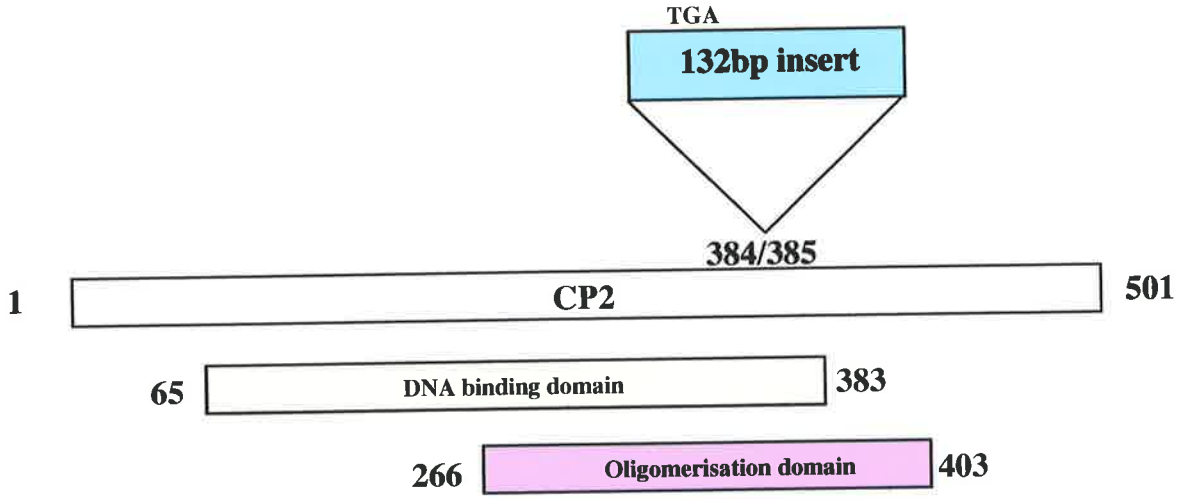
FIGURE 3.14

***altCP2* contains an additional 132 bp insert resulting in truncation of the altCP2 amino acid sequence.**

A) Schematic representation of the relative position of the additional 132 bp insert of *altCP2* in the CP2 amino acid sequence and regions conserved in CP2 analogous to the DNA binding domain and dimerisation domain of LBP-1c.

B) Sequence alignment of the predicted CP2 and altCP2 amino acid sequences. Details as described for figure 3.6.

A



B

```
CP2          1 MAWALKLPLADEVIESGLVQDFDASLSGIGQELGAGAYSMSDVLALPIFKQEESSLPPDN
alt CP2      1 MAWALKLPLADEVIESGLVQDFDASLSGIGQELGAGAYSMSDVLALPIFKQEESSLPPDN

CP2         61 ENEILPFQYVLCAATSPAVKLHDETLTYLNQGQSYEIRMLDNRKLGELPELNGKLVKSIF
alt CP2     61 ENEILPFQYVLCAATSPAVKLHDETLTYLNQGQSYEIRMLDNRKLGELPELNGKLVKSIF

CP2        121 RVVFHDRRLQYTEHQQLEGWRWNRPGDRILDIDIPMSVGVIDPRANPTQLNTVEFLWDPS
alt CP2    121 RVVFHDRRLQYTEHQQLEGWRWNRPGDRILDIDIPMSVGVIDPRANPTQLNTVEFLWDPS

CP2        181 KRTSVFIQVHCISTEFTMRKHGGEKGVPFRVQIDTFKENGNGEYTEHLHSASCQIKVFKP
alt CP2    181 KRTSVFIQVHCISTEFTMRKHGGEKGVPFRVQIDTFKENGNGEYTEHLHSASCQIKVFKP

CP2        241 KGADRKQKIDREKMEKRTPHEKEKYQPSYETTILTECSPWPEITYVNNSPSPGENSSHSS
alt CP2    241 KGADRKQKIDREKMEKRTPHEKEKYQPSYETTILTECSPWPEITYVNNSPSPGENSSHSS

CP2        301 FSLGEGNGSPNHQPEPPPPVTDNLLPTTTPQEAQQWLHRNRFSTFTRLFTNFSGADLLKL
alt CP2    301 FSLGEGNGSPNHQPEPPPPVTDNLLPTTTPQEAQQWLHRNRFSTFTRLFTNFSGADLLKL

CP2        361 TRDDVIQICGPADGIRLFNALKGRMVRPRLTIYVCQESLQLREQQPQPQPQPQKQEDGDS
alt CP2    361 TRDDVIQICGPADGIRLFNALKGRCVLSVEMPMKVDSK----------------------

CP2        421 NGTFFVYHAIYLEELTAVELTEKIAQLFSISPHQISQIYKQGPTGIHVVISDEMVQNFQE
alt CP2        ------------------------------------------------------------

CP2        481 EACFILDTMEAETSDSYHVILK
alt CP2        ----------------------
```

3.7.5 Mapping of the *altCP2* Inserted Sequence to the Mouse Genome.

CP2 has been mapped to the distal tip of mouse chromosome 15 and is spread over approximately 30 kb consisting of 16 exons (Swendeman *et al.*, 1994). Comparison of the unique 132bp inserted sequence in altCP2 with the mouse genome sequence at NCBI using BlastN (2.3.1.16) identified a region showing 100% identity located at the distal tip of chromosome 15. The altCP2 132 bp insert was identified as a direct extension of exon 12, designated exon 12v (Figure 3.15a). Sequence at the 5' boundary of exon 12v and the 3' end of exon 12, GGCCG/GTGTGT, conforms to the consensus for splice donor sites, CorAAG/GTAorGAGT (Mount, 1982). Splicing at this point generates CP2. At the 3'end of exon 12v is a poor consensus splice donor sequence, AAAGG/TACTAA, which is utilised to produce altCP2 (Figure 3.15b). The poor consensus of the exon 12v splice donor sequence provides a molecular explanation for low-level expression of altCP2 (3.7.3). Furthermore, expression of altCP2 appears to be regulated, in contrast to the ubiquitously expressed CP2, suggesting that factors regulating alternative splicing of the CP2 pre-mRNA may also be regulated.

3.8 DISCUSSION.

3.8.1 CRTR-1 is a Novel Member of the CP2 Family.

The compiled *CRTR-1* cDNA sequence was isolated as a number of individual clones derived from 5 independent library screens using a Lambda ZAP II ES D3 cDNA library (Clontech). The complete *CRTR-1* cDNA sequence is 9405 bp consisting of a 91 bp 5'UTR confirmed by two independent techniques, a 1446 bp ORF with a predicted protein sequence of 481 amino acids and a 7868 bp 3'UTR determined by the presence of a poly(A) tail.

Searches of the Blast databases at NCBI demonstrated that CRTR-1 shares significant identity to the CP2 family of transcription factors. Alignment between CRTR-1 and CP2 family members showed extensive conservation across the full length of the

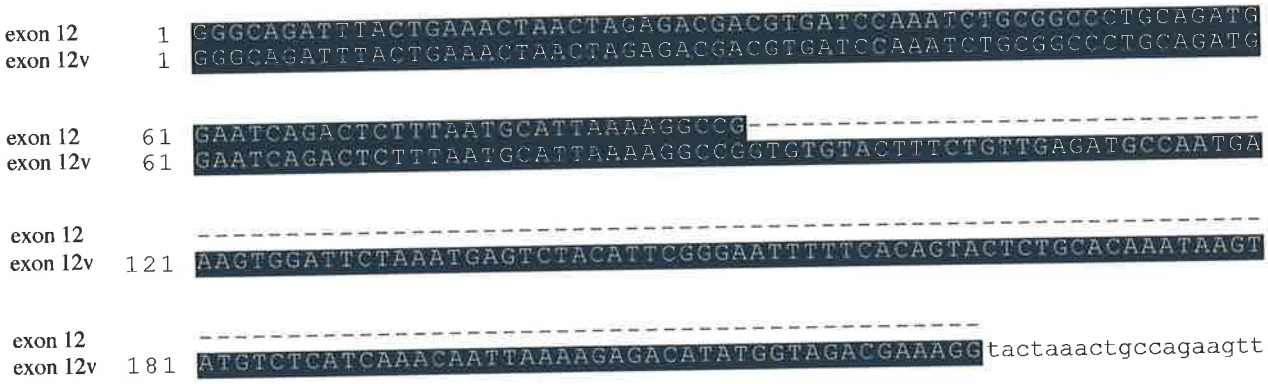
FIGURE 3.15

***altCP2* is alternatively spliced from a poor consensus splice donor site.**

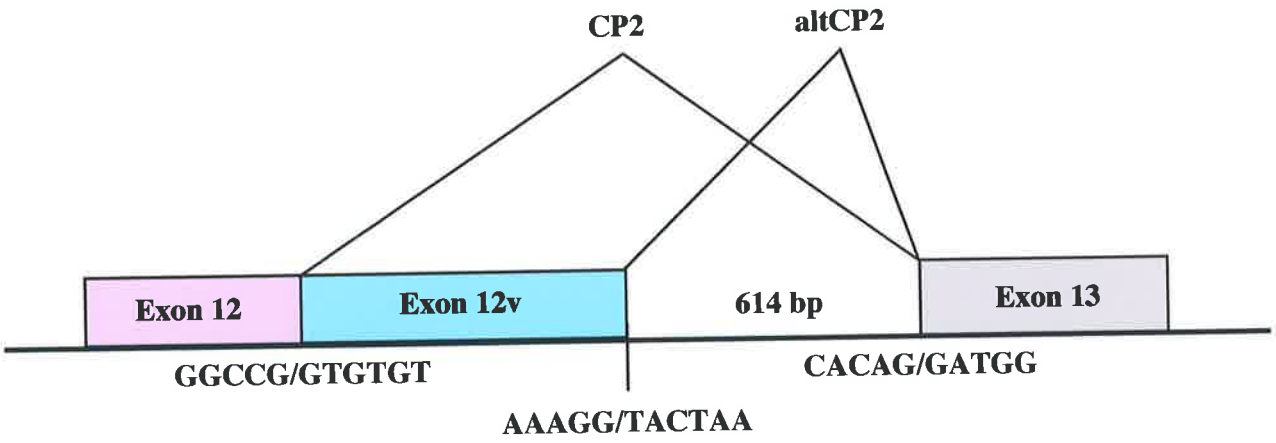
A) Alignment of exon 12 and exon 12v nucleotide sequences. Alignment carried out using ClustalW (2.3.1.16). Lower case letters represent intron nucleotide sequence.

B) Schematic representation of the genomic structure and splicing of the CP2 locus around exons 12, 12v and 13. The splice donor sequences for exon 12 and 12v and the splice acceptor sequence for exon 13 are indicated. The consensus splice donor sites sequence is CorAAG/GTAorGAGT (Mount 1982).

A


exon 12 1 GGGCAGATTTACTGAAACTAACTAGAGACGACGTGATCCAAATCTGCGGCCCTGCAGATG
exon 12v 1 GGGCAGATTTACTGAAACTAACTAGAGACGACGTGATCCAAATCTGCGGCCCTGCAGATG
exon 12 61 GAATCAGACTCTTTAATGCATTAAAAGGCCG----------
exon 12v 61 GAATCAGACTCTTTAATGCATTAAAAGGCCGGTGTGTACTTTCTGTTGAGATGCCAATGA
exon 12 ----------
exon 12v 121 AAGTGGATTCTAAATGAGTCTACATTCGGGAATTTTTCACAGTACTCTGCACAAATAAGT
exon 12 ----------
exon 12v 181 ATGTCTCATCAAACAATTAAAAGAGACATATGGTAGACGAAAGG tactaaactgccagaagtt

B


CP2
altCP2
Exon 12
Exon 12v
614 bp
Exon 13
GGCCG/GTGTGT
AAAGG/TACTAA
CACAG/GATGG

protein with the exception of amino acids 1-47 and 381-401, which were conserved only with LBP-9 (Figure 3.6 and 3.7). Homology between CRTR-1 and CP2 family members was specific for the ORF with no conservation in the 5' or 3' UTR nucleic acid sequence being observed. The level of conservation of amino acid sequence between CP2 and CRTR-1 defines CRTR-1 as a novel member of the CP2 family. Furthermore, the level of identity shared between CRTR-1 and LBP-9, including regions in CRTR-1 that were divergent from CP2, suggest that LBP-9 may be the human homologue of CRTR-1.

3.8.2 CRTR-1 Contains a Conserved DNA Binding Domain and Oligomerisation Domain.

Members of the CP2 family of transcription factors bind a consensus DNA sequence consisting of a direct bipartite repeat, CNRG-N_6-CNRG (Lim *et al.*, 1993; Murata *et al.*, 1998). N- and C-terminal truncation studies have defined the minimal DNA binding region of LBP-1c between amino acids 65 and 383 (Shirra and Hansen, 1998) (Figure 1.13). Amino acids 63-270 of CP2 share 32% sequence identity with the region required for DNA binding in *GRH* (Lim *et al.*, 1992; Wilanowski *et al.*, 2002). This region is conserved in CRTR-1 and shows identity of 72% to LBP-1c and 24% to GRH DNA binding regions (Figure 3.8a), suggesting that CRTR-1 (Figure 3.8a) will be able to bind CP2 consensus DNA response elements.

The region required for homomeric interaction of CP2 family members has been localised by truncation studies to amino acids 266-403 of LBP-1c (Shirra and Hansen, 1998) (Figure 1.13). Furthermore, LBP-1c amino acids 260-502 can bind non-CP2 cellular proteins (Tuckfield *et al.*, 2002b; Zhou *et al.*, 2000). Conservation of these regions in CRTR-1 (Figures 3.6 and 3.8b) suggests that CRTR-1 will be able to form homomeric complexes, heteromeric complexes with other CP2 family members and heteromeric complexes with unrelated cellular proteins.

3.8.3 CRTR-1 Does Not Contain Conserved Transcriptional Regulatory Regions.

CP2 family members are generally described as transcriptional activators, with the exception of LBP-9, which has been shown to antagonise the transcriptional activation ability of LBP-1b from the P450scc promoter by an unknown mechanism (Huang and Miller, 2000). Consistent with this role, CRTR-1 contains the SPXX motifs that characterise many DNA binding proteins and are present in CP2 family members. However, the glutamine or glutamine/proline repeats C-terminal to the SPXX motifs in LBP-1c and CP2 repectively, suggested to act as transcriptional activation domains (Lim *et al.*, 1992) were not contained in CRTR-1. Recently, it has been shown that the N-terminal 40 amino acids of CP2 are required and sufficient for transcriptional activation (Ramamurthy *et al.*, 2001; Tuckfiled, 2002a). This region is conserved between CP2 family members, with the exception of LBP-9 and CRTR-1, which are 97% identical across this region (Figures 3.6 and 3.8). The absence of amino acid similarity between CP2 and CRTR-1 within regions that have been shown to be important for transcriptional activation suggest that CRTR-1 may have a different transcriptional regulatory role from other CP2 family members.

Determination of the ability of CRTR-1 to regulate transcription, bind DNA and interact with other CP2 family members and unrelated cellular proteins would confirm this protein as a member of the CP2 family and provide insight into the biological role of CRTR-1 as a possible regulator of transcriptional activity, particularly in the regulation and/or maintenance of pluripotency in the early mouse embryo and the development and physiology of the kidney.

3.8.4 Alternative Splice Variants of *NF2D9* and *CP2*.

PCR isolation of full length and dimerisation domain equivalents of *NF2d9* and *CP2* led to the identification of alternatively spliced *NF2d9* and *CP2* variants, referred to as *altNF2d9* and *altCP2*, respectively.

altNF2d9 contains a 108 bp insert that contributes an additional 36 amino acids to the NF2d9 ORF between amino acids 274-275. *altNF2d9* is the mouse equivalent of LBP-1b, which is produced by alternative splicing of the *LBP-1a* mRNA (Yoon *et al.*, 1994). The region encompassing the 36 amino acid shares amino acid sequence conservation to regions in LBP-1c important for both DNA binding (amino acids 65-383) and oligomerisation (amino acids 266-403) (Figure 3.11a). This suggests that altNF2d9 may function differently from NF2d9 in its ability to bind DNA and interact with other proteins. LBP-1b, however, has been shown to activate transcription from a CP2 responsive promoter and bind DNA as a homomeric complex and as a participant in heteromeric DNA binding complexes with LBP-1a and LBP-1c (Yoon *et al.*, 1994). These observations suggest that altNF2d9 will also be able to bind DNA and other proteins in a manner analogous to NF2d9. This, however, does not exclude the possibility that altNF2d9 may play a role in the ability to bind DNA response elements and/or other proteins that are distinct from NF2d9 and other CP2 family members. The hypothesis that altNF2d9 plays a significant biological role distinct from NF2d9 is supported by conservation of the alternative splicing event in humans.

The existence of altCP2 was confirmed by three different methods, RT-PCR, ribonuclease protection and sequence mapping to the mouse genome. altCP2 contains an additional 132 bp insert, termed exon 12v, that encodes an in frame stop codon truncating the altCP2 ORF at amino acid 384. The novel sequence is the product of alternative splicing from a poor splice donor site at the 3' end of exon 12v.

Truncation studies have defined the minimal DNA binding and oligomerisation regions for LBP-1c to amino acids 65-383 and 266-403, repectively. However, DNA binding and oligomerisation comparable to the full-length protein was only observed when amino acids 65-502 and 266-502 of LBP-1c were present, respectively. While the minimal functional regions of LBP-1c homologous to altCP2 are preserved, altCP2 lacks the C-terminal 103 amino acids of CP2. This suggests that altCP2 may differ from CP2 in its ability to bind CP2 consensus DNA response elements and/or interact with other CP2 family members and unrelated cellular proteins. An LBP-1c equivalent to altCP2 has not been described. A possible unique function of altCP2 may therefore be at the level of negative regulation of CP2 family members by blocking protein/protein and/or DNA/protein complex formation. Expression of altCP2 was regulated, detected only in the mouse testis and 16.5 d.p.c embryonic brain, and at low levels in comparison to the CP2 transcript suggesting that factors regulating the alternative splicing of the CP2 transcript may also be regulated.

In sight into the biological roles of CRTR-1, altNF2d9 and altCP2 requires determination of their DNA binding ability and ability to form protein/protein interactions with other CP2 family members and other non-related cellular proteins. Molecular charatcerisation at this level would also provide information about the possible regulatory interplay between CP2 family members and give an overall better understanding of transcriptional regulation by the CP2 family.

CHAPTER 4

CRTR-1 acts as a transcriptional repressor.

4.1 INTRODUCTION

Members of the CP2 family of transcription factors are generally described as transcriptional activators (Bing *et al.*, 1999; Huang and Miller, 2000; Kim *et al.*, 1990; Kim *et al.*, 1987; Lim *et al.*, 1993; Lim *et al.*, 1992; Murata *et al.*, 1998), with the exception of LBP-9, which has not been analysed in detail. There has been some discussion over the position of the transcriptional activation domain. A glutamine/proline repeat in CP2 and a 10 amino acid glutamine repeat at the analogous position in LBP-1c are homologous to sequences found in other transcription factors such as Sp1 which form part of a transactivation domain (Courey *et al.*, 1989; Mitchell and Tjian, 1989). These conserved regions in the CP2 family have not yet been functionally investigated. More recently, it has been shown that the N-terminal 40 amino acids of CP2 are required and sufficient for the transcriptional activation ability of LBP-1c (Ramamurthy *et al.*, 2001; Tuckfiled, 2002a). This region is highly conserved between CP2 family members with the exception of CRTR-1 and LBP-9, which are homologous with each other but not other family members.

Transfection of JEG-3 cells with increasing amounts of LBP-9 suppressed LBP-1b-mediated reporter activation from the -155/-131 region of the human P450scc promoter (Huang and Miller, 2000). The mechanism of this inhibition was not resolved. Amino acid sequence homology shared between CRTR-1 and CP2 family members suggest that CRTR-1 will function as a transcription factor while homology shared between LBP-9 and CRTR-1 over the N-terminal amino acids, demonstrated to contain the CP2 transcriptional activation domain, suggest that CRTR-1 may have a transcriptional regulatory ability distinct from other CP2 family members, consistent with LBP-9. These features prompted analysis of the transcriptional regulatory ability of CRTR-1.

4.2 REGULATION OF MAMMALIAN TRANSCRIPTION BY CRTR-1.

Use of Gal4-DNA binding domain (Gal4-DBD) (Ma and Ptashne, 1987) fusion proteins in transcriptional regulation assays permits investigation into the transcriptional

regulatory ability of a protein by tethering it to a heterologous promoter via binding to the Gal4 DNA binding response element. As the Gal4-DBD is sufficient for DNA binding (Ma and Ptashne, 1987), this approach enables dissection of proteins into functional domains for characterisation, independent of their DNA binding, and can be used for the identification of transcriptional regulatory domains.

Transcriptional regulation activity of CRTR-1 was assessed as a fusion protein with amino acids 1-174 of the Gal4-DNA-binding domain (DBD) (Ma and Ptashne, 1987). As the transcriptional activation domain for CP2 has been localised to the N-terminal 40 amino acids (Ramamurthy *et al.*, 2001; Tuckfiled, 2002a), constructs which direct the expression of the Gal4-DBD fused to amino acid regions 1-52 and 47-481 were used for localisation of the regulatory domain. pTK-MH100x4-LUC (Kang *et al.*, 1993), which contains a luciferase gene regulated by the thymidine kinase (Tk) promoter and four upstream tandem copies of the Gal4 binding site, was used as a reporter.

4.2.1 Generation of GAL4-DBD.CRTR-1 Fusion Protein Expression Constructs.

In-frame fusions between the Gal4-DBD and *CRTR-1* fragments were generated in the plasmid pGalO (Chen *et al.*, 2000; Dowhan and Muscat, 1996; Kato *et al.*, 1990; Lau *et al.*, 1999; Sartorelli *et al.*, 1997) (2.2.6.1), which contains amino acids 1-147 of the Gal4-DBD (Kang *et al.*, 1993) (Figures 4.1a and 4.2a). A fragment encoding the full length CRTR-1 ORF was excised from pGEM-T-CRTR-1 (3.3.1) by digestion with *Sal*I and *Sac*I (2.3.11), and cloned into *Sal*I/*Sac*I digested pGalO, generating pGalO.CRTR-1. cDNA fragments encoding the N-terminal 52 amino acids and C-terminal 435 amino acids of CRTR-1 were amplified by PCR from pGEM-T-CRTR-1 using Pfu Turbo (Stratagene, 2.3.1.13) with the primer combinations SR1/SR4 and SR3/SR2 (2.2.7.2), respectively. PCR products were digested with *Sal*I and *Sac*I, gel purified (2.3.1.3) and cloned into *Sal*I/*Sac*I digested pGalO generating pGalO.CRTR-1(1-52) and pGalO.CRTR-1(47-481),

FIGURE 4.1

Construction of pGalO.CRTR-1, pGalO.CRTR-1(47-481) and pGalO.CRTR-1(1-52)

A) pGalO contains the Gal4-DBD, amino acids 1-147 of Gal4, under the control of an SV40 promoter. cDNAs for full length CRTR-1, CRTR-1(47-481) and CRTR-1(1-52) were cloned into pGalO generating plasmids pGalO.CRTR-1, pGalO.CRTR-1(47-481) and pGalO.CRTR-1(1-52) which direct expression of CRTR-1 cDNAs as Gal4-DBD fusion proteins.

B) $3x10^5$ COS-1 cells, grown in 60 mm diameter tissue culture dishes, were transfected with 2μg of pGalO.CRTR-1, pGalO.CRTR-1(47-481) and pGalO.CRTR-1(1-52) (2.4.5). Whole cell extracts were produced 24 hrs post transfection and separated by 10% SDS-PAGE (2.3.6.4) prior to western analysis (2.3.6.6). The filter was probed with rabbit anti-Gal4-DBD antibody and visualised using chemiluminescence following incubation with horseradish peroxidase (HRP)- conjugated goat anti-rabbit secondary antibody (2.2.8). Markers; 10 μL of Benchmark prestained protein ladder (2.2.14).

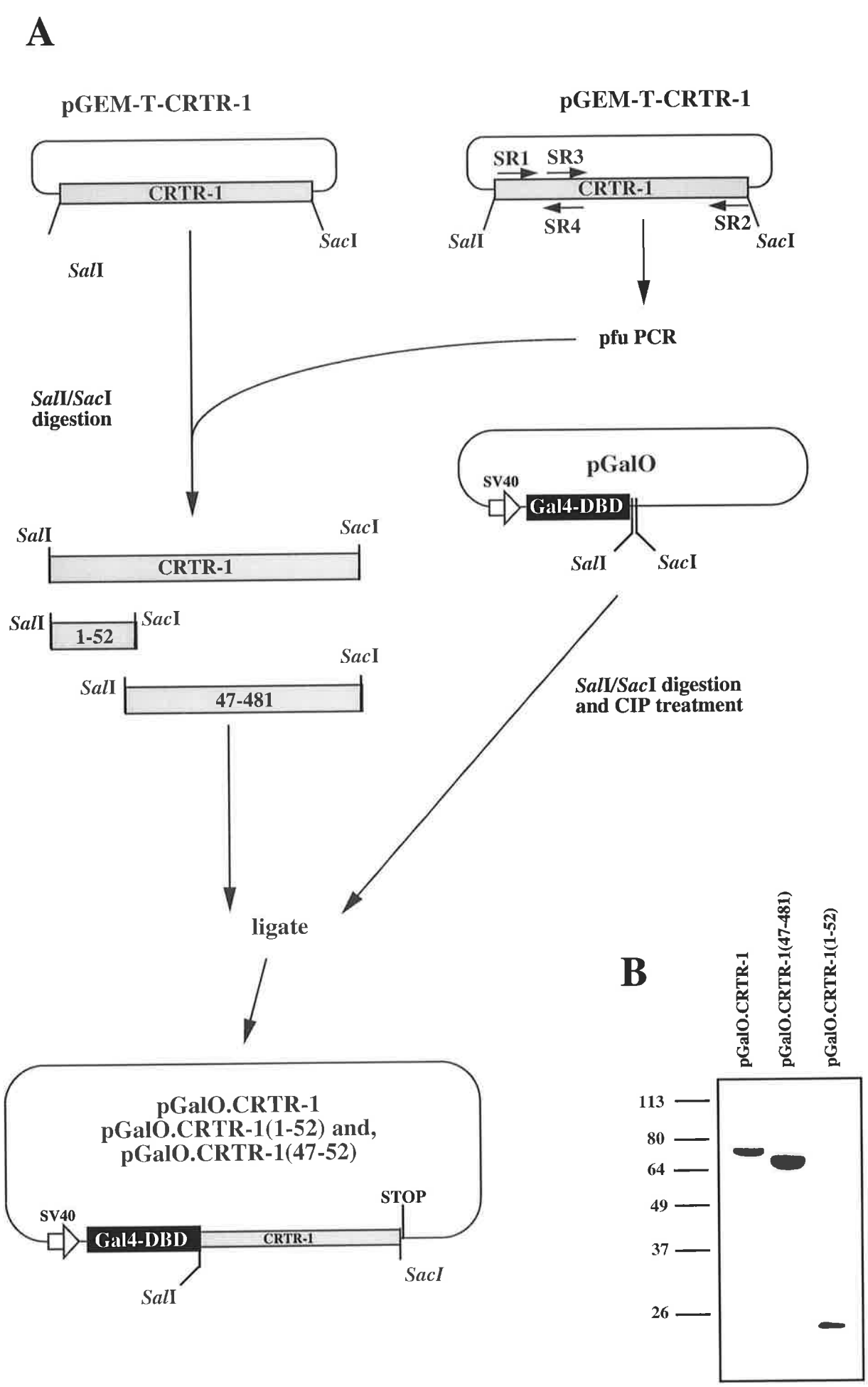
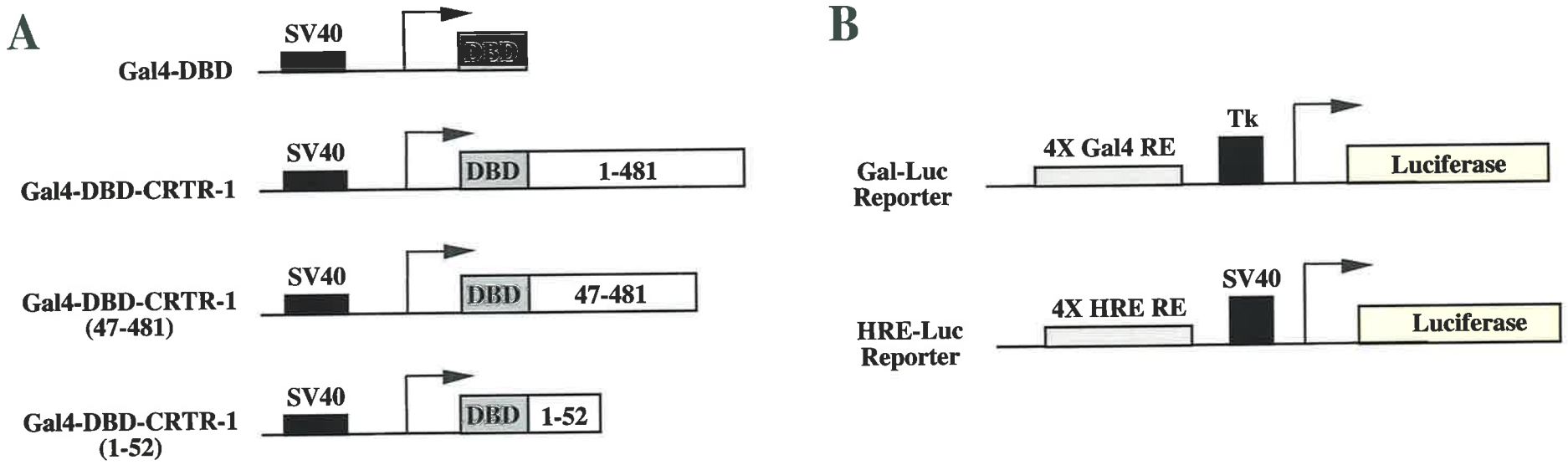

A
pGEM-T-CRTR-1
CRTR-1
SacI
SalI
pGEM-T-CRTR-1
SR1
SR3
CRTR-1
SR4
SR2
SalI
SacI
pfu PCR
SalI/SacI
digestion
pGalO
SV40
Gal4-DBD
SalI
SacI
SalI
SacI
CRTR-1
SalI
SacI
1-52
SacI
SalI
47-481
SalI/SacI digestion
and CIP treatment
ligate
pGalO.CRTR-1
pGalO.CRTR-1(1-52) and,
pGalO.CRTR-1(47-52)
STOP
SV40
Gal4-DBD
CRTR-1
SacI
SalI
B
pGalO.CRTR-1
pGalO.CRTR-1(47-481)
pGalO.CRTR-1(1-52)
113
80
64
49
37
26

FIGURE 4.2

Schematic representation of Gal4-DBD fusion protein expression and luciferase reporter constructs.

A) Gal4-DBD.CRTR-1, Gal4-DBD.CRTR-1(47-481) and Gal4-DBD.CRTR-1(1-52) were expressed under control of the SV40 early promoter from pGalO.CRTR-1, pGalO.CRTR-1(47-481) and pGalO.CRTR-1(1-52), respectively.

B) pTK-MH100x4-LUC, containing four copies of the Gal4-DBD response element upstream of a Tk promoter driving luciferase expression, and pHRE-Luc, containing 3 copies of the hypoxia inducible factor response element upstream of an SV40 promoter driving luciferase expression, were used as reporter constructs in co-transfection experiments with plasmids described in **(A)** and Figure 4.1.


A
SV40
Gal4-DBD
DBD
SV40
Gal4-DBD-CRTR-1
DBD
1-481
SV40
Gal4-DBD-CRTR-1
(47-481)
DBD
47-481
SV40
Gal4-DBD-CRTR-1
(1-52)
DBD
1-52
B
Tk
4X Gal4 RE
Gal-Luc
Reporter
Luciferase
SV40
4X HRE RE
HRE-Luc
Reporter
Luciferase

respectively. Correct clones were confirmed by diagnostic digest and BDT automated sequencing (2.3.1.14).

Expression of the Gal4-DBD fusion proteins was tested by transfection of $3x10^6$ COS-1 cells (2.4.1), grown overnight in a 60mm diameter tissue culture dish, with 2 μg of each vector using Fugene 6 (2.4.5). Whole cell protein extracts were made (2.3.6.2) 24hrs post-transfection, separated by 10% SDS-PAGE (2.3.6.4) and transferred to protran nylon filters for western analysis (2.3.6.6). Membranes were probed with a rabbit α-Gal4-DBD poly-clonal antibody (2.2.8) and demonstrated bands at the expected sizes of 72 kDa, 67 kDa, and 23 kDa corresponding to Gal4-DBD.CRTR-1, Gal4-DBD.CRTR-1(47-481) and Gal4-DBD.CRTR-1(1-52) respectively (Figure 4.1b).

4.2.2 CRTR-1 Acts as a Transcriptional Repressor in a Variety of Cell Types.

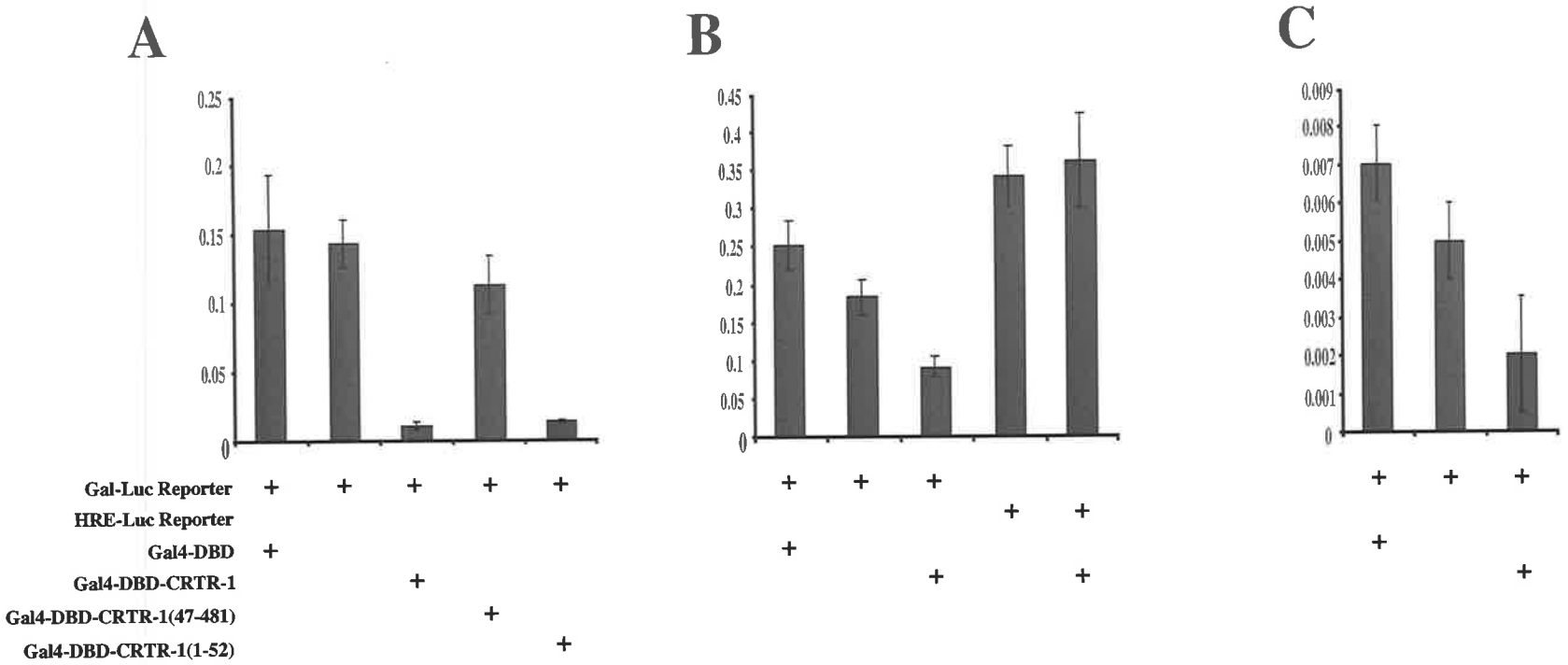
pTK-MH100x4-LUC (Figure 4.2b) was transfected in combination with pGalO-CRTR-1, pGalO.CRTR-1(47-481) and pGalO.CRTR-1(1-52) into COS-1 cells (2.4.5). Levels of luciferase activity were analysed in cell extracts 36 hours post-transfection using the dual luciferase reporter system (2.2.3, 2.4.9). Luciferase activity was normalised to expression of Renilla luciferase expressed from pRLTK.

Co-transfection of pGalO (23-27) with pTK-MH100x4-LUC did not alter the levels of luciferase activity (Figure 4.3a, column 1) compared to pTK-MH100x4-LUC alone (Figure 4.3a, column 2). Co-transfection of pGalO.CRTR-1 with pTK-MH100x4-LUC resulted in a 10-15 fold reduction in luciferase activity (Figure 4.3a, column 3). CRTR-1-mediated transcriptional repression was also demonstrated in 293T (Figure 4.3b) and ES cells (Figure 4.3c) where expression of the Gal4-DBD-CRTR-1 fusion protein reduced luciferase expression 2.5 and 3.5 fold, respectively. This transcriptional repression was specific for the reporter plasmid pTK-MH100x4-LUC and not a result of general transcriptional toxicity of the Gal4-DBD-CRTR-1 fusion protein by normalisation of

FIGURE 4.3

CRTR-1 represses transcription from a basal promoter.

COS-1 cells (**A**), 293T cells (**B**) and ES cells (**C**), were transfected (2.4.5) with expression vectors for Gal4-DBD-CRTR-1 fusion proteins, pGalO.CRTR-1, pGalO.CRTR-1(47-481) and pGalO.CRTR-1(1-52), and the reporter plasmids, pTk-MH100x4-LUC (Gal-Luc Reporter) and pHRE-Luc (HRE-Luc Reporter), as indicated. Luciferase activity was assayed (2.4.9) 36 hours post transfection using a TD-20/20 luminometer and normalised against expression of Renilla luciferase expressed from the co-transfected plasmid pRLTK. The mean and standard deviation of three independent experiments is represented.


A
B
C
0.25
0.2
0.15
0.1
0.05
0
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
Gal-Luc Reporter + + + + +
HRE-Luc Reporter
Gal4-DBD +
Gal4-DBD-CRTR-1 +
Gal4-DBD-CRTR-1(47-481) +
Gal4-DBD-CRTR-1(1-52) +
+ + + + +
+ + +
+ + +
+ + +
+ +

luciferase activity against Renilla luciferase expression from the thymidine kinase promoter. Further, expression of luciferase from pHRE-Luc (Figure 4.2b) (Ema *et al.*, 1997; Lando *et al.*, 2000), in which expression of luciferase is controlled by the SV40 promoter and 3 upstream copies of the hypoxia inducible factor response element, was not altered by co-transfection with pGalO.CRTR-1 (Figure 4.3b, columns 4 and 5). These results demonstrate that CRTR-1 acts as a transcriptional repressor in a variety of cell types including ES cells and 293T cells, representative of sites of expression in pluripotent cells and kidney *in vivo*.

4.2.3 The Ability of CRTR-1 to Repress Transcription Resides in an N-terminal Repression Domain.

Co-transfection of COS-1 cells (2.4.5) with pGalO.CRTR-1(47-481) and pTK-MH100x4-LUC did not affect levels of luciferase activity (Figure 4.3a, column 4) compared to transfection of pTK-MH100x4-LUC alone (Figure 4.3a, column 2), or co-transfection of pGalO and pTK-MH100x4-LUC (Figure 4.3a, column 1). However, co-transfection of pGalO.CRTR-1(1-52) and pTK-MH100x4-LUC resulted in 10-15 fold reduction in luciferase activity (Figure 4.3a, column 5) consistent with the level of repression resulting from co-transfection with full length CRTR-1 (Figure 4.3a, column 3).

CRTR-1 mediated transcriptional repression was not the result of steric hindrance caused by Gal4-DBD-CRTR-1 protein fusions because Gal4-DBD.CRTR-1(47-481) had no transcriptional regulatory effect on expression of luciferase from pTK-MH100x4-LUC (Figure 4.3a, column 4). This demonstrates that the N-terminal 52 amino acids of CRTR-1 are both necessary and sufficient for the transcriptional repression exerted through CRTR-1 and suggest that this region encodes a transcriptional repression domain.

4.3 DISCUSSION

4.3.1 CRTR-1 is a Novel Transcriptional Repressor

Results presented in this chapter confirm the prediction based on amino acid sequence homology that CRTR-1 functions as a transcription factor. Members of the CP2 family have been generally reported to act as transcriptional activators in both *in vitro* (Kim *et al.*, 1990; Lim *et al.*, 1993) and *in vivo* (Bing *et al.*, 1999; Huang and Miller, 2000; Lim *et al.*, 1993; Murata *et al.*, 1998; Yoon *et al.*, 1994) transcription assays. CRTR-1, however, was shown to repress transcription from a heterologous promoter when expressed as a Gal4-DBD fusion protein. While the extent of repression varied from 2.5-15 fold in different cell types (Figure 4.3), conservation of this activity in different cell lines suggests that these results are indicative of normal CRTR-1 activity. This is supported by the fact that 293T and ES cells, in which repression was demonstrated, are representative of *in vivo* expression sites in kidney and pluripotent cells respectively.

Activity as a transcriptional repressor is consistent with the lack of amino acid conservation between CRTR-1 and other CP2 family members at the N-terminus which contains the activation domain in CP2 (Ramamurthy *et al.*, 2001; Tuckfiled, 2002a), and with the lack of polyglutamine and glutamine/proline rich sequences suggested as activation domains in the LBP-1c and CP2 sequences respectively (Lim *et al.*, 1992). CRTR-1 shares greatest homology with LBP-9, identified as a sequence specific binding protein on the -155/-131 region of the P450scc promoter, which also exhibits unusual transcriptional activity. While LBP-1b, which also binds this sequence, activated transcription of a linked reporter gene 21 fold in JEG-3 cells, LBP-9 did not activate transcription in the same system (Huang and Miller, 2000). Transfection of cells with increasing amounts of LBP-9 suppressed the LBP-1b-mediated reporter activation to basal levels. The mechanism of inhibition was not resolved, and could result from direct repression of transcription, steric exclusion of LBP-1b from the DNA-binding site, or displacement of LBP-1b from the promoter by formation of complexes with LBP-9. Direct

repression of transcription from a heterologous promoter reported for CRTR-1 may therefore provide a mechanistic explanation for the suppression of LBP-1b-mediated transcription activation by LBP-9.

It is interesting that expression of LBP-9, like CRTR-1, is developmentally regulated in contrast to other CP2 family members. Participation of CRTR-1 and LBP-9 in heteromeric protein complexes with other CP2 family members might facilitate dominant repression of CP2 family regulated promoters, providing a mechanism for developmental regulation of ubiquitously expressed CP2 family transcriptional activators.

4.3.2 CRTR-1 Contains a Novel Repression Domain.

The transcriptional repression activity of CRTR-1 was found to be localised to the N-terminal 52 amino acids. This region was necessary and sufficient for CRTR-1 mediated transcriptional repression and did not show significant homology to members of the CP2 family that activate transcription.

Transcriptional repression can be mediated through several different mechanisms such as interference with assembly of the transcriptional machinery (Johnson, 1995) or recruitment of co-repressors including histone deacetylases (Ng and Bird, 2000). Protein sequence motifs present in DNA-binding transcriptional repressors that mediate interaction with co-repressor proteins include PXDLS in the Ikaros protein (Koipally and Georgopoulos, 2000; Schaeper *et al.*, 1995; Schaeper *et al.*, 1998), WRPW in Hairy-related bHLH proteins (Castella *et al.*, 2000; Chen *et al.*, 1999a) and a Gly/Arg-rich sequence present in the transcription factor YY1 (Yang *et al.*, 1996). Further, a histone deacetylase-independent mechanism of transcriptional repression has been described for methyl-CpG-binding protein 2 that is dependent on the presence of a conserved 30 amino acid sequence that contains two clusters of basic amino acids (Yu *et al.*, 2000). These motifs could not be identified within the N-terminal 52 amino acids of CRTR-1, suggesting a novel mechanism

of transcriptional repression for this protein. Conservation of repressor activity in cell lines of diverse origin and properties such as ES cells, 293T cells and COS-1 cells suggests that factors required for CRTR-1-mediated repression are widely expressed.

CHAPTER 5

Interactions between CRTR-1 and CP2 family members.

5.1 INTRODUCTION

Formation of homomeric complexes by CP2 family members has been described for CP2 (Uv *et al.*, 1994), LBP-1c (Zhong *et al.*, 1994) and chicken CP2 (Murata *et al.*, 1998). Similarly, electrophoretic mobility shift assays (EMSA) demonstrated that LBP-1c can bind DNA as a dimer (Jane *et al.*, 1995; Shirra *et al.*, 1994; Zhong *et al.*, 1994) or tetramers (Murata *et al.*, 1998; Shirra and Hansen, 1998; Shirra *et al.*, 1994). It has been proposed that tetramer formation from a dimer of dimers that exists in solution may facilitate a stable DNA interaction (Shirra and Hansen, 1998).

Formation of hetero-multimers between CP2 family members has also been demonstrated. Specifically, LBP-1a, b, and c have been shown to interact with each other in DNA binding protein complexes *in vitro* (Yoon *et al.*, 1994). Formation of complexes by LBP-1d and other CP2 family members has not been investigated directly, however, interaction between LBP-1d and LBP-1a has been implied by a reduction in LBP-1a DNA binding in the presence of excess LBP-1d (Yoon *et al.*, 1994).

The ability of CRTR-1 to interact with other CP2 family members would result in the formation of protein complexes containing both transcriptional repressors and activators. The outcome of this possible interaction could provide a mechanism for regulation of CP2 family member transcriptional activation.

5.2 DIRECT INTERACTION OF CRTR-1 WITH LBP-1c AND LBP-1a.

Several studies have defined a dimerisation domain of LBP-1c and LBP-1 between amino acids 260 and 247 to the C-terminus (referred to as LBP-1c-DD and LBP-1a-DD) that can be used to investigate the protein binding abilities of CP2 family members by GST-pull down and yeast-2-hybrid (Ramamurthy *et al.*, 2001; Tuckfield *et al.*, 2002b; Wilanowski *et al.*, 2002; Zhou *et al.*, 2000).

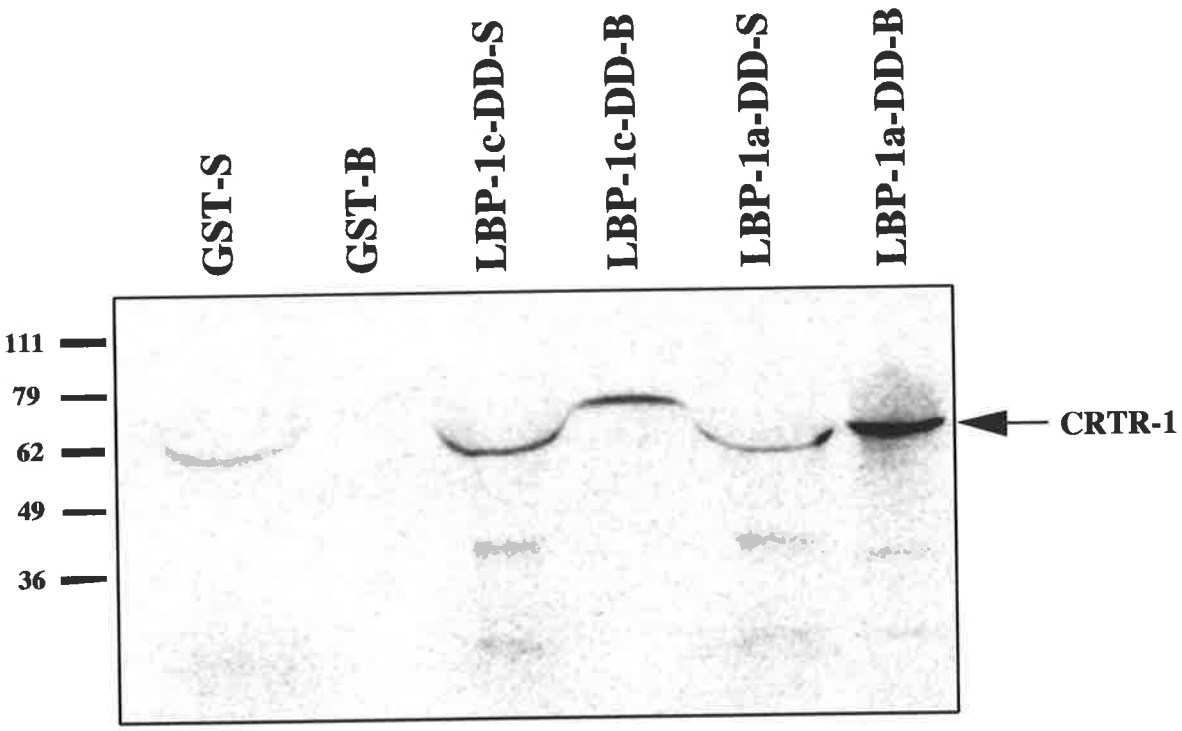
GST-pull down assays were carried out to investigate interactions between CRTR-1 with LBP-1c and LBP-1a, which are both activators of transcription (Huang and Miller, 2000; Kim *et al.*, 1988; Kim *et al.*, 1990; Kim *et al.*, 1987; Lim *et al.*, 1993; Lim *et al.*, 1992). BL21 *E.coli* were obtained from Dr. Stephen Jane (Rotary bone marrow research laboratories, Royal Melbourne Hospital, Victoria, Australia) transformed with pGEX-6P2, pGEX-6P2-LBP-1c-DD and pGEX-6P2-LBP-1a-DD, constructs for expression of GST and the dimerisation domains of LBP-1c and LBP-1a as GST fusion proteins, respectively. Expression of GST fusion proteins was induced with 0.1 mM IPTG for 2hrs at 37°C (2.3.6.1.5). Soluble lysates of induced bacteria were made (2.3.6.1.5) and 1 ml samples of each were incubated with 25 µL of pre-equilabrated glutathione sepharose beads at room temperature for 30 mins (2.3.6.8) to purify GST proteins. Beads were resuspended in 1 ml of binding buffer and mixed with 2 µL of ^{35}S-methionine labelled IvTT CRTR-1 (3.3.2 and Figure 3.8) for 1hr at room temperature. 20 µL samples of the binding reactions containing bound and unbound IvTT CRTR-1, referred to as the soup samples, were added to 20 µL of 2xSDS-gel loading buffer and retained for PAGE analysis. Beads were extensively washed with NETN buffer (2.2.5, 2.3.6.8) to remove unbound proteins and resuspended in 2xSDS-gel loading buffer (2.2.5). Proteins bound to the beads were separated by 10% SDS-PAGE (2.3.6.4) together with soup samples (Figure 5.1).

The presence of a band at 64 kDa, representative of IvTT CRTR-1, for binding reactions containing GST-LBP-1c-DD (Figure 5.1, lane 4) and GST-LBP-1a-DD (Figure 5.1, lane 6) immobilised to glutathione beads, illustrating that IvTT CRTR-1 was immobilised with GST-LBP-1c-DD and GST-LBP-1b-DD demonstrates the formation of protein/protein interactions between these proteins. The specificity of this interaction was confirmed by the absence of bound IvTT CRTR-1 for reactions containing GST immobilised to glutathione beads (Figure 5.1, lane 2). These results also demonstrate that

FIGURE 5.1

CRTR-1 interacts with LBP-1c and LBP-1a.

BL21 *E.coli* transformed with pGEX-6P2, pGEX-6P2-LBP-1c-DD or pGEX-6P2-LBP-1a-DD were induced to express GST fusion proteins by addition of IPTG (2.3.6.1.5). Expressed proteins, GST, GST-LBP-1c-DD and GST-LBP-1a-DD, were immobilised to glutathione beads (2.3.6.8) and incubated at room temperature for 1 hr with 2 µL of ^{35}S-methionine labelled IvTT CRTR-1. 20 µL samples of the binding reactions containing bound and unbound IvTT CRTR-1, referred to as the soup samples, were collected. Beads were then washed extensively, resuspended in 2xSDS-looad buffer and bound proteins were separated by 10% SDS-PAGE (2.3.6.4) together with the soup samples. Markers; 10 µL of Benchmark pre-stained protein ladder (2.2.14). S, soup samples; B, bound proteins.


GST-S
GST-B
LBP-1c-DD-S
LBP-1c-DD-B
LBP-1a-DD-S
LBP-1a-DD-B
111
79
62
49
36
CRTR-1

the region required for LBP-1c and LBP-1a to interact with CRTR-1 is equivalent to that demonstrated for their ability to interact with other CP2 family members.

5.3 YEAST-2-HYBRID ANALYSIS OF CP2 FAMILY PROTEIN INTERACTIONS.

Investigation into the ability of two proteins to interact by yeast-2-hybrid analysis has been used widely for the determination of binding partners for many protein families including the CP2 family (Uv *et al.*, 1994). Yeast-2-hybrid experiments conducted throughout this thesis used the Matchmaker Gal4 two-hybrid system 3 (Clontech). pGBKT7 (2.2.6.1) contains the Gal4 DNA binding domain (DBD), amino acids 1-147, linked to a multiple cloning site for in-frame insertion of cDNAs encoding the 'bait' protein. This vector contains a tryptophan selection marker. pGADT7 (2.2.6.1) contains the Gal4 transcriptional activation domain (AD), amino acids 768-881, linked to a multiple cloning site for in-frame insertion of cDNAs encoding the 'prey' protein. This vector contains a leucine selection marker. Both vectors contain T7 polymerase promoters allowing IvTT to be carried out, confirming expression of full length proteins. The matchmaker system uses yeast strain AH109 (2.2.11) which contains four reporter genes under the exclusive control of heterologous Gal4 up-stream activation sequences (UAS): *his3* (required for histidine biosynthesis), *ade2* (required for adenine biosynthesis) and *mel1* and *lacZ* encoding α-galactosidase and β-galoctosidase, respectively.

AH109 yeast co-transformed with pGBKT7 and pGADT7 based vectors can be selected for growth on minimal media plates lacking leucine (-leu) and tryptophan (-trp). Interaction between the Gal4-AD fusion protein and the Gal4-DBD fusion protein which occurs if the bait and prey proteins can interact, positions the Gal4-AD at the promoter of the reporter genes, thereby activating expression of reporter genes and enabling growth of the yeast at medium, -leu/-trp/-his, or stringent, -leu/-trp/-his/-ade, selection pressures (Figure 5.2a).

FIGURE 5.2

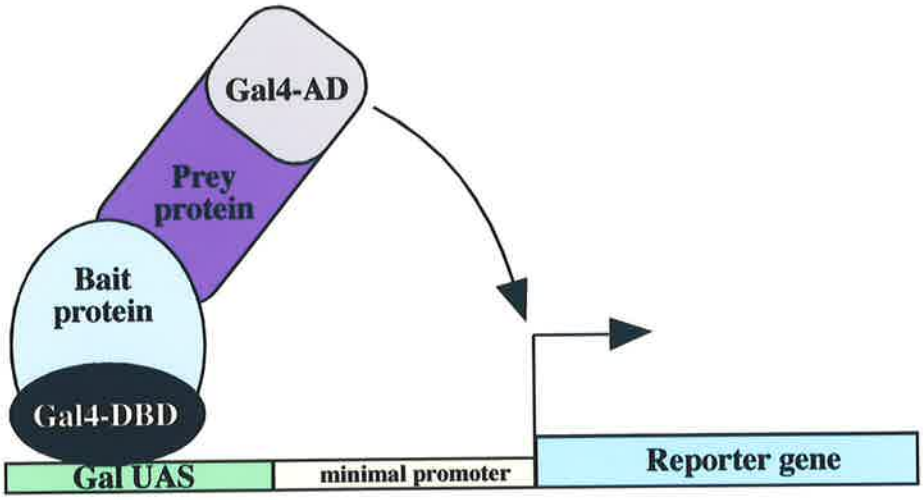
Overview of the Matchmaker Gal4 two-hybrid system 3 and validation of pGB-CRTR-1(47-481) and pGAD-CRTR-1(47-481) by IvTT.

A) The Gal4-DNA binding domain (amino acids 1-147), which binds to the heterologous Gal4 UAS upstream of reporter genes, is expressed as a fusion with the 'bait' protein. The Gal4-activation domain (amino acids 768-881) is expressed as a fusion with the prey protein. Interaction of the bait and prey proteins positions the Gal4-activation domain at the promoter where it activates transcription of the gene and growth of yeast.

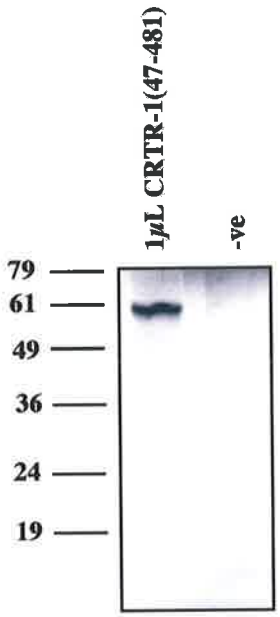
Modified from 'Matchmaker Gal4 two-hybrid system 3 and libraries user manual' (Clontech)

B) CRTR-1(47-481) was synthesised and ^{35}S-methionine labelled by IvTT (2.3.6.7) from 1 µg of pGB-CRTR-1(47-481). 1 µL of IvTT CRTR-1(47-481) separated by 10% SDS-PAGE (2.3.6.4), dried down and visualised by autoradiography. –ve, 3µL negative control IvTT reaction without DNA. Markers, 10 µL of benchmark pre-stained protein ladder (2.2.14),

A


Gal4-AD
Prey protein
Bait protein
Gal4-DBD
Gal UAS
minimal promoter
Reporter gene

B


1μL CRTR-1(47-481)
-ve
79
61
49
36
24
19

5.3.1 Construction of CP2 Family Expression Vectors for Yeast-2-Hybrid Analysis.

The region required for LBP-1c to activate transcription has been localised to the N-terminal 40 amino acids (Ramamurthy *et al.*, 2001; Tuckfiled, 2002a). As this region is likely to form the transcriptional regulatory region of CRTR-1, constructs for the expression of CRTR-1 fusion proteins excluded this domain but included the regions implicated in DNA binding and oligomerisation in CP2 family members.

A cDNA fragment encoding amino acids 47-481 of CRTR-1 was obtained by *Sal*I/*Sac*I digestion (2.3.1.1) of pGalO.CRTR-1(47-481) (4.2.1) which was blunt ended (2.3.1.4) and cloned into pGBKT7 and pGADT7 vectors digested with *Sma*I and CIP treated (2.3.1.5) generating, pGB-CRTR-1(47-481) and pGAD-CRTR-1(47-481), respectively. In frame fusions were determined by *Eco*RI digestion and automated BDT sequencing (2.3.1.14) using the RACE primer (2.2.7.2).

Cloned cDNA fragments for CP2-DD, NF2d9-DD and altNF2d9-DD were excised from pGEM-T-CP2-DD, pGEM-T-NF2d9-DD and pGEM-T-altNF2d9-DD, respectively, by *Sal*I/*Hind*III digestion (2.3.1.1). Fragments were then blunt ended (2.3.1.4) and cloned into pGBKT7 and pGADT7 that had been digested with *Sma*I and CIP treated (2.3.1.5) generating, pGB-CP2-DD, pGB-NF2d9-DD, pGB-altNF2d9-DD, pGAD-CP2-DD, pGAD-NF2d9-DD and pGAD-altNF2d9-DD. The altCP2-DD cDNA fragment was PCR amplified using platinum Taq (2.3.1.12) and pGEM-T-altCP2-DD as the template with primers CP2SR8 and CP2SR9 (2.2.7.2). The 880bp PCR product was isolated, cloned into pGEM-T easy, excised by *Eco*RI digestion, isolated by gel purification (2.3.1.2) and cloned into pGBKT7 and pGADT7 vectors that had been digested with *Eco*RI and CIP treated to generate pGB-altCP2-DD and pGAD-altCP2-DD.

IvTT of pGB-CRTR-1(47-481) was carried out to ensure translation of the entire ORF. 1 μg of plasmid DNA was used in a 40 μl TNT Quick coupled IvTT reaction (2.3.6.7) and 1 μl of the reaction was separated on a 10% SDS-PAGE (2.3.6.4) (Figure 5.2b). A single band at the approximate expected size of 50KDa was observed (lanes 1) dependent on priming of the IvTT reaction with pGB-CRTR-1(47-481) as no product was observed in the negative IvTT control reaction without DNA (lane 2), confirming that CRTR-1(47-481) was translated to full length. Equivalent results were obtained for IvTT of pGAD-CRTR-1(47-481), pGB-CP2-DD, pGB-altCP2-DD, pGB-NF2d9-DD and pGB-altNF2d9-DD (data not shown).

AH109 yeast were transformed (2.3.4.3) with pGB-CRTR-1(47-481) and plated onto -trp selection plates. After 4 days culture at 30°C colonies were picked and streaked onto -trp/-his, -trp/-ade and -trp/-his/-ade yeast selection plates (2.2.12) and grown at 30°C for 5 days. No growth was observed for yeast transformed with pGB-CRTR-1(47-481) on any of the selection plates (Figure 5.3) demonstrating that amino acids 47-481 of CRTR-1 does not contain a region capable of auto-activating the growth selection marker genes. Equivalent results were obtained for transformation of AH109 yeast with pGB-CP2-DD, pGB-altCP2-DD, pGB-NF2d9-DD and pGB-altNF2d9-DD (data not shown).

Each pGAD construct was co-transformed (2.3.4.3) with pLAM 5'-1 (2.2.6.1, Clontech), a negative control vector expressing a Gal4-DBD human lamin C fusion protein into AH109 yeast, and plated on -leu/-trp selection plates. After 4 days culture at 30°C, colonies were streaked onto -leu/-trp/-his selection plates and cultured for a further 4 days at 30°C. The absence of yeast growth confirmed that Gal4-AD fusion proteins did not interact with lamin C, as expected, and therefore yeast growth observed by co-transformation with pGB vectors would be indicative of specific protein interactions (data not shown).

FIGURE 5.3

Gal4-DBD-CRTR-1(47-481) does not auto-activate AH109 reporter genes.

A) AH109 yeast were transformed (2.3.4.3) with pGB-CRTR-1(47-481), plated on -trp selection plates and incubated at 30°C for 5 days. Colonies were then streaked onto -trp, -trp/-his, -trp/-ade and -trp/-his/-ade selection plates and incubated at 30°C for 5 days.

B) AH109 yeast were co-transformed with pTD1-1/pVA3-1, pGB-CRTR-1(47-481)/pGADT7, and pGBKT7/pGADT7 plated on -leu/-trp selection plates and incubated at 30°C for 5 days. pTD1-1 and pVA3-1 expressing Gal4-AD-SV40 large T-antigen and Gal4-DBD-p53 fusion proteins, respectively (Clontech) known to efficiently interact, were used as a positive control confirming the yeast growth selection pressure.

Plates **(A)** and **(B)** were scanned using a HP scanjet 7400c scanner.

A

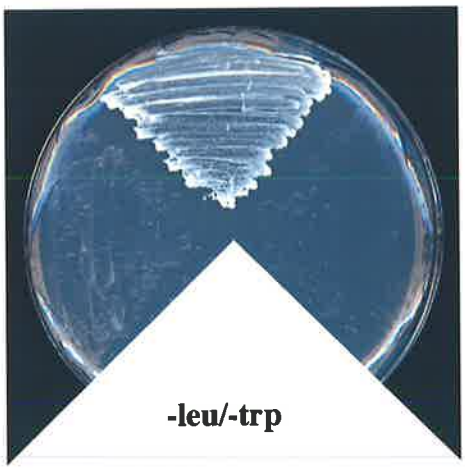
-trp

-trp/-his

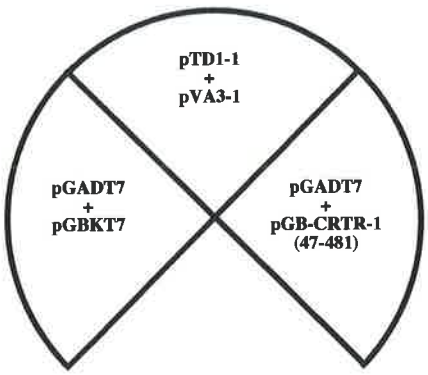
-trp/-ade

-trp/-his/-ade

B



-leu/-trp



5.3.2 Specific Interactions Between CP2 Family Members.

pGB and pGAD constructs were co-transformed (2.3.4.3) in various combinations into AH109 yeast and plated onto -leu/-trp selection plates (2.2.12) and incubated at 30°C for 4 days. Colonies from each plate were streaked onto -leu/-trp/-his and -leu/-trp/-his/-ade selection plates (2.2.12) and incubated at 30°C for a further 5 days. Results for these experiments are presented in Figures 5.4-5.8 and summarised in Figure 5.9.

5.3.2.1 CRTR-1 protein interactions.

Yeast co-transformed (2.3.4.3) with pGB-CRTR-1(47-481) and pGAD-CRTR-1(47-481), pGAD-CP2-DD, pGAD-altCP2-DD, pGAD-NF2d9-DD or pGAD-altNF2d9-DD grew on -leu/-trp/-his and -leu/-trp/-his/-ade plates (2.2.12) (Figure 5.4) demonstrating that CRTR-1 can form homomeric interactions and heteromeric interactions with CP2, altCP2, NF2d9 and altNF2d9. Further, this verifies that the region of CRTR-1 required for interaction with other CP2 family members is contained within amino acids 47-481. Yeast co-transformed with pGB-CRTR-1(47-481) and pGAD-CP2-DD or pGAD-altCP2-DD formed dense pink colonies. The change of colour of yeast colonies from white to pink when plated on -ade selection plates is indicative of a deficiency in adenine, suggesting that the ade selection gene in these yeast was not fully activated. This observation could be representative of weaker CRTR-1/CP2 and CRTR-1/altCP2 protein interaction compared to CRTR-1/CRTR-1, CRTR-1/NF2d9 and CRTR-1/altNF2d9.

5.3.2.2 CP2 and altCP2 protein interactions.

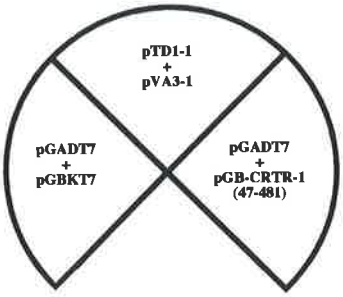
Yeast co-transformed (2.3.4.3) with pGB-CP2-DD and pGAD-CP2-DD, pGAD-altCP2-DD, pGAD-NF2d9-DD, pGAD-altNF2d9-DD or pGAD-CRTR-1(47-481) grew on -leu/-trp/-his plates (Figure 5.5) demonstrating that CP2 can form homomeric interactions and heteromeric interactions with CRTR-1, altCP2, NF2d9 and altNF2d9. Streaking co-

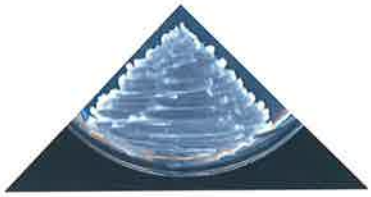
FIGURE 5.4

CRTR-1 forms homomeric and heteromeric protein interactions with members of the CP2 family.

AH109 yeast were co-transformed (2.3.4.3) with pGB and pGAD based vectors as indicated (right) and plated on -leu/-trp selection plates and incubated at 30°C for 5 days. Colonies were then streaked onto -leu/-trp/-his and -leu/-trp/-his/-ade selection plates as indicated (left) and incubated at 30°C for 5 days. Plates were scanned using a HP scanjet 7400c scanner.

-leu/-trp/-his

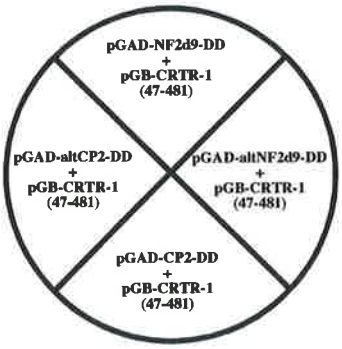




-leu/-trp/-his

-leu/-trp/-his/-ade

-leu/-trp/-his



-leu/-trp/-his/-ade

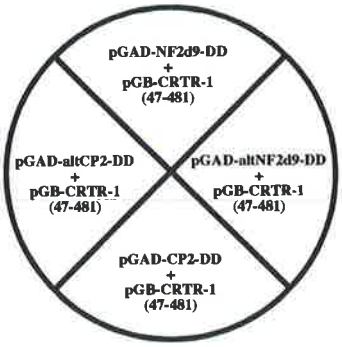
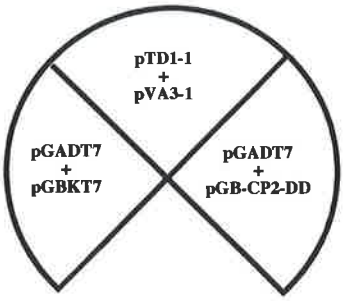


FIGURE 5.5

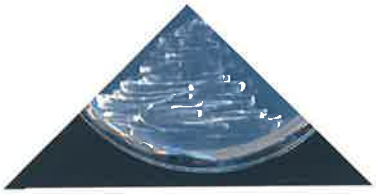
CP2 forms homomeric and heteromeric protein interactions with members of the CP2 family.

AH109 yeast were co-transformed (2.3.4.3) with pGB and pGAD vectors as indicated (right). Yeast were grown and analysed as described for figure 5.4.

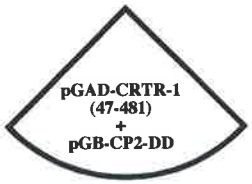
-leu/-trp/-his



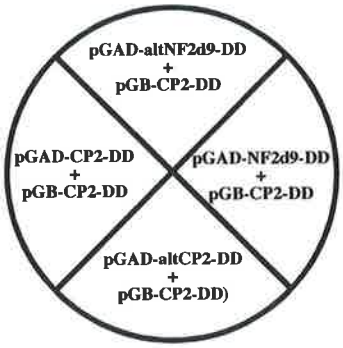
-leu/-trp/-his



-leu/-trp/-his/-ade



-leu/-trp/-his



-leu/-trp/-his/-ade

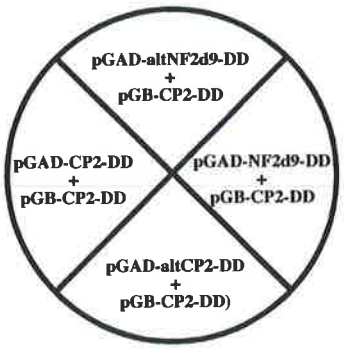
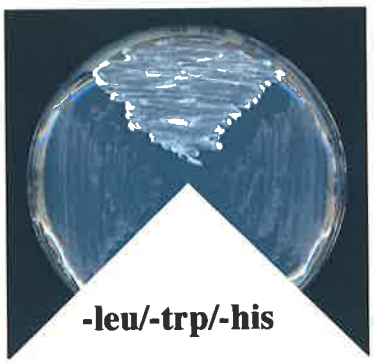


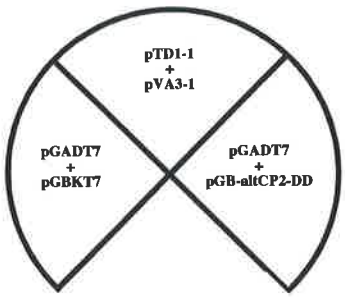
FIGURE 5.6

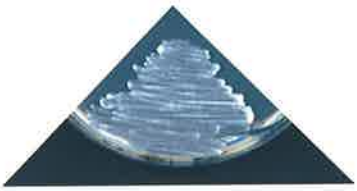
altCP2 forms homomeric and heteromeric protein interactions with members of the CP2 family.

AH109 yeast were co-transformed (2.3.4.3) with pGB and pGAD vectors as indicated (right). Yeast were grown and analysed as described for figure 5.4.

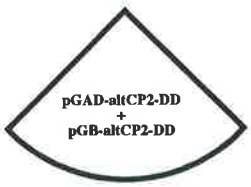


-leu/-trp/-his

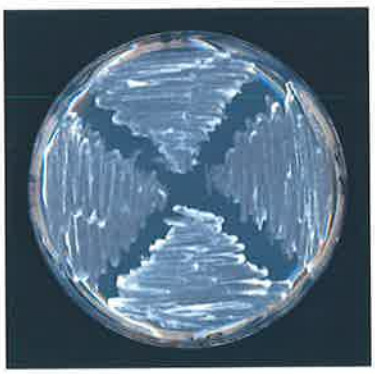




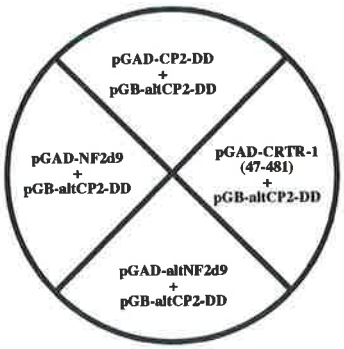
-leu/-trp/-his



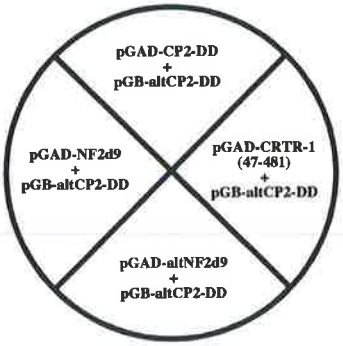
-leu/-trp/-his/-ade



-leu/-trp/-his



-leu/-trp/-his/-ade



transformed yeast on -leu/-trp/-his/-ade selection plates (2.2.12) resulted in formation of dense white colonies for yeast co-transformed with pGB-CP2-DD and pGAD-CP2-DD, pGAD-altCP2-DD and pGAD-altNF2d9-DD confirming the interaction between CP2/CP2, CP2/altCP2 and CP2/altNF2d9. Yeast co-transformed with pGB-CP2-DD and pGAD-CRTR-1(47-481) or pGAD-NF2d9-DD showed sparse pale and dark pink colonies, respectively, suggesting weaker CP2/CRTR-1 and CP2/NF2d9 protein interactions. This observation was consistent with results in 5.3.2.1.

Similar observations were made for yeast co-transformed with pGB-altCP2-DD and pGAD-CP2-DD, pGAD-altCP2-DD, pGAD-NF2d9-DD, pGAD-altNF2d9-DD or pGAD-CRTR-1(47-481) (Figure 5.6). This demonstrates that the absence of amino acids at the C-terminus of altCP2, present in CP2, does not affect the protein/protein interaction ability of altCP2 with other CP2 family members.

5.3.2.3 NF2d9 and altNF2d9 protein interactions.

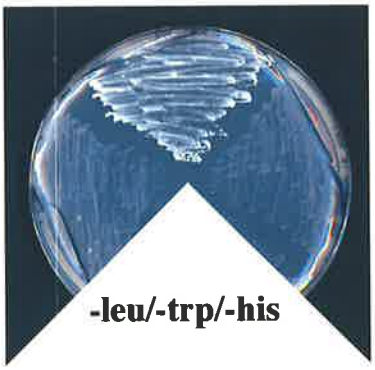
Yeast co-transformed (2.3.4.3) with pGB-NF2d9-DD and pGAD-CP2-DD, pGAD-altCP2-DD, pGAD-NF2d9-DD, pGAD-altNF2d9-DD or pGAD-CRTR-1(47-481) grew on -leu/-trp/-his plates (2.2.12) (Figure 5.7), however growth was restricted compared to other combinations. This result demonstrates that NF2d9 can form homomeric and heteromeric interactions with CRTR-1. Restricted growth of yeast co transformed with pGB-NF2d9-DD and pGAD-CP2-DD, pGAD-altCP2-DD or pGAD-altNF2d9-DD suggested the formation of weak heteromeric protein interactions between NF2d9 and CP2, altCP2 and altNF2d9.

Streaking co-transformed yeast on -leu/-trp/-his/-ade selection plates resulted in formation of dense white colonies for yeast co-transformed with pGB-NF2d9-DD and pGAD-CRTR-1(47-481) and pGAD-NF2d9-DD confirming the strength of the NF2d9/CRTR-1 and NF2d9/NF2d9 interaction (Figure 5.7). Yeast co-transformed with pGB-NF2d9 and pGAD-altNF2d9-DD, pGAD-CP2-DD and pGAD-altCP2-DD showed

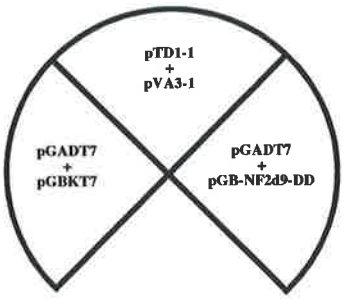
FIGURE 5.7

NF2d9 forms homomeric and heteromeric protein interactions with members of the CP2 family.

AH109 yeast were co-transformed (2.3.4.3) with pGB and pGAD vectors as indicated (right). Yeast were grown and analysed as described for figure 5.4.



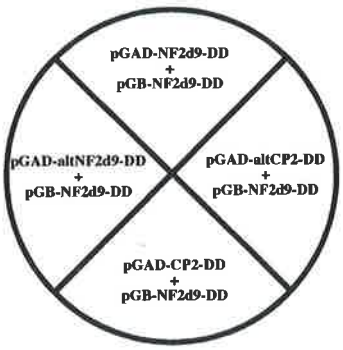
-leu/-trp/-his



-leu/-trp/-his

-leu/-trp/-his/-ade

-leu/-trp/-his



-leu/-trp/-his/-ade

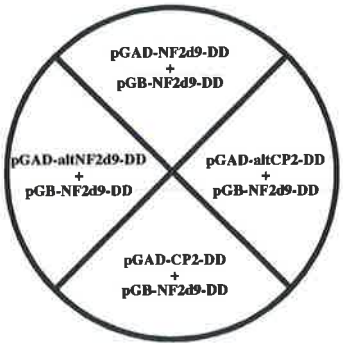
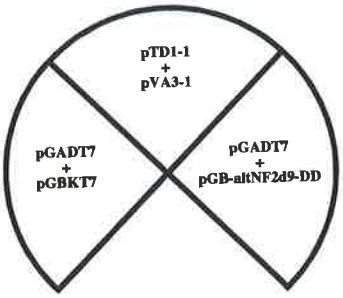


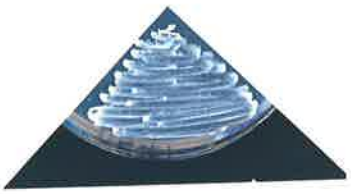
FIGURE 5.8

altNF2d9 forms homomeric and heteromeric protein interactions with members of the CP2 family.

AH109 yeast were co-transformed (2.3.4.3) with pGB and pGAD vectors as indicated (right). Yeast were grown and analysed as described for figure 5.4.

-leu/-trp/-his

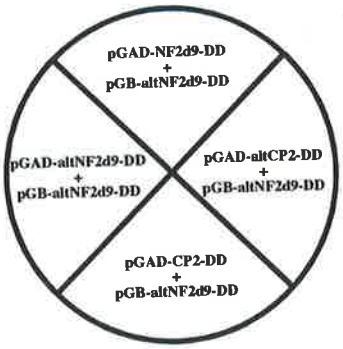




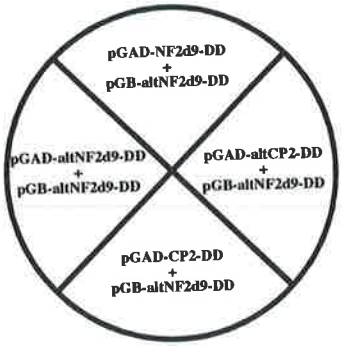
-leu/-trp/-his

-leu/-trp/-his/-ade

-leu/-trp/-his



-leu/-trp/-his/-ade



very little growth, confirming the weak interaction between NF2d9/altNF2d9 and NF2d9/altCP2 and suggesting that the NF2d9/CP2 interaction may also be weak.

Similar observations were made for yeast co-transformed with pGB-altNF2d9-DD and pGAD-CP2-DD, pGAD-altCP2-DD, pGAD-NF2d9-DD, pGAD-altNF2d9-DD or pGAD-CRTR-1(47-481) (Figure 5.8). This demonstrates that the additional 36 amino acids to the central region altNF2d9, not present in NF2d9, does not affect the protein/protein interaction ability of altNF2d9 with other CP2 family members.

5.4 CO-PURIFICATION OF CRTR-1 AND CP2 FAMILY MEMBERS.

Proposed interactions between CRTR-1 and CP2 family members were tested using co-purification experiments in COS-1 cells to validate the specificity of protein interactions deduced from yeast-2-hybrid experiments.

5.4.1 Generation of Tagged Fusion Proteins for Purification and Identification.

To enable purification and identification, CP2 family members were fused to 6xHis (Crowe *et al.*, 1995) and FLAG (Brizzard *et al.*, 1994) moieties expressed at the N- and C-terminus of ORFs respectively. Specific detection of CRTR-1 was achieved by N-terminal fusion with a Haemaglutinin epitope (Howard *et al.*, 1995).

Plasmids encoding full length CP2 and altCP2 ORFs fused with N-terminal 6xHis and C-terminal FLAG epitopes were generated by platinum Taq PCR (2.3.1.12) using primers CP2SR10 and CP2SR11 (2.2.7.2) on pGEM-CP2-FL and pGEM-altCP2-FL (3.6). Plasmids encoding full length NF2d9 and altNF2d9 ORFs fused with N-terminal 6xHis and C-terminal FLAG epitopes were generated by platinum Taq PCR (2.3.1.12) using primers NF2d9SR10 and NF2d9SR11 (2.2.7.2) on pGEM-NF2d9-FL and pGEM-altNF2d9-FL (3.6). PCR products were cloned into pGEM-T easy (2.2.6.1, Promega). CP2 and altCP2 fragments were excised from pGEM-T easy by *Eco*RI digestion (2.3.1.1) and cloned into pXMT2 *Eco*RI digested and CIP treated (2.3.1.5) generating pXMT2-6xHis-CP2-FLAG

FIGURE 5.9

Summary of CP2 family homomeric and heteromeric protein interactions analysed by yeast-2-hybrid.

Comparative assessment of growth of AH109 yeast co-transformed with pGB and pGAD vectors, as indicated (Figures 5.4-5.8). +++++; strong growth, +: poor growth.

	pGB-CP2(250-501)	pGB-altCP2(250-501)	pGB-NF2d9(247-504)	pGB-altNF2d9(247-504)	pGB-CRTR-1(47-481)
pGAD-CP2(250-501)	+++++	+++++	+++	+++++	+++
pGAD-altCP2(250-501)	+++++	+++++	++++	++++	++++
pGAD-NF2d9(247-504)	+	+	+++++	+++	+++++
pGAD-altNF2d9(247-504)	+	+	+	+++++	+++++
pGAD-CRTR-1(47-481)	+++	+++	+++++	+++++	+++++

and pXMT2-6xHis-altCP2-FLAG. NF2d9 and altNF2d9 fragments were excised from pGEM-T easy by *Xho*I digestion and cloned into pXMT2 *Xho*I digested and CIP treated, generating pXMT2-6xHis-NF2d9-FLAG and pXMT2-6xHis-altNF2d9-FLAG. The identity of clones was confirmed by BDT automated sequencing (2.3.1.14) across the multiple cloning site.

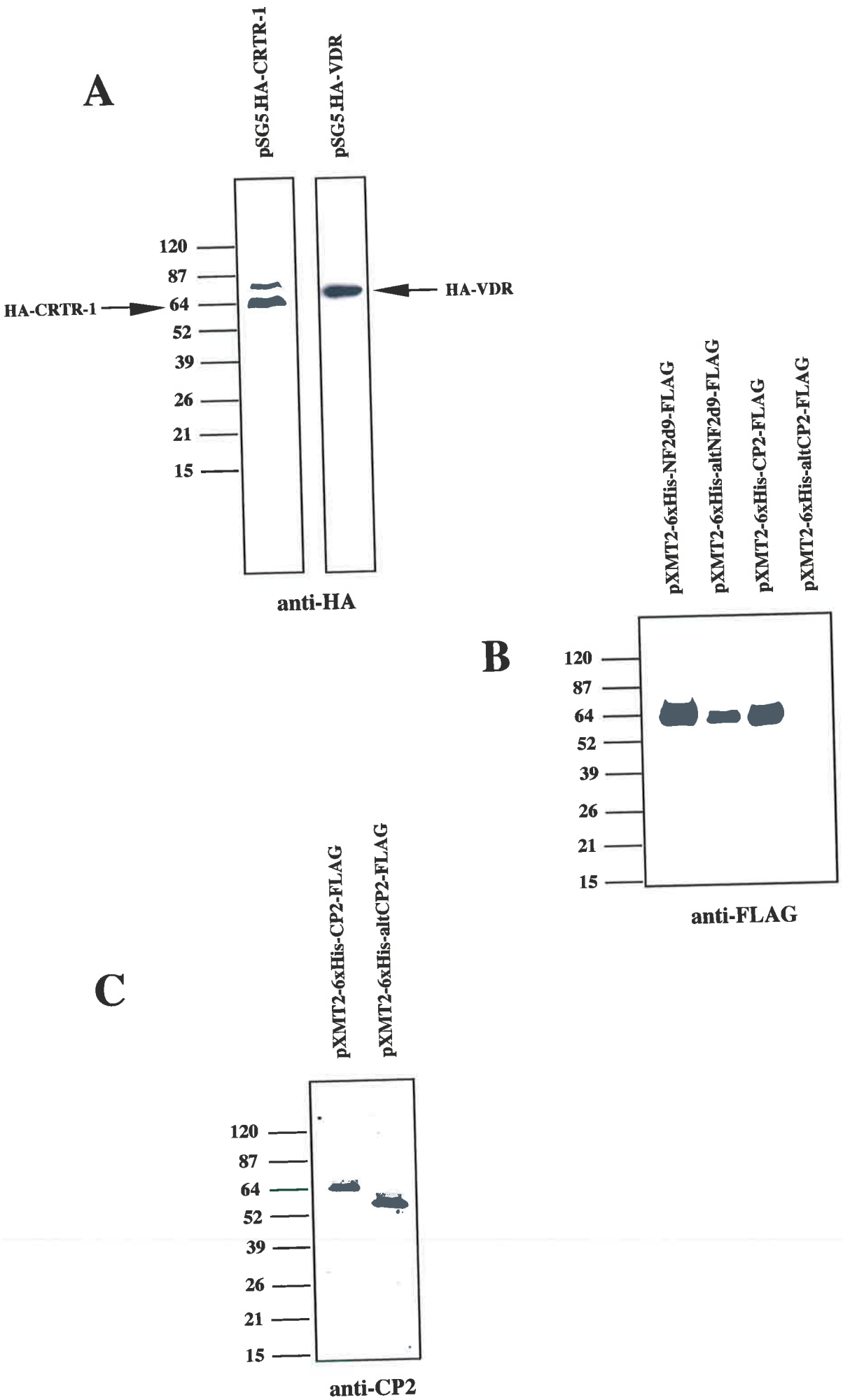
A plasmid encoding full length CRTR-1 ORF fused N-terminally with the HA epitope was generated by platinum Taq PCR (2.3.1.12) with primers SR28 and SR2 (2.2.7.2) on pGEM-T-*CRTR-1* (3.3.1). The PCR product was purified (2.3.1.3), cloned into pGEM-T easy, excised by *Xho*I/*Hind*III digestion, blunt ended (2.3.1.4) and cloned into pSG5.HA (2.2.6.1) (Chen *et al.*, 1999b) cut with *Xho*I, blunt ended (2.3.1.4) and CIP treated (2.3.1.5), generating pSG5.HA-CRTR-1. Clones were confirmed by BDT automated sequencing (2.3.1.14). pSG5.HA-VDR (kindly provided by Dr. Brian May, Department of Molecular Biosciences, The University of Adelaide), which directs expression of HA tagged vitamin D-receptor was used as a negative control.

To test the expression of fusion proteins, $3x10^6$ COS-1 cells, grown in a 60 mm diameter tissue culture dish, were transfected with 2 μg of each vector (2.4.5). Whole cell protein extracts were made 24 hours post-transfection (2.3.6.2), separated by 10% SDS-PAGE (2.3.6.4) and transferred to protran nylon filters for western analysis (2.3.6.6). Membranes probed with the mouse anti-HA monoclonal antibody (2.2.8) detected the expected bands at 64 kDa for HA-CRTR-1 and 70 kDa for HA-VDR (Figure 5.10a). Significance of the less abundant, 74 kDa band produced in pSG5.HA-CRTR-1 transfected cells is unclear but may represent a post-translationally modified form of CRTR-1. Membranes probed with the mouse anti-FLAG monoclonal antibody (2.2.8) detected expected bands of 64 kDa for 6xHis-NF2d9-FLAG, 67 kDa for 6xHis-altNF2d9-FLAG and 64 kDA for 6xHis-CP2-FLAG (Figure 5.10b). Membranes probed with the polyclonal rabbit anti-CP2 antibody (2.2.8) raised from immunisation of a rabbit with the region of

FIGURE 5.10

Validation of expression constructs generated for co-purification experiments.

$3x10^5$ COS-1 cells, grown in 60 mm diameter tissue culture dishes, were transfected with 2 μg of pSG5.HA-CRTR-1, pSG5.HA-VDR, pXMT2-6xHis-NF2d9-FLAG, pXMT2-6xHis-altNF2d9-FLAG, pXMT2-6xHis-CP2-FLAG or pXMT2-6xHis-altCP2-FLAG as indicated (2.4.5). Whole cell extracts were produced 24 hrs post transfection (2.3.6.2) and separated by 10% SDS-PAGE (2.3.6.4) prior to western analysis (2.3.6.6). Filters were probed with mono-clonal mouse anti-HA (A), mono-clonal mouse anti-FLAG (B) and poly-clonal rabbit anti-CP2 (C) antibodies prior to incubation with horse radish peroxidase (HRP) conjugated rabbit anti-mouse (A) and (B) or HRP conjugated goat anti-rabbit (C) secondary antibody (2.2.8). Filters were visualised using chemiluminescence (2.3.6.6). Markers; 10μL of Benchmark pre-stained protein ladder (2.2.14)


A
pSG5.HA-CRTR-1
pSG5.HA-VDR
120
87
64
52
39
26
21
15
HA-CRTR-1
HA-VDR
anti-HA
B
pXMT2-6xHis-NF2d9-FLAG
pXMT2-6xHis-altNF2d9-FLAG
pXMT2-6xHis-CP2-FLAG
pXMT2-6xHis-altCP2-FLAG
120
87
64
52
39
26
21
15
anti-FLAG
C
pXMT2-6xHis-CP2-FLAG
pXMT2-6xHis-altCP2-FLAG
120
87
64
52
39
26
21
15
anti-CP2

CP2 encompassing amino acids 133-395 (Lim *et al* 1992) which is common to both CP2 and altCP2, showed bands at 64 kDa and 54 kDa consistent with the expected sizes of 6xHis-CP2-FLAG and 6xHis-altCP2-FLAG, respectively (Figure 5.10c).

5.4.2 Co-purification of CRTR-1 with CP2 Family Members.

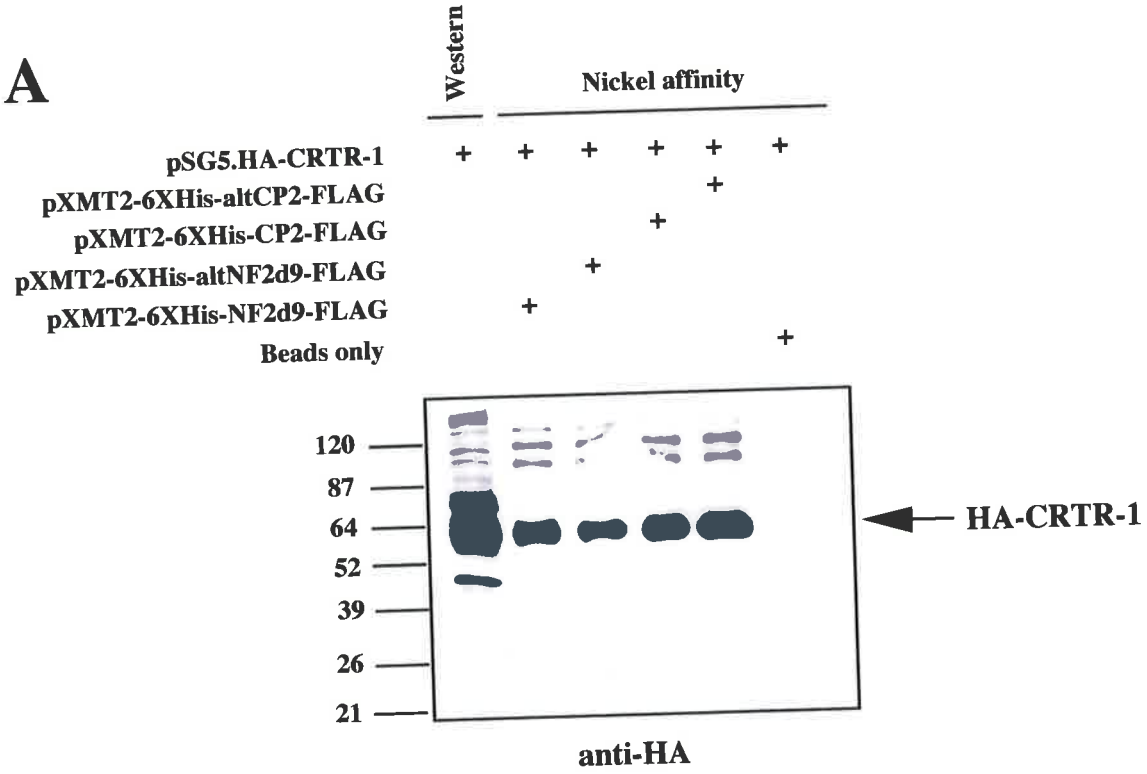
pSG5.HA-CRTR-1 was co-transfected into $3x10^6$ COS-1 cells cultured in a 60 mm diameter tissue culture dish with pXMT2-6xHis-CP2-FLAG, pXMT2-6xHis-altCP2-FLAG pXMT2-6xHis-NF2d9-FLAG or pXMT2-6xHis-altNF2d9-FLAG (2.4.5). 24 hours post transfection, whole cell extracts were produced (2.3.6.2) and nickel affinity purification of CP2 family members by the addition of talon beads (Clontech) in the presence of 5 mM imidazole was carried out (2.3.6.3). Beads were washed several times with 1ml volumes of lysis buffer and resuspended in 40 µL 2xSDS load buffer (2.2.5), separated by 10% SDS-PAGE (2.3.6.4) and transferred to protran nylon filters for western analysis (2.3.6.6) with appropriate antibodies (2.2.8). Co-transfection of 6xHis- and FLAG- tagged CP2 family members with pSG5.HA-VDR was used as a negative control.

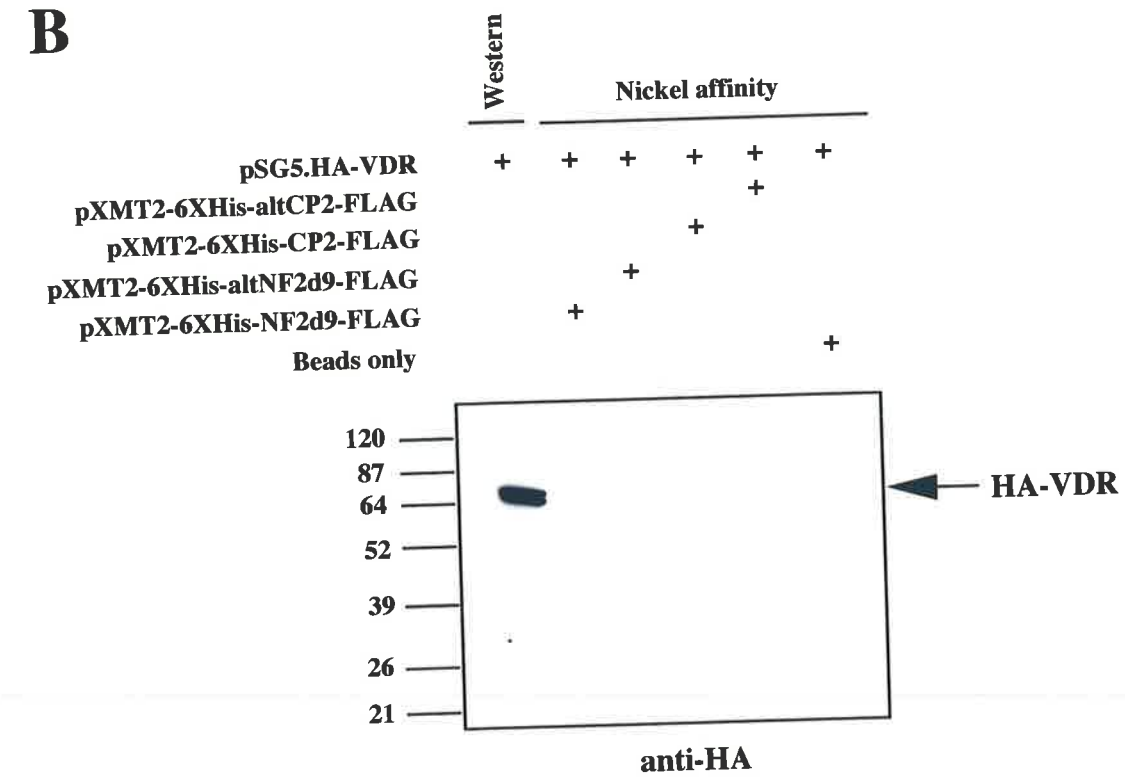
Probing of extracts from cells co-transfected with pSG5.HA-CRTR-1 and pXMT2-6xHis-NF2d9-FLAG, pXMT2-6xHis-altNF2d9-FLAG, pXMT2-6xHis-CP2-FLAG and pXMT2-6xHis-altCP2-FLAG with the anti-HA monoclonal antibody (2.2.8) revealed a band at 64 kDa consistent with the size predicted for HA-CRTR-1 (Figure 5.11a, lanes 1-5) indicative for co-purification of HA-CRTR-1 with 6xHis tagged proteins, confirming the ability of these proteins to interact in solution in transfected cells. No bands were detected in cell extracts from transfected COS-1 cells with pSG5.HA-CRTR-1 alone mixed with nickel affinity beads (lane 6) or HA-VDR co-transfected with 6XHis-NF2d9-FLAG, 6XHis-altNF2d9-FLAG, 6XHis-CP2-FLAG and 6XHis-altCP2-FLAG (Figure 5.11b). These observations confirm the specificity of the observed CRTR-1 co-purification with CP2 family members and demonstrate that CRTR-1 interacts with CP2, altCP2, NF2d9 and

FIGURE 5.11

Co-purification of CRTR-1 with CP2 family members.

2 μg of pSG5.HA-CRTR-1 (A) and pSG5.HA-VDR (B) were transfected in combination with 2 μg of pXMT2-6xHis-NF2d9-FLAG, pXMT2-6xHis-altNF2d9-FLAG, pXMT2-6xHis-CP2-FLAG or pXMT2-6xHis-altCP2-FLAG as indicated, into $3x10^5$ COS-1 cells grown in 60 mm diameter tissue culture dishes (2.4.4). Whole cell extracts were produced 24 hrs post transfection, nickel affinity purification of 6xHis tagged proteins carried out (2.3.6.3) and proteins separated by 10% SDS-PAGE (2.3.6.4) prior to western analysis (2.3.6.6). Filters were probed with monoclonal mouse anti-HA prior to incubation with horseradish peroxidase (HRP) conjugated rabbit anti-mouse secondary antibody (2.2.8) and visualised using chemiluminescence (2.3.6.6). Extracts of COS-1 cells transfected with pSG5.HA-CRTR-1 or pSG5.HA-VDR alone were included in the analysis as controls. Markers; 10 μL of Benchmark pre-stained protein ladder (2.2.14).


A
Western
Nickel affinity
pSG5.HA-CRTR-1
pXMT2-6XHis-altCP2-FLAG
pXMT2-6XHis-CP2-FLAG
pXMT2-6XHis-altNF2d9-FLAG
pXMT2-6XHis-NF2d9-FLAG
Beads only
120
87
64
52
39
26
21
HA-CRTR-1
anti-HA


B
Western
Nickel affinity
pSG5.HA-VDR
pXMT2-6XHis-altCP2-FLAG
pXMT2-6XHis-CP2-FLAG
pXMT2-6XHis-altNF2d9-FLAG
pXMT2-6XHis-NF2d9-FLAG
Beads only
120
87
64
52
39
26
21
HA-VDR
anti-HA

altNF2d9, consistent with results obtained by yeast-2-hybrid (5.3.2.1) and GST-pull down (5.2).

The higher band of 74 kDa present in the HA-CRTR-1 lane was not co-precipitated with CP2 family members suggesting a specific interaction with the 64 Kda HA-CRTR-1. The higher band observed may not be specific for HA-CRTR-1, or may represent a post-translationally modified form of CRTR-1 that is not able to bind CP2 family members.

5.5 DISCUSSION.

5.5.1 CRTR-1 Interacts with Members of the CP2 Family.

CRTR-1 has been shown to form heteromeric complexes efficiently with all members of the CP2 family by three different methods; GST pull down (Figure 5.1), yeast-2-hybrid (Figure 5.4) and co-purification from transfected COS-1 cells (Figure 5.11). Formation of such complexes is consistent with the homology shared between CRTR-1 and members of the CP2 family over the oligomerisation domain, amino acids 266-403 of LBP-1c (Shirra and Hansen, 1998). Furthermore, formation of heteromeric complexes not require the N-terminal 46 amino acids of CRTR-1 which were not present in pGB-CRTR-1(47-481) or pGAD-CRTR-1(47-481) used for yeast-2-hybrid analysis.

The ability of CRTR-1 to bind CP2 family members suggests that CRTR-1 exists within the cell in protein complexes containing other CP2 family members, which are expressed ubiquitously. The function of CRTR-1 may be to influence the overall biological activity of the CP2 family member complexes, perhaps at the level of DNA binding or transcriptional regulation. CRTR-1 has been shown to repress transcription (4.2.2), suggesting that interaction of CRTR-1 with other CP2 family members would result in formation of protein complexes containing both transcriptional repressors and activators. The outcome of this interaction could provide a mechanism for regulation of CP2 family member transcriptional activation. Conversely, the ability of CRTR-1 to form homomeric interactions, demonstrated by yeast-2-hybrid (Figure 5.4), suggests that CRTR-1

multimeric complexes may have a biological activity independent of their ability to bind other CP2 family members. Elucidation of biological function of both CRTR-1 heteromeric and homomeric protein interactions requires the DNA binding ability of CRTR-1 to be determined.

5.5.2 altCP2 and CP2 form Equivalent Heteromeric and Homomeric Protein Interactions.

LBP-1c truncated C-terminally from amino acid 403 (LSFΔ403) forms multimeric complexes but is unable to bind DNA (Shirra and Hansen, 1998). Identification of the alternatively spliced isoform of CP2 (501 amino acids), altCP2 (398 amino acids), truncates the protein C-terminally from amino acid 384 and is therefore also potentially able to form protein/protein interactions. This hypothesis was confirmed by yeast-2-hybrid analysis which demonstrated the formation of homomeric and heteromeric protein interactions for both altCP2 and CP2 (Figures 5.5 and 5.6).

It would be expected, by analogy with LSFΔ403, that altCP2 will not be able to bind DNA. This, together with the observation that altCP2 can participate in the formation of homomeric and heteromeric complexes with CP2 and other family members, suggests that altCP2 may act as a dominant negative form of CP2. Elucidation of the biological function of altCP2 as a dominant negative form of CP2 requires the DNA binding ability of altCP2 to be determined.

5.5.3 Variation in Heteromeric Protein Interactions Between CP2 Family Members.

Investigation into the formation of protein/protein interactions of NF2d9 and altNF2d9 by yeast-2-hybrid revealed differences in the ability of NF2d9 and altNF2d9 to interact with other CP2 family members (Figures 5.7 and 5.8). NF2d9 and altNF2d9 were able to form homomeric interactions and heteromeric interactions with CRTR-1. However,

only weak interactions were observed between NF2d9 or altNF2d9 with CP2 and altCP2 and between NF2d9 with altNF2d9.

Despite extensive amino acid sequence alignment between the dimerisation domains of NF2d9 and other CP2 family members, differences in what might be key residues could direct the specificity of these protein/protein interactions and could represent a mechanism for specification of interactions between CP2 family members. The presence of the additional 36 amino acids in altNF2d9 did not alter its ability to form homomeric interactions or heteromeric interactions with CRTR-1, CP2 or altCP2 in comparison to the interactions observed for NF2d9. However, a weak interaction between NF2d9 and altNF2d9 was observed in comparison to the homomeric interactions for each protein suggesting that the additional 36 amino acids present in altNF2d9 may influence the ability of NF2d9 and its alternatively spliced isoform, altNF2d9, to interact with each other. The biological consequence of differences in the ability of NF2d9 to interact with other CP2 family members, including altNF2d9, could provide a possible mechanism for the determination of promoter specificity by regulation of DNA response element binding in target genes. The ability of NF2d9 and altNF2d9 to interact with each other and with CP2 and altCP2 would need to be confirmed by co-purification of each protein as part of a heteromeric complex from extracts of transfected tissue culture cell lines in a series of experiment analogous to those described for CRTR-1 (5.4.2).

CHAPTER 6

DNA binding of CRTR-1 and CP2 family members.

6.1 INTRODUCTION.

Members of the CP2 family of transcription factors bind a consensus DNA sequence consisting of a direct bipartite repeat sequence, CNRG-N_6-CNRG (Lim *et al.*, 1993; Murata *et al.*, 1998), which has been described in a wide range of cellular and viral promoters (Table 1.1). N- and C-terminal truncation studies of LBP-1c have defined the minimum DNA binding region between amino acids 65 and 383 (Shirra and Hansen, 1998) (Figure 1.13). This is consistent with the region of LBP-1c (amino acids 63-270) which shares 32% identity and 52% sequence similarity with the DNA binding domain from *grainyhead*, (*GRH*; amino acids 631-833) (Lim *et al.*, 1992; Wilanowski *et al.*, 2002). LBP-1d, which is translated from an alternatively spliced form of LBP-1c lacking amino acids 189-239, is unable to bind the LBP-1c DNA binding sequence (Shirra *et al.*, 1994; Yoon *et al.*, 1994) highlighting the importance of this region for DNA binding. The presumptive DNA binding region is highly conserved in CP2 family members including CRTR-1 (Figure 3.6) suggesting that it will be able to bind DNA, potentially a CP2 consensus DNA response element. Determination of the ability of CRTR-1 to bind CP2 consensus DNA response elements would have implications for CRTR-1 as a regulator of promoters controlled by CP2 and for the identification of putative response elements in other cellular and viral promoters.

Experiments demonstrating the ability of CP2 family members to bind DNA by electrophoretic mobility shift assay (EMSA) have used CP2 family proteins from a variety of sources. CP2 was originally identified in nuclear extracts of murine erythroleukemia (MEL) cells as a protein able to bind a region in the α-globin promoter overlapping the CCAAT box (Kim *et al.*, 1988; Kim *et al.*, 1990; Lim *et al.*, 1993; Lim *et al.*, 1992). Further studies investigating DNA binding ability and specificity have used CP2 family proteins expressed in nuclear extracts of cell lines (Zhong *et al.*, 1994), as fusion proteins

with GST (Jane *et al.*, 1995; Lim *et al.*, 1992; Shirra *et al.*, 1994; Yoon *et al.*, 1994; Zhong *et al.*, 1994) and IvTT proteins (Shirra and Hansen, 1998; Yoon *et al.*, 1994) in EMSA.

6.2 CRTR-1 BINDS A CONSENSUS CP2 DNA RESPONSE ELEMENT.

The DNA binding response element for CRTR-1 is not known. In order to test the ability of CRTR-1 to bind DNA, the α-globin DNA binding response element was used as a potential candidate CP2 consensus binding sequence.

Oligonucleotides for wild type α-globin and mutant-α-globin (Lim *et al.*, 1993) CP2 DNA binding response elements (Figure 6.1a) were annealed and labelled with ^{32}P-dCTP by an end fill reaction generating probes used for EMSA (2.3.1.17). Labelled probes were purified and samples separated by non-denaturing 16% PAGE (2.3.1.19) (Figure 6.1b).

Consistent with the ubiquitous expression of CP2 family members, a cell line that does not express CP2 family members has not been described. To ensure that DNA binding activity was specific for CRTR-1, CRTR-1 protein was synthesised by IvTT. Full length CRTR-1 was produced and ^{35}S-methionine labelled from 1 μg of pGB-CRTR-1 (7.3) in a 40 μL IvTT reaction (2.3.6.7). 3 μL of each reaction was separated by 10% SDS-PAGE (2.3.6.4) to demonstrate full-length transcription and translation of the 64 kDa CRTR-1 protein (Figure 6.2a). 2 μL of the IvTT CRTR-1 was incubated with 100,000 cpm of wild type and mutant probes (2.3.1.18), equivalent to 0.2 ng, prior to analysis by non-denaturing PAGE (2.3.1.19) (Figure 6.2b). EMSA analysis of the wild type α-globin probe showed that addition of CRTR-1 resulted in formation of a specific retarded complex (lane 4) that was not observed when mutant α-globin probe was incubated with CRTR-1 (lane 3). These results demonstrate that CRTR-1 binds the wild type α-globin specifically in a manner analogous to CP2.

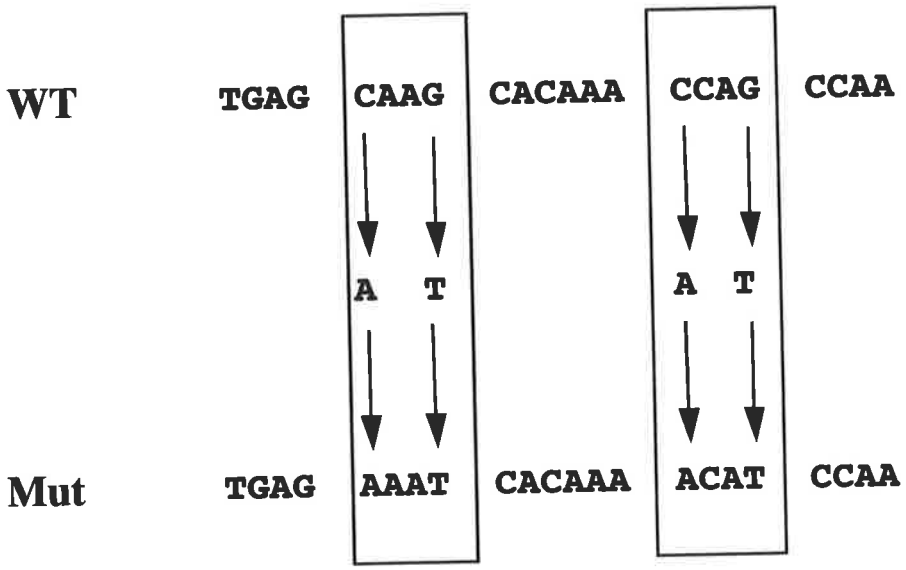
FIGURE 6.1

Wild type and mutant CP2 response elements from the α-globin promoter.

A) The wild type (WT) CP2 binding sequence for the α-globin promoter is shown together with the nucleotide sequence of a mutant (Mut) oligonucleotide sequence shown not to bind CP2 (Lim *et al.*, 1993). Boxed are the CP2 binding half sites.

B) Oligonucleotide for the wild type (WT) and mutant (Mut) α-globin CP2 response element were annealed and ^{32}P-dATP labelled by end fill reaction (2.3.1.4). Labelled oligonucleotides were purified, separated by 16% PAGE (2.3.1.19) and visualised by autoradiography.

A


WT
TGAG
CAAG
CACAAA
CCAG
CCAA
A
T
A
T
Mut
TGAG
AAAT
CACAAA
ACAT
CCAA

B

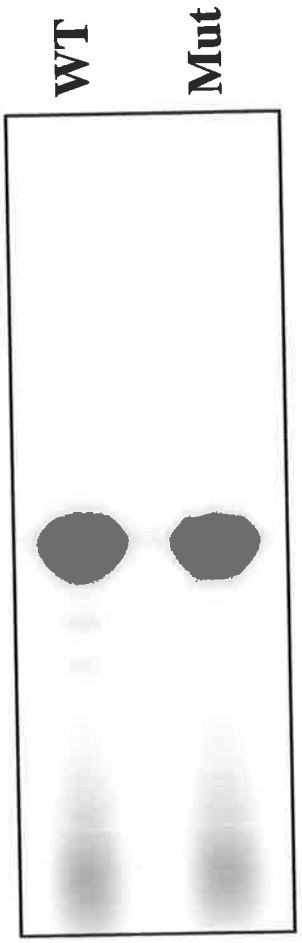

WT
Mut

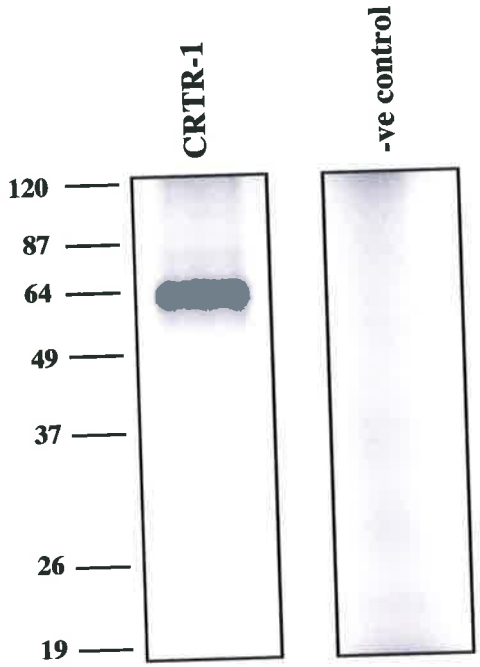
FIGURE 6.2

CRTR-1 specifically binds the wild type α-globin CP2 response element.

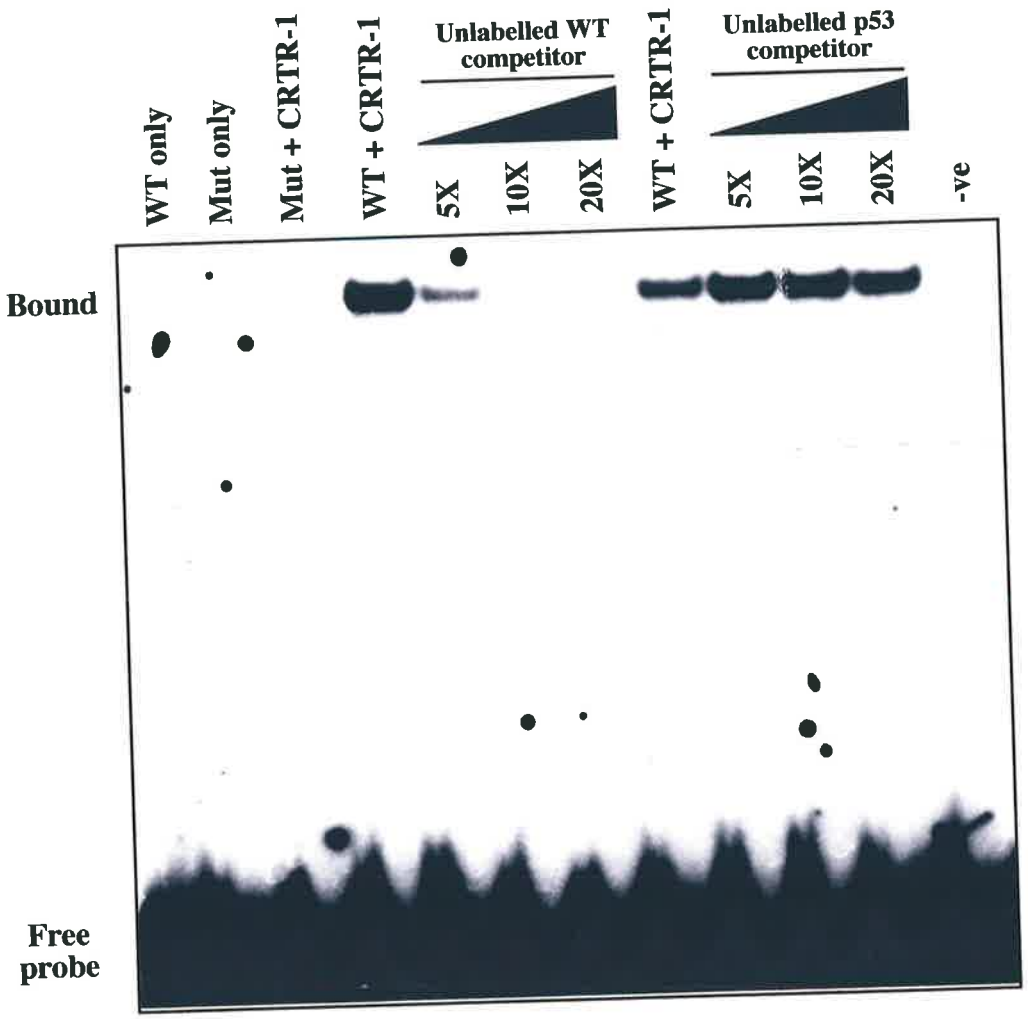
A) Full length CRTR-1 was synthesised and ^{35}S-methionine labelled by IvTT (2.3.6.7) from 1 μg of pGB-CRTR-1 in a 40 μL TNT Quickcoupled *in vitro* transcription translation reaction (Promega). 3 μL of IvTT CRTR-1 was separated by 10% SDS-PAGE (2.3.6.4), dried down and visualised by autoradiography. –ve control, negative control IvTT carried out in the absence of DNA; Markers, 10 μL of Benchmark pre-stained protein ladder (2.2.14).

B) EMSA analysis of IvTT CRTR-1 **(A)** using the wild type and the mutant α-globin CP2 DNA binding sequences. EMSA reactions were carried out for 20 minutes at room temperature using 2 μl of CRTR-1 with 100,000 cpm (equivalent to 0.2ng) of each probe (2.3.1.18). Binding of the wild type sequence was also titrated against unlabelled wild type oligonucleotide and unlabelled p53 binding oligonucleotide sequences at 5X, 10X and 20X, equivalent to 1, 2 and 4ng, respectively. Binding reactions were separated by non-denaturing 5.5% PAGE, dried down and visualised by autoradiography. Positions of free probe and CRTR-1 containing complexes are indicated. WT, wild type probe; Mut, Mutant probe; -ve, EMSA reaction carried out for 20 mins out at room temperature using 2 μL of negative control IvTT **(A)** with 100,000 cpm of the WT probe.

A


CRTR-1
-ve control
120
87
64
49
37
26
19

B


WT only
Mut only
Mut + CRTR-1
WT + CRTR-1
Unlabelled WT competitor
5X
10X
20X
WT + CRTR-1
Unlabelled p53 competitor
5X
10X
20X
-ve
Bound
Free probe

Titration of the CRTR-1/DNA complex with unlabelled wild-type α-globin probe at 5, 10 and 20 fold greater concentrations, equivalent to 1, 2 and 4 ng respectively, competed the complex resulting in a reduction in the CRTR-1/DNA binding complex (Figure 6.2b, lanes 5-7). By contrast, this complex was not competed by a p53 specific (Foord *et al.*, 1993) unlabelled probe (lanes 8-11), confirming the binding specificity of CRTR-1 for the wild type α-globin CP2 response element sequence.

Similar analysis demonstrated specific interaction between CRTR-1 and the SV40 LBP-1c response element (acagCTGGttctttCCGCtcaga) (Huang *et al.*, 1990) (data not shown). CRTR-1 is therefore able to bind the α-globin and SV40 CP2 family consensus DNA binding response elements.

6.3 CRTR-1 BINDS DNA AS A MULTIMERIC COMPLEX.

CP2 family members bind DNA as dimers and tetramers (Jane *et al.*, 1995; Murata *et al.*, 1998; Shirra and Hansen, 1998; Shirra *et al.*, 1994; Zhong *et al.*, 1994). In order to investigate whether CRTR-1 binds DNA as a multimeric complex, an alternative molecular weight CRTR-1 that would enable distinction between of homomeric and heteromeric complexes was produced by IvTT of a CRTR-1 ORF lacking the first 46 amino acids, deduced by homology to encode the CRTR-1 DBD and oligomerisation domain (3.5).

CRTR-1(47-481) was synthesised and ^{35}S-methionine labelled by IvTT (2.3.6.7) using pGB-CRTR-1(47-481) (5.3.1) (Figure 6.3a, lane 2). 2 μL of IvTT CRTR-1(47-481) was incubated with 100,000 cpm of the wild type and mutant α-globin probes (2.3.1.18) and analysed by non-denaturing PAGE (2.3.1.19). Like CRTR-1, CRTR-1(47-481) was able to form complexes with the wild type (Figure 6.3b, lane 2) but not the mutant α-globin probes (Figure 6.3b, lane 1). As expected, the mobility of the retarded complex formed with the lower molecular weight CRTR-1(47-481) (Figure 6.3c lane 3) differed from the mobility of the retarded complex formed with the full length CRTR-1 (Figure 6.3c, lane 2).

FIGURE 6.3

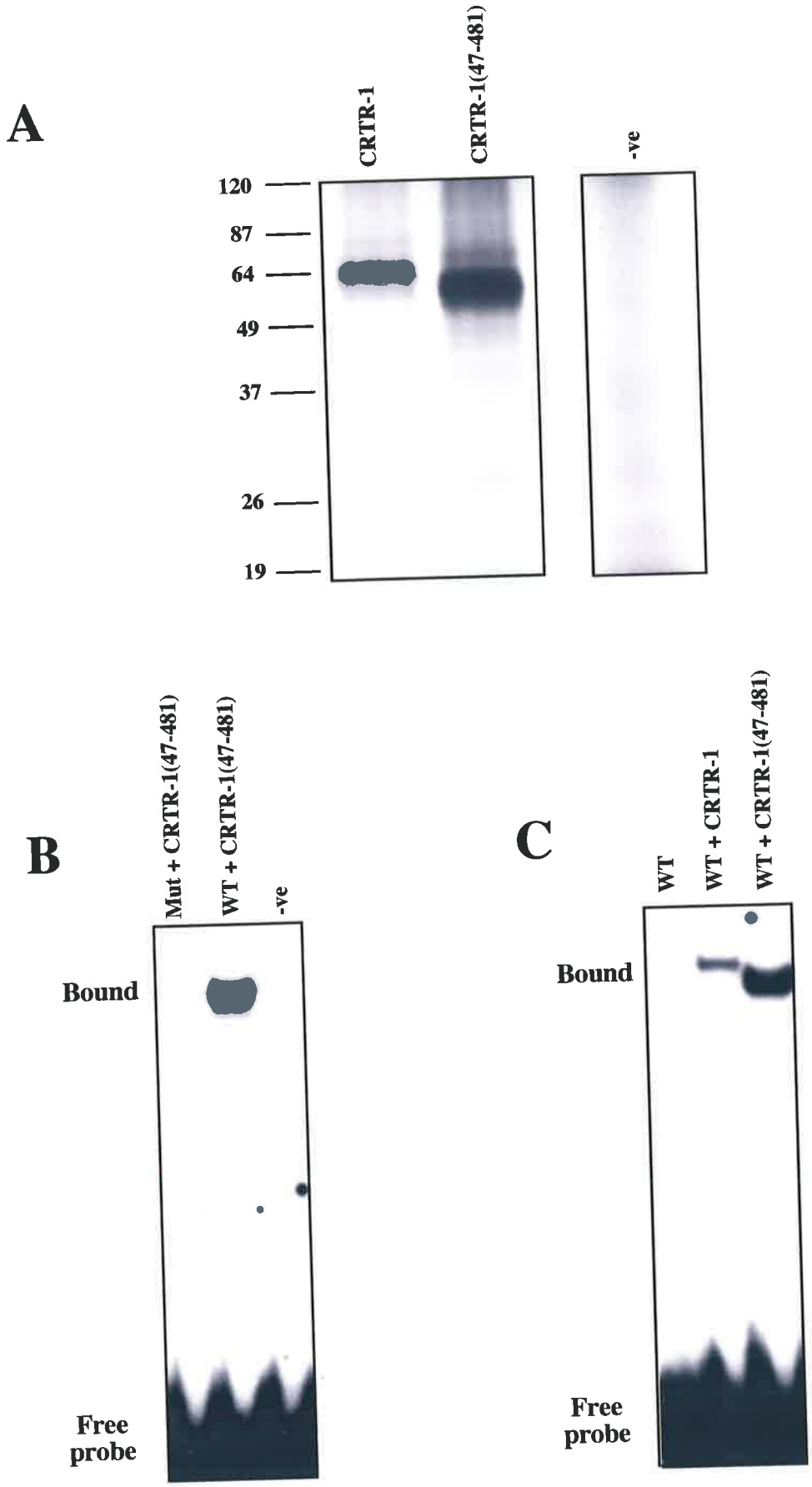
CRTR-1(47-481) specifically binds the wild type α-globin CP2 response element.

A) Full length CRTR-1 and CRTR-1(47-481) were synthesised and ^{35}S-methionine labelled by IvTT (2.3.6.7) from 1 μg of pGB-CRTR-1 and pGB-CRTR-1(47-481) respectively. IvTT reactions were analysed as described in figure 6.2A.

B) EMSA analysis using 2 μL of IvTT CRTR-1(47-481) **(A)** with 100,000 cpm of the wild type (WT) and the mutant (Mut) α-globin CP2 DNA binding sequences.

C) EMSA analysis using 2 μL of IvTT CRTR-1 and CRTR-1(47-481) **(A)** with 100,000 cpm of the wild type (WT) α-globin CP2 DNA binding sequence.

Details for Binding reaction analysis for **(B)** and **(C)** are as described in figure 6.2B.


A
CRTR-1
CRTR-1(47-481)
-ve
120
87
64
49
37
26
19
B
Mut + CRTR-1(47-481)
WT + CRTR-1(47-481)
-ve
Bound
Free probe
C
WT
WT + CRTR-1
WT + CRTR-1(47-481)
Bound
Free probe

To establish whether CRTR-1 binds DNA as a multimer, 2 µl each of IvTT full-length CRTR-1 and IvTT CRTR-1(47-481) (2.3.6.7) were incubated with 100,000 cpm of the wild type α-globin probe (2.3.1.18). Non-denaturing PAGE analysis (2.3.1.19) (Figure 6.4a, lane 2) revealed a DNA/protein complex that showed a shift in mobility greater than that observed for CRTR-1(47-481) alone (lane 1) but less than that observed for full length CRTR-1 (lane 3). This is interpreted as representing a heterologous intermediate complex containing both full length CRTR-1 and CRTR-1(47-481). This result suggests that CRTR-1 binds the α-globin probe as a complex containing more than one CRTR-1 molecule. Titration of CRTR-1(47-481) 1:1 and 1:2 by CRTR-1 in EMSA (Figure 6.4b, lanes 2 and 3) resulted in the formation of complexes of progressively increased mobility but still intermediate between those formed in the presence of CRTR-1(47-481) (lane 1) or CRTR-1 alone (lane 4). This was interpreted as evidence that the multimeric DNA binding CRTR-1 complex is greater than a dimer.

6.4 DNA BINDING OF CP2, NF2D9 AND THEIR ISOFORMS.

The DNA binding ability of CP2, NF2d9 and their isoforms to the α-globin CP2 response element was investigated to establish the relative DNA binding abilities possible functions of altCP2 and altNF2d9 (3.6).

6.4.1 Binding of CP2 and altCP2 to the CP2 Consensus Sequence.

Full length CP2 and altCP2 were synthesised and ^{35}S-methionine labelled by IvTT (2.3.6.7) of 1 µg pGEM-T-CP2 and pGEM-T-altCP2 (3.6). 3 µL samples of each reaction were separated out by 10% SDS-PAGE (2.3.6.4) and the production of full-length proteins of 64 KDa and 54 KDa, representing CP2 and altCP2 respectively, confirmed (Figure 6.5a). 2 µL of each reaction was incubated with 100,000 cpm of wild type and mutant α-globin

FIGURE 6.4

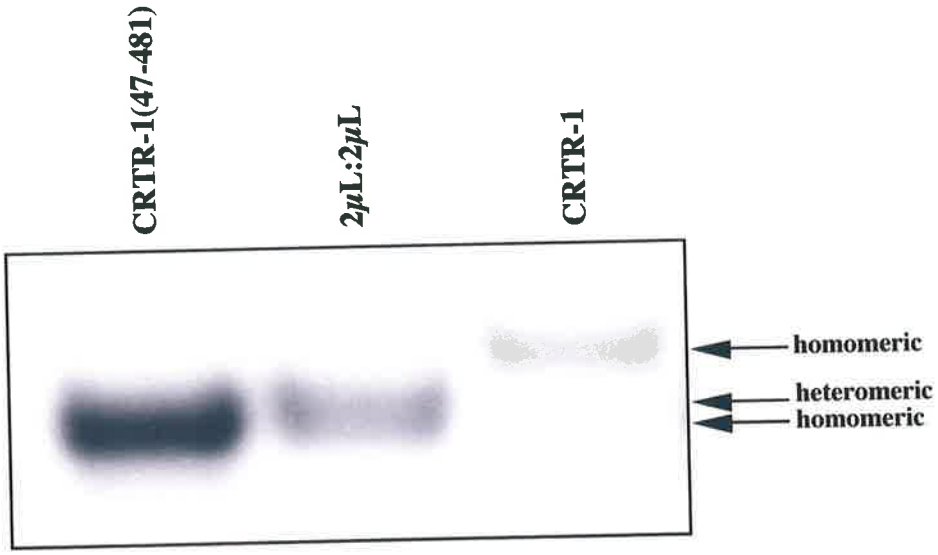
CRTR-1 binds DNA as a homo-multimeric complex.

A) EMSA analysis using 2 μL IvTT CRTR-1(47-481), 2 μL IvTT CRTR-1 and 2μL:2μL CRTR-1(47-481):CRTR-1 with 100,000 cpm of the wild type α-globin CP2 DNA binding sequences.

B) EMSA analysis using 2 μL of IvTT CRTR-1, 2 μL IvTT CRTR-1(47-481), 2 μL:2 μL of CRTR-1(47-481):CRTR-1 and 2 μL:4 μL CRTR-1:CRTR-1(47-481) with 100,000 cpm of the wild type α-globin CP2 DNA binding sequences.

Binding reactions **(A)** and **(B)** were analysed as described in figure 6.2B. Arrows indicate DNA/protein complexes.

A


CRTR-1(47-481)
2μL:2μL
CRTR-1
homomeric
heteromeric
homomeric

B

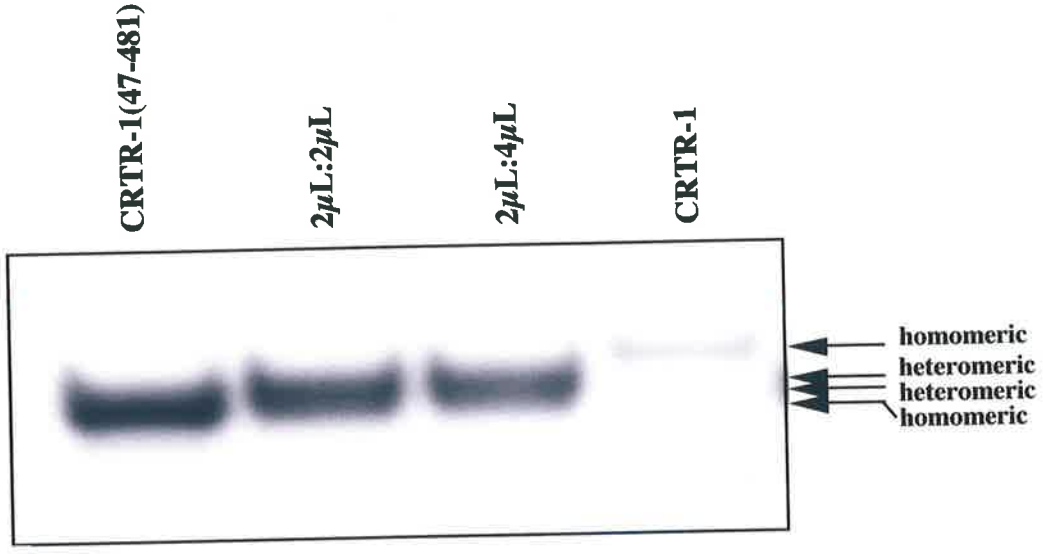

CRTR-1(47-481)
2μL:2μL
2μL:4μL
CRTR-1
homomeric
heteromeric
heteromeric
homomeric

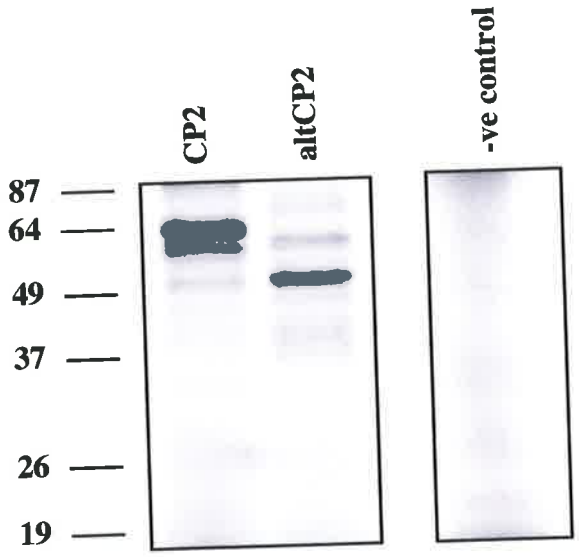
FIGURE 6.5

Differential DNA binding ability of CP2 and altCP2.

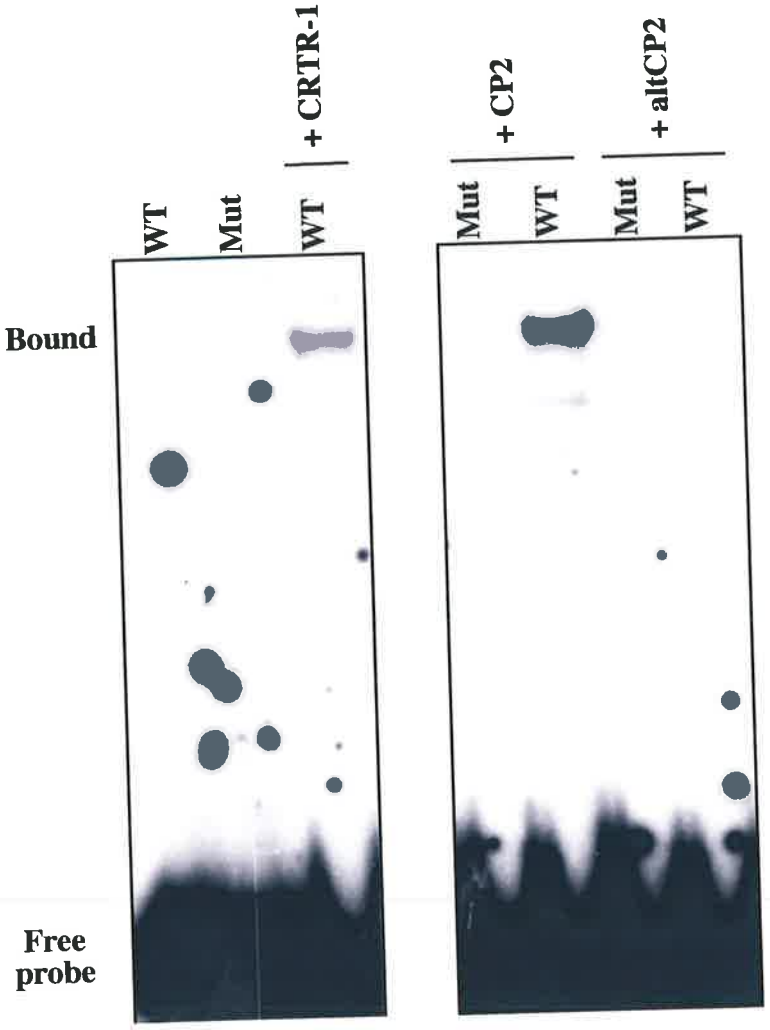
A) Full length CP2 and altCP2 were synthesised and ^{35}S-methionine labelled by IvTT (2.3.6.7) from 1μg of pGEM-T-CP2 and pGEM-T-altCP2, respectively. IvTT reactions were analysed as described in figure 6.2A.

B) EMSA analysis using 2μL of IvTT CRTR-1, 2 μL of IvTT CP2 and 2 μL IvTT altCP2 with 100,000 cpm of the wild type (WT) and mutant (Mut) α-globin probes. Binding reactions were analysed as described in figure 6.2B.

A


CP2
altCP2
-ve control
87
64
49
37
26
19

B


+ CRTR-1
+ CP2
+ altCP2
WT
Mut
WT
Mut
WT
Mut
WT
Bound
Free
probe

probe (2.3.1.18) and analysed by non-denaturing PAGE (2.3.1.19) (Figure 6.5b). Consistent with previous reports (Lim *et al.*, 1993), EMSA analysis of the wild type α-globin probe incubated with CP2 showed a band with retarded mobility (lane 5) indicating the formation of a protein/DNA complex. The specificity of this complex was indicated by failure to form when mutant α-globin probe was incubated with IvTT CP2 (lane 4). No retarded complex was seen in EMSA analysis of the wild type and mutant α-globin probes incubated with altCP2 (lanes 7 and 6), demonstrating that altCP2 was not able to bind either probe and that the C-terminal 100 amino acids of CP2, absent in altCP2, are required for DNA binding.

Mixing of 2 µl IvTT CP2 and 2 µL IvTT altCP2 with 100,000 cpm of the wild type α-globin probe (2.3.1.18) resulted in a significant decrease in the amount of wild type α-globin/CP2 complex formed (Figure 6.6a lane 2), but did not alter the mobility of this complex compared to that formed in the presence of CP2 alone (lane 1). All DNA binding activity in the CP2/altCP2 mixture therefore appears to be composed exclusively of CP2. The ability of altCP2 to interact with CP2 has been previously demonstrated in 5.3.2.2. The biological consequence of this interaction between altCP2 and CP2 can therefore be deduced to prevent the formation of stable DNA binding complexes. The reduction in DNA binding activity would result from formation of CP2:altCP2 protein complexes that are unable to bind DNA thereby reducing the number of protein complexes competent for DNA binding. Reduced DNA/protein complex formation observed for mixed CP2 and altCP2 could not be attributed to proteins present in the IvTT mixture or dilution of the EMSA reaction as addition of 2 µL CP2 with 2 µL negative IvTT (IvTT reaction performed in the absence of DNA) (Figure 6.6b, lane 2) did not reduce the DNA binding ability of CP2 to the wild type α-globin probe (compare lanes 1 and 2). Together, these results demonstrate the requirement for DNA binding regions in each component of the protein complex to bind DNA.

FIGURE 6.6

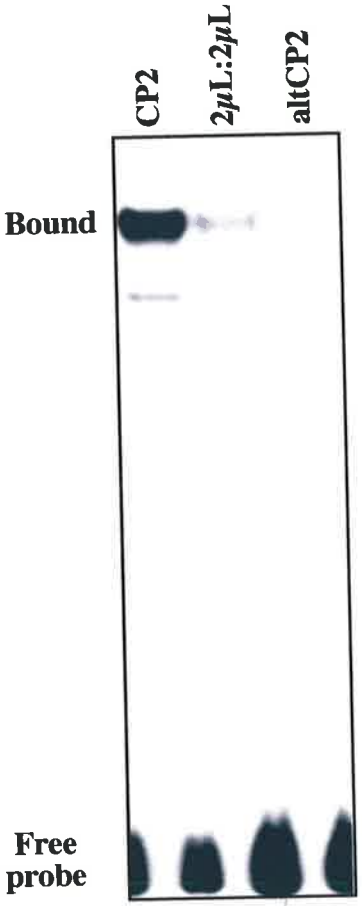
altCP2 abrogates CP2 DNA binding.

A) EMSA analysis using 2 μL of IvTT CP2, 2 μL IvTT altCP2 and 2μL:2μL of CP2:altCP2 with 100,000 cpm of the wild type α-globin probe.

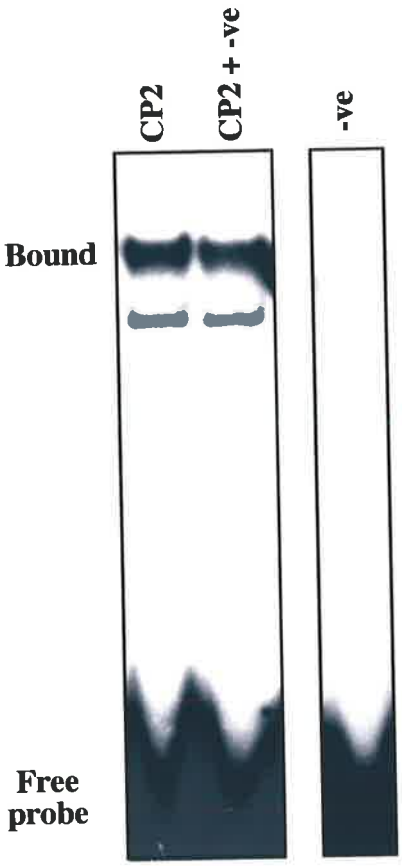
B) EMSA analysis using, 2 μL IvTT CP2 and 2 μL:2 μL of CP2:negative control with 100,000 cpm of the wild type α-globin probe.

Binding reactions **(A)** and **(B)** were analysed as described in figure 6.2B.

A


CP2
2μL:2μL
altCP2
Bound
Free probe

B


CP2
CP2 + -ve
-ve
Bound
Free probe

6.4.2 Binding of NF2d9 and altNF2d9 to the CP2 Consensus Sequence.

Full length NF2d9 and altNF2d9 were synthesised and ^{35}S-methionine labelled by IvTT (2.3.6.7) of 1μg pGEM-T-NF2d9 and pGEM-T-altNF2d9 (3.6) respectively. 3 μL samples of each reaction were separated by 10% SDS-PAGE (2.3.6.4) and confirmed expression of full-length proteins, 64 kDa and 67 kDa, representing NF2d9 and altNF2d9 respectively (Figure 6.7a). 2 μL of each reaction was incubated with 100,000 cpm of the wild type and mutant α-globin probes (2.3.1.18) and analysed by non-denaturing PAGE (2.3.1.19) (Figure 6.7b). Addition of both NF2d9 and altNF2d9 resulted the formation of protein/DNA complexes as assessed by the presence of retarded bands of lower mobility when incubated with the wild type (lanes 4 and 6) but not mutant α-globin probes (lanes 5 and 7), indicating that NF2d9 and altNf2d9 therefore interact specifically with the α-globin CP2 response element. The additional 36 amino acids specific for altNF2d9 present in a region of overlapping functional importance for DNA binding and oligomerisation (Figure 3.11) do not inhibit the DNA binding ability of altNF2d9.

EMSA reactions (Figure 6.7c) containing 2 μl each of NF2d9 and altNF2d9 with 100,000 cpm of the wild type α-globin probe (2.3.1.18) revealed formation of a DNA/protein complex (lane 2) with a greater mobility intermediate between that formed in the presence of NF2d9 (lane 1) or altNF2d9 alone (lane 3). This was interpreted as representing a heterologous intermediate complex containing full length NF2d9 and altNF2d9 bound to the α-globin probe, and indicates that NF2d9 and altNF2d9 can bind the α-globin oligonucleotide as a heteromeric complex containing both protein species. The difference in intensity of bands representative of DNA/protein complexes for NF2d9 compared with altNF2d9 could be indicative of more efficient altNF2d9 DNA binding and may represent a biological relevance for the additional 36 amino acids in altNF2d9.

FIGURE 6.7

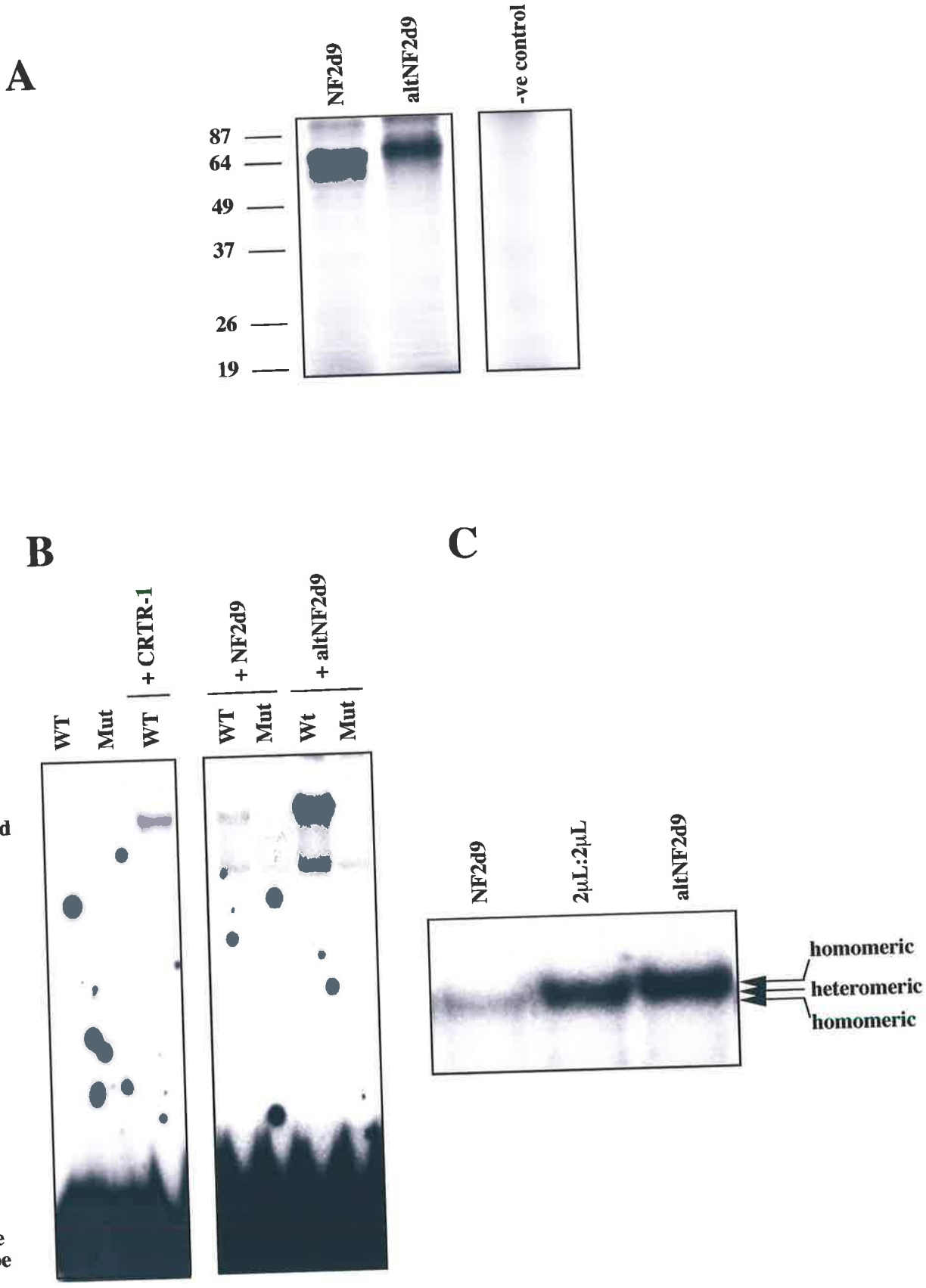
NF2d9 and altNF2d9 specifically bind the wild type α-globin CP2 response element.

A) Full length NF2d9 and altNF2d9 were synthesised and ^{35}S-Methionine labelled by IvTT (2.3.6.7) from 1 μg of pGEM-T-NF2d9 and pGEM-T-altNF2d9, respectively. IvTT reactions were analysed as described in figure 6.2A.

B) EMSA analysis using 2 μL of IvTT CRTR-1, 2 μL IvTT NF2d9 and 2 μL IvTT altNF2d9 with 100,000 cpm of the wild type (WT) and mutant (Mut) α-globin probes.

C) EMSA analysis using 2 μL of IvTT NF2d9, 2 μL IvTT altNF2d9 and 2μL:2μL of NF2d9:altNF2d9 using 100,000cpm of the wild type α-globin probe.

Binding reactions **(B)** and **(C)** were analysed as described in figure 6.2B. Arrows in **(C)** indicate DNA/protein complexes.


A
NF2d9
altNF2d9
-ve control
87
64
49
37
26
19
B
+ CRTR-1
+ NF2d9
+ altNF2d9
WT
Mut
WT
WT
Mut
Wt
Mut
Bound
Free probe
C
NF2d9
2μL:2μL
altNF2d9
homomeric
heteromeric
homomeric

6.5 FORMATION OF HETEROMERIC DNA BINDING COMPLEXES AMONGST CP2 FAMILY MEMBERS.

The ability of CRTR-1 to bind DNA as part of a heteromeric protein complex with other CP2 family members would position both a transcriptional repressor and a transcriptional activator at promoter elements and could have implications for control of CP2 family regulated promoters. The formation of heteromeric complexes containing CRTR-1, CP2, NF2d9 and their isoforms on the α-globin CP2 response element was investigated.

2 µl of IvTT CRTR-1(47-481) or CP2 were mixed with 2 µL IvTT NF2d9 and 100,000 cpm of the wild type α-globin probe and analysed by EMSA (2.3.1.18). DNA binding complexes that showed an intermediate mobility (Figure 6.8a and c, lane 2) between complexes containing CRTR-1(47-481) (Figure 6.8 a and b, lane 1) or CP2 (Figure 6.9c and d, lane 1) alone, and those containing NF2d9 (Figure 6.8a and c, lane 3) alone were observed. Similar results were obtained for EMSA in which altNF2d9 replaced NF2d9 (Figure 6.8b and d, lane 2). These results demonstrate that CRTR-1(47-481) and CP2 are able to form protein complexes with NF2d9 and altNF2d9 that are capable of binding the α-globin CP2 response element. The additional 36 amino acids present in altNF2d9 therefore did not inhibit protein/protein interactions required for formation of heteromeric DNA binding protein complexes.

Titration of CRTR-1(47-481) 1:1 or 1:2 with NF2d9 or altNF2d9 in EMSA reactions (2.3.1.18) resulted in the formation of complexes (Figure 6.9a and b, lanes 2 and 3) with progressively increasing intermediate mobility between those formed in the presence of CRTR-1(47-481) (Figure 6.9a and b, lane 1), NF2d9 (Figure 6.9a, lane 4) or altNF2d9 (Figure 6.9b, lane 4) alone. This indicates that the DNA binding CRTR-1/NF2d9 or CRTR-1/altNF2d9 complex is multimeric and likely to be greater than a dimer. Bands of equivalent mobility to CRTR-1(47-481) (Figure 6.9a and b, lane 1), NF2d9 (Figure 6.9a, lane 4) or altNF2d9 (Figure 6.9b, lane 4) homomeric DNA binding complexes were not

FIGURE 6.8

CRTR-1(47-481) and CP2 form DNA binding heteromeric complexes with NF2d9 and altNF2d9.

A) EMSA analysis using 2 μL of IvTT CRTR-1(47-481), 2 μL IvTT NF2d9 and 2 μL:2 μL of CRTR-1(47-481):NF2d9 with 100,000 cpm of the wild type α-globin probe.

B) EMSA analysis using 2 μL of IvTT CRTR-1(47-481), 2 μL IvTT altNF2d9 and 2 μL:2 μL of CRTR-1(47-481):altNF2d9 with 100,000 cpm of the wild type α-globin probe.

C) EMSA analysis using 2 μL of IvTT CP2, 2 μL IvTT NF2d9 and 2 μL:2 μL of CP2:NF2d9 with 100,000 cpm of the wild type α-globin probe.

D) EMSA analysis using 2 μL of IvTT CP2, 2 μL altNF2d9 and 2 μl:2 μL of CP2:altNF2d9 with 100,000 cpm of the wild type α-globin probe.

Binding reactions **(A)**, **(B)**, **(C)**, and **(D)** were analysed as described in figure 6.2B. Arrows indicate DNA/protein complexes.

A
CRTR-1(47-481)
2μL:2μL
NF2d9
homomeric
heteromeric
homomeric
B
CRTR-1(47-481)
2μL:2μL
altNF2d9
homomeric
heteromeric
homomeric
C
CP2
2μL:2μL
NF2d9
homomeric
heteromeric
homomeric
D
CP2
2μL:2μL
altNF2d9
homomeric
heteromeric
homomeric

FIGURE 6.9

CRTR-1(47-481) binds DNA as a hetero-multimeric complex with NF2d9 and altNF2d9.

A) EMSA analysis using 2 μL of IvTT CRTR-1(47-481), 2 μL IvTT NF2d9, 2 μL:2 μL and 2 μL:4 μL of CRTR-1(47-481):NF2d9 with 100,000 cpm of the wild type α-globin probe.

B) EMSA analysis using 2 μL of IvTT CRTR-1(47-481), 2 μL IvTT altNF2d9, 2 μL:2 μL and 2 μL:4 μL of CRTR-1(47-481):altNF2d9 with 100,000 cpm of the wild type α-globin probe.

Binding reactions **(A)** and **(B)** were analysed as described in figure 6.2B. Arrows indicate DNA/protein complexes.

A

CRTR-1(47-481)
2μL:2μL
2μL:4μL
NF2d9
homomeric
heteromeric
heteromeric
homomeric

B

CRTR-1(47-481)
2μL:2μL
2μL:4μL
altNF2d9
homomeric
heteromeric
heteromeric
homomeric

observed in lanes containing mixtures of CRTR-1(47-481) with NF2d9 (Figure 6.9 a, lanes 2 and 3) or altNF2d9 (Figure 6.9 b, lanes 2 and 3), suggesting that the formation of heteromeric protein complexes is favorable for the formation of stable DNA binding complexes.

EMSA reactions containing 2 µl of CRTR-1(47-481) and 2 µL of CP2 with 100,000 cpm of the wild type α-globin oligonucleotide (2.3.1.18) revealed a DNA binding complex (Figure 6.10a, lane 2) with mobility intermediate between complexes formed in the presence of CRTR-1(47-481) (Figure 6.10a, lane 1) or CP2 (Figure 6.10a, lane 3) alone. This altered mobility was interpreted as representative of formation of a CRTR-1/CP2 heteromeric DNA binding complex. Consistent with observations described for combinations of CRTR-1/NF2d9 and CRTR-1/altNF2d9, homomeric DNA binding complexes were not observed in lanes containing mixtures of CRTR-1(47-481) with CP2 (Figure 6.10a, lane 2) suggesting that the formation of heteromultmeric complexes is favored. Complexes observed for EMSA reactions containing 2 µL of CRTR-1(47-481) and 2 µL of altCP2 (Figure 6.10b lane 2) demonstrated equivalent mobility to those formed in the presence of CRTR-1(47-481) alone (lane 1) but with a reduced intensity. The decreased intensity of the retarded band may represent sequestration of CRTR-1(47-481) in heteromeric protein complexes that are unable to bind DNA (6.4.1). Reduced DNA/protein complex formation observed for mixed CRTR-1 and altCP2 could not be attributed to proteins present in the IvTT mixture or dilution of the EMSA reaction as addition of 2 µL CRTR-1 with 2 µL negative IvTT (IvTT reaction performed in the absence of DNA) (Figure 6.10c, lane 2) did not reduce the DNA binding ability of CP2 to the wild type α-globin probe (compare lanes 1 and 2). Together, these results were consistent with observations for EMSA of CP2/altCP2 and demonstrate the requirement for DNA binding regions in each component of the protein complex to bind DNA.

FIGURE 6.10

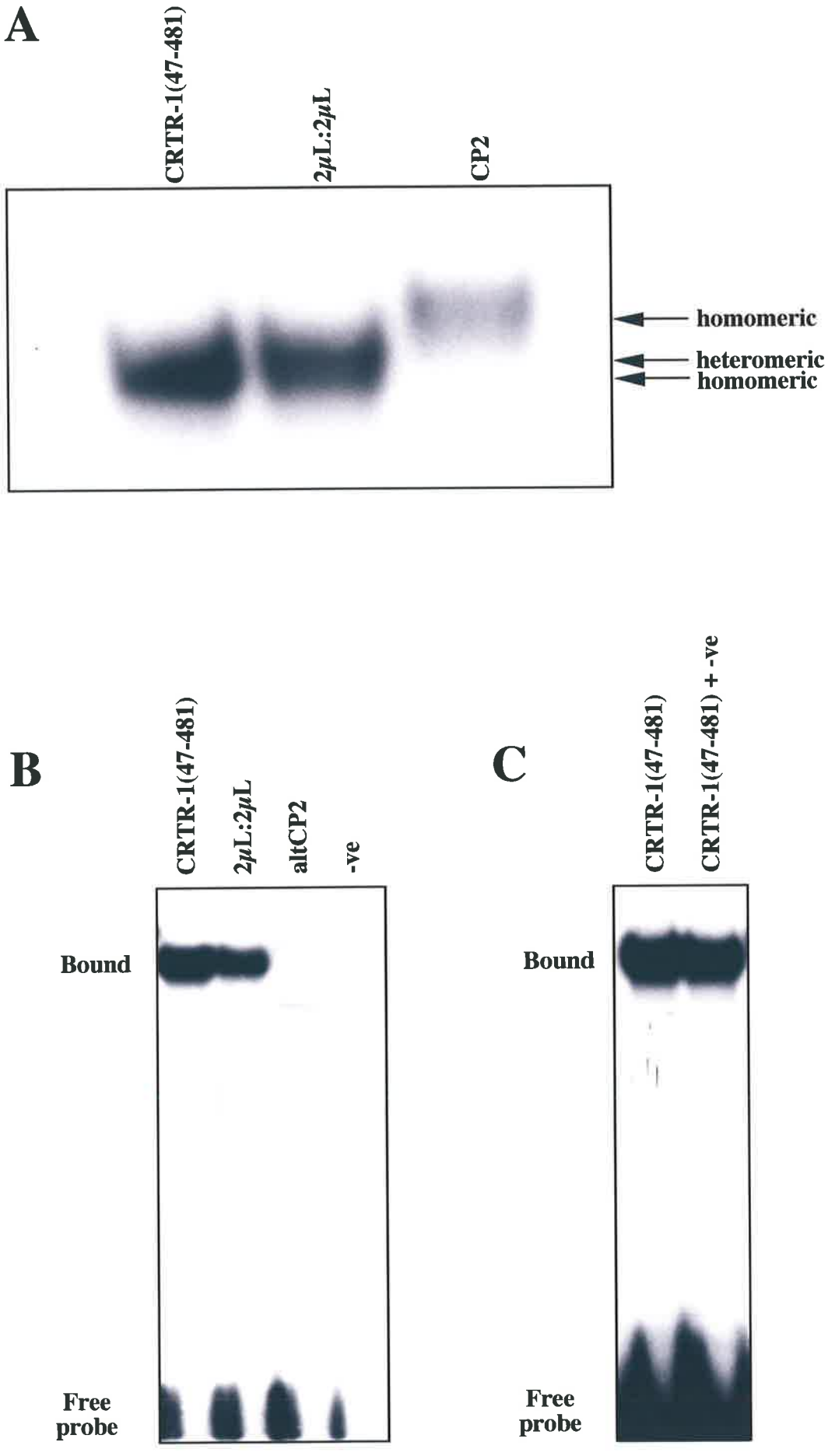
Differential DNA binding ability of CRTR-1(47-481) heteromultimerised with CP2 and altCP2.

A) EMSA analysis using 2 μL of IvTT CRTR-1(47-481), 2 μL IvTT CP2 and 2 μL:2 μL of CRTR-1(47-481):CP2 with 100,000 cpm of the wild type α-globin probe.

B) EMSA analysis using 2 μL of IvTT CRTR-1(47-481), 2 μL IvTT altCP2 and 2 μL:2 μL of CRTR-1(47-481):altCP2 with 100,000 cpm of the wild type α-globin probe.

C) EMSA analysis using, 2 μL IvTT CRTR-1(47-481) and 2 μL:2 μL of CRTR-1(47-481):negative control with 100,000 cpm of the wild type α-globin probe.

Binding reactions **(A)**, **(B)** and **(C)** were analysed as described in figure 6.2B. Arrows in **(A)** indicate DNA/protein complexes.


A
CRTR-1(47-481)
2μL:2μL
CP2
homomeric
heteromeric
homomeric
B
CRTR-1(47-481)
2μL:2μL
altCP2
-ve
Bound
Free probe
C
CRTR-1(47-481)
CRTR-1(47-481) + -ve
Bound
Free probe

6.6 DISCUSSION.

6.6.1 CRTR-1 Binds Consensus CP2 DNA Response Element Sequences.

CRTR-1 was shown to bind both the α-globin (Figure 6.2b, lane 4) and the SV40 (data not shown) CP2 consensus DNA response elements. Specificity of this interaction was demonstrated by the inability of CRTR-1 to bind a mutant α-globin sequence (Figure 6.1a and 6.2b, lane 3) and by competition for complex formation by unlabelled wild type α-globin probe but not unlabelled p53 binding probe (Figure 6.2b). Inability of CRTR-1 to bind the mutant α-globin probe indicates that nucleotides important for CP2 DNA binding, defined as the CP2 consensus recognition sequence CNRG-N_6-CNRG, are also required by CRTR-1 for DNA binding. The ability of CRTR-1 to bind CP2 consensus DNA response elements *in vitro* could have implications for the regulation of CP2 family activated promoters by CRTR-1 mediated transcriptional repression (4.2.2) *in vivo*. Furthermore, identification of a CRTR-1 DNA binding sequences facilitates the identification of other putative target genes by identification of potential response elements in their promoter regions. Several studies have identified CP2 consensus response elements in the promoters of genes such as *Interleukin-4* (Casolaro *et al.*, 2000), *thymidylate synthase* (Powell *et al.*, 2000) and *Uroporphyrinonen III synthase* (Solis *et al.*, 2001), which may be CRTR-1 regulated. Of particular interest is the putative CP2 response element found in the *Wilm's Tumor 1* (*WT1*) gene (Bing *et al.*, 1999). *WT1* encodes a tumor supressor transcription factor which is required for regulating the early response of the kidney mesenchyme induction and has been implicated to play many roles during the course of renal epithelial cell development (Dressler, 1995). The ability of CRTR-1 to regulate transcription from this promoter would be consistent with the expression of CRTR-1 during the later stages of kidney development and would provide information about the biological function of CRTR-1.

6.6.2 CRTR-1 Binds DNA as Part of a Multimeric Protein Complex.

Combination of CRTR-1 with CRTR-1(47-481) in EMSA demonstrated the formation of a protein complex containing both forms of CRTR-1 (Figure 6.5a). This suggests that, like other CP2 family members, CRTR-1 binds DNA as a protein complex and not as a monomer. Titration of CRTR-1 against CRTR-1(47-481) resulted in a progressive increase in size of the DNA binding complex toward the CRTR-1 alone band (Figure 6.5b) suggesting that the DNA binding protein complex was greater than a dimer. Similar observations were made for complexes containing CRTR-1(47-481) with NF2d9 (Figure 6.9a) and CRTR-1(47-481) with CP2 (Figure 6.11a). This indicates that CRTR-1 can form homo- and hetero-meric protein DNA binding complexes and is consistent with reports demonstrating that LBP-1c (Shirra and Hansen, 1998) and chicken CP2 (Murata *et al.*, 1998) bind DNA as tetramers. The change in mobility observed for CRTR-1(47-481)/CP2 DNA binding complexes (Figure 6.11a) was not as great as observed for CRTR-1(47-481)/NF2d9 (Figure 6.9a) or CRTR-1(47-481)/altNF2d9 (Figure 6.9b) DNA binding complexes, suggesting that CRTR-1(47-481)/CP2 DNA binding protein complexes may contain a different stoichiometry of proteins compared with CRTR-1(47-481)/NF2d9 and CRTR-1(47-481)/altNF2d9 DNA binding complexes and may be reflective of differences in the ability of proteins to interact demonstrated by yeast-2-hybrid (5.3.2.1 and Figure 5.4). The ability of CRTR-1 to interact with other CP2 family members as heteromeric DNA binding complexes positions both a transcriptional repressor and a transcriptional activator together at the promoter of CP2 family regulated genes *in vivo*. This could have implications for the control of CP2 family regulated promoters through the formation of heteromeric DNA binding complexes that contain different transcriptional regulatory abilities, where one activity may be dominant over the other.

6.6.3 Preferential Formation of CP2 Family Member Heteromeric DNA Binding Complexes.

Combination of CRTR-1(47-481) with NF2d9 (Figure 6.8a and 6.9a), altNF2d9 (Figure 6.8b and 6.9b) or CP2 (Figure 6.10a) and combination of CP2 with NF2d9 (Figure 6.8c) or altNF2d9 (Figure 6.8d) demonstrated formation of complexes with mobility intermediate between those formed by homomeric interaction of CRTR-1(47-481), NF2d9, altNF2d9 and CP2. Failure to form homomeric protein complexes suggests that CP2 family members bind DNA preferentially as a heteromeric complex. This observation has been previously reported for LBP-1a, LBP-1b and LBP-1c in analogous experiments (Yoon *et al.*, 1994) and is consistent with a variety of other multimeric transcription factors. For example, MyoD, a helix-loop-helix transcription factor, is able to bind DNA as a homodimer but has preferential and higher affinity DNA binding as part of a heteromeric complex with another helix-loop-helix transcription factor, E2A (Sun and Baltimore, 1991). Another example is c-Jun and c-Fos where c-Jun binds the AP-1 DNA response element as a homodimer while c-Fos does not bind this element, however combination of c-Jun and c-Fos heterodimers bind with a 25 fold higher affinity than c-Jun homodimers (Halazonetis *et al.*, 1988).

6.6.4 Structural Implications of DNA Binding by CP2 Family Members.

The ability of altNF2d9 to bind DNA as a homomeric complex and as a participant in the formation of heteromeric DNA binding complexes demonstrates that the additional 36 amino acids present in altNF2d9, absent in NF2d9, do not inhibit DNA binding or the ability to form heteromeric protein/protein interactions. This is consistent with yeast-two-hybrid results (5.3.2.3 and Figures 5.7-8) and previous reports for LBP-1b (Yoon *et al.*, 1994). The ability of altNF2d9 to bind DNA and other CP2 family members suggests that the additional 36 amino acids does not change the tertiary structure of the protein in regions required for these functions.

altCP2 was not able to bind the wild type or mutant α-globin probes (Figure 6.6b) demonstrating that the C-terminal 117 amino acids of CP2 are required for DNA binding. This observation is consistent with previous reports demonstrating that LBP-1c C-terminally truncated from amino acid 403 was not able to bind DNA (Shirra and Hansen, 1998). These observations suggest that the C-terminal 117 amino acids of CP2 contribute to the tertiary structure of the protein to enable it to bind DNA. The ability of altCP2 to interact with other CP2 family members indicates that the regions of tertiary structure required for protein binding are distinct from DNA binding.

Mobility shift assays combining CP2 with altCP2 demonstrated a substantial reduction in DNA binding but no change in the mobility of the residual DNA binding protein complex was observed compared to CP2 alone (Figure 6.7a). This demonstrated that all DNA binding activity in the CP2/altCP2 mixture is exclusively CP2 homomeric complexes and suggests that binding of altCP2 to CP2 prevents the formation of stable DNA binding complexes. altCP2 has been shown previously (5.3.2.2 and Figures 5.5-6) to be able to form protein/protein interactions with CP2 and other CP2 family members by yeast-2-hybrid experiments in a fashion consistent with CP2. This observation is also consistent with previous reports demonstrating that C-terminally truncated LBP-1c, from amino acid 403, is able to oligomerise (Shirra and Hansen, 1998).

Contribution of altCP2 to CP2 protein complexes at a 1:3, 2:2 or 3:1 ratio abrogate DNA binding (Figure 6.5), demonstrating that both half sites of the response element are required. Furthermore, each response element must be occupied by protein complex were all components of the complex are capable of DNA binding to confer competence of DNA binding for the complex as a whole. Consistent with this notion is the observation that mutation of one of the CNRG half sites in the α-globin CP2 binding response element abolishes all CP2 DNA binding (Lim *et al.*, 1993), illustrating the importance of stable DNA/protein interactions with both CNRG half sites.

The ability of altCP2 to abrogate CP2 DNA binding suggests that altCP2 may have a biological role as a dominant negative CP2 isoform. This, together with regulated expression of altCP2 (3.7.3), could provide a mechanism for regulated control of promoters activated by CP2 family members, which are expressed ubiquitously.

CHAPTER 7

Negative regulation of CP2 activated transcription by dominant repression.

7.1 INTRODUCTION.

CRTR-1, the likely LBP-9 homologue, has been shown to contain a novel N-terminal repression domain (4.2.3), which shares 97% identity with LBP-9 (Figure 3.8). The ability of CRTR-1 to repress transcription (4.2.2), interact with other CP2 family members (5.3.2.1) and bind a consensus CP2 response element (6.2) suggests that CRTR-1 may have the potential to act as a dominant repressor of CP2 family activated transcription. Suppression of LBP-1b-mediated transcriptional activation by co-transfection of cells with increasing amounts of LBP-9 provides support for the hypothesis that CRTR-1 and LBP-9 may act as dominant repressors of transcription.

altCP2 has been shown to bind other CP2 family members consistent with CP2 (5.3.2.2) but, in contrast to CRTR-1, was not capable of binding a CP2 consensus DNA response element (6.4.1). Formation of heteromeric protein complexes with CP2 abrogated CP2 DNA binding (Figure 6.6) providing an alternative mechanism for dominant repression of CP2 mediated gene expression.

The ability to act as dominant repressors of transcription together with developmentally regulated expression of CRTR-1 and altCP2 would provide a mechanism for developmental regulation of the ubiquitously expressed CP2 family transcriptional activators. These observations prompted investigation into the ability of CRTR-1 and altCP2 to regulate transcriptional activity by CP2 family members from a CP2 activated promoter.

7.2 TRANSCRIPTIONAL REGULATION FROM A CP2 CONSENSUS BINDING SITE PROMOTER IN MAMMALIAN CELLS.

The transcriptional regulatory ability of CRTR-1 and altCP2 was investigated from a luciferase reporter under the control a CP2 responsive promoter.

7.2.1 Generation of CP2 Responsive Luciferase Reporter Constructs.

Constructs in which expression of luciferase is regulated by four copies of the CP2 wild type or mutant α-globin CP2 response element were generated using a modified pTK-MH100x4-LUC (Figure 7.1a). Sets of complementary oligonucleotides (2.2.7.2) were annealed (2.3.1.17), kinase treated (2.3.1.20) and ligated (2.3.16) to make a DNA fragment consisting of 4 wild type or mutant copies of the α-globin CP2 response element. pTK-MH100x4-LUC was digested with *Hind*III/*Bam*HI to release the Gal4 responsive element and CIP treated (2.3.1.5). Linearised vector was then used in ligation reactions with wild type and mutant DNA fragments generating clones pTK-CP2WTx4-LUC and pTK-CP2Mutx4-LUC. Correct clones were selected by *Hind*III/*Bam*HI digestion and BDT automated sequencing (2.3.1.14).

7.2.2 CRTR-1 Transcriptional Regulation from a CP2 Consensus Binding Site in Mammalian Cells.

pTK-CP2WTx4-LUC and pTK-CP2Mutx4-LUC were co-transfected in combination with pSG5.HA-CRTR-1 (5.4.1) and pSG5.HA-VDR (5.4.1) into COS-1 cells (2.4.5). Levels of luciferase activity were analysed in cell extracts 36 hours post-transfection using the dual luciferase reporter system (2.4.9) and normalised to expression of Renilla luciferase. Co-transfection of pSG5.HA-VDR with pTK-CP2WTx4-LUC did not alter levels of luciferase activity (Figure. 7.1b, column 3) compared to pTK-CP2WTx4-LUC alone (Figure 7.1b, column 1). Co-transfection of pSG5.HA-CRTR-1 with pTK-CP2WTx4-LUC resulted in 3-6.5 fold reduction in luciferase activity (Figure 7.1b, column 2). The specificity of this response was shown by the fact that expression of luciferase from

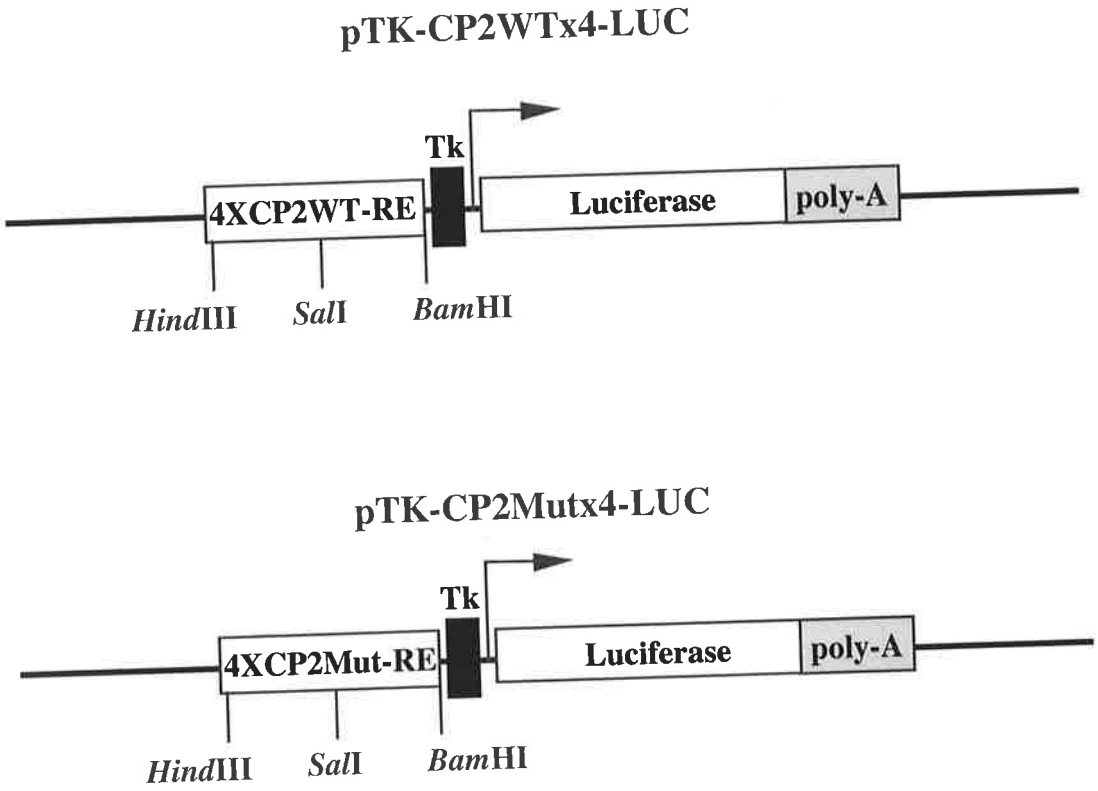
FIGURE 7.1

CRTR-1 represses transcription from a CP2 consensus DNA binding site in mammalian cells.

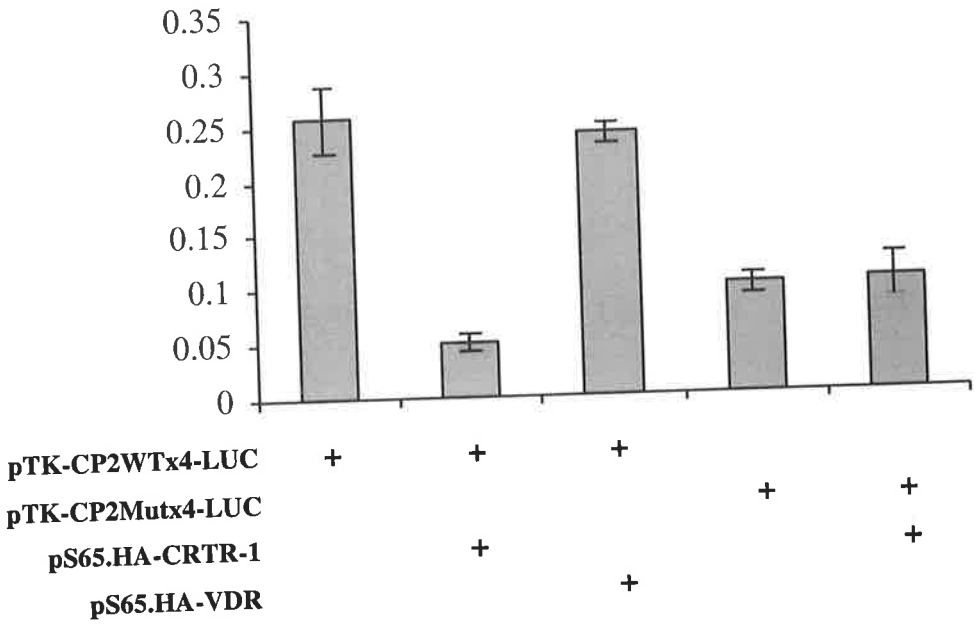
A) Schematic representation of pTK-CP2WTx4-LUC and pTK-CP2Mutx4-LUC, which contain a luciferase reporter gene regulated by a Tk promoter and four upstream copies of the wild type (WT) or mutant (Mut) α-globin CP2 response element, respectively, used as reporter constructs for experiments presented in **(B)** and Figure 7.3.

B) COS-1 cells were transfected (2.4.5) with expression vectors for HA-CRTR-1 and HA-VDR and the reporter plasmids, pTK-CP2WTx4-LUC and pTK-CP2Mutx4-LUC, as indicated. Luciferase activity was assayed (2.4.9) using a TD-20/20 luminometer and normalised against expression of Renilla luciferase expressed from the co-transfected plasmid pRLTK. The mean and standard deviation of three independent experiments is represented.

A


pTK-CP2WTx4-LUC
Tk
4XCP2WT-RE
Luciferase
poly-A
HindIII
SalI
BamHI
pTK-CP2Mutx4-LUC
Tk
4XCP2Mut-RE
Luciferase
poly-A
HindIII
SalI
BamHI

B


0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
pTK-CP2WTx4-LUC
pTK-CP2Mutx4-LUC
pS65.HA-CRTR-1
pS65.HA-VDR

pTK-CP2Mutx4-LUC was not altered by co-transfection with pSG5.HA-CRTR-1 (Figure 7.1b, columns 4, 5). These results demonstrate that CRTR-1 is able to repress transcription from a promoter containing CP2 consensus response elements, and that the mechanism for repression requires DNA binding.

7.2.3 CRTR-1 Can Act as a Dominant Repressor of CP2 Mediated Activation in Mammalian Cells.

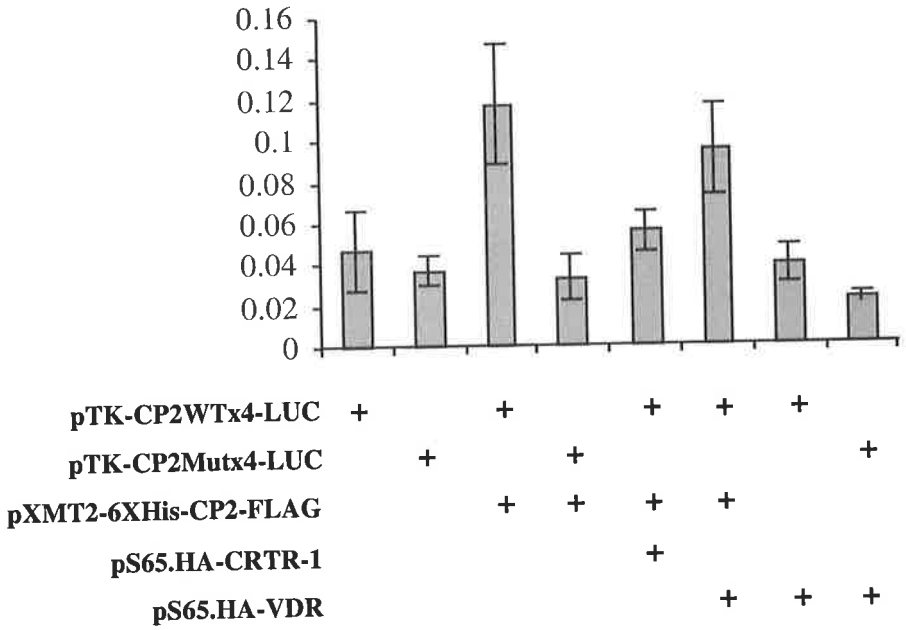
Activation of transcription by CP2 following binding to CP2 consensus sequences has been demonstrated for a variety of promoters (Bing *et al.*, 1999; Huang and Miller, 2000; Kim *et al.*, 1990; Kim *et al.*, 1987; Lim *et al.*, 1993; Lim *et al.*, 1992; Murata *et al.*, 1998). Co-transfection of pXMT2-6XhisCP2-FLAG (5.4.1) with pTK-CP2WTx4-LUC in COS-1 cells resulted in a 1.4-5 fold increase in luciferase activity (Figure 7.2, columns 1 and 3). This transcriptional activation resulted from CP2 binding to the CP2 response elements and activating luciferase expression because expression of luciferase from pTK-CP2Mutx4-LUC was not affected by co-transfection with pXMT2-6XhisCP2-FLAG (Figure 7.2, columns 2 and 4). Co-transfection of pSG5.HA-CRTR-1 and pXMT2-6XhisCP2-FLAG with pTK-CP2WTx4-LUC resulted in a 1.3-3 fold reduction in 6XHis-CP2-FLAG activation (Figure 7.2, column 5). Reduction in 6xHis-CP2-FLAG mediated transcriptional activation was specific for co-expression of HA-CRTR-1, as co-expression of HA-VDR did not affect expression of pTK-CP2WTx4-LUC in the presence 6xHis-CP2-FLAG (Figure 7.2, column 6).

These results demonstrate that CP2 is able to activate transcription from the α-globin CP2 response element, consistent with previous reports using *in vitro* and *in vivo* expcrimental techniques (Lim *et al.*, 1993). However, co-expression of CRTR-1 with CP2 reduced CP2 mediated activation of transcription indicating that CRTR-1 can act as a dominant repressor of CP2 mediated transcription.

FIGURE 7.2

Dominant transcriptional regulation of a CP2 responsive promoter by CRTR-1.

COS-1 cells were transfected with expression vectors for 6xHis-CP2-FLAG, HA-CRTR-1 and HA-VDR and the reporter plasmids, pTK-CP2WTx4-LUC and pTK-CP2Mutx4-LUC, as indicated. Luciferase activity was assayed using a TD-20/20 luminometer and normalised against expression of Renilla luciferase expressed from the co-transfected plasmid pRLTK. The mean and standard deviation of three independent experiments is represented.


0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
pTK-CP2WTx4-LUC + + + + +
pTK-CP2Mutx4-LUC + + +
pXMT2-6XHis-CP2-FLAG + + + +
pS65.HA-CRTR-1 +
pS65.HA-VDR + + +

7.3 DOMINANT REPRESSION OF TRANSCRIPTION BY CRTR-1 IN YEAST.

Properties of the Gal4-AD in yeast have been well characterised (Ma and Ptashne, 1987). Investigation into the ability of the CRTR-1 repression domain to can repress Gal4 activated transcription could provide insight into the mechanism employed by CRTR-1 to mediate dominant transcriptional repression. It was therefore of interest to determine if the presence of the CRTR-1 repression domain, expressed as a Gal4-AD fusion protein, would be able to abrogate transcriptional activation of growth selection genes mediated by Gal4-AD

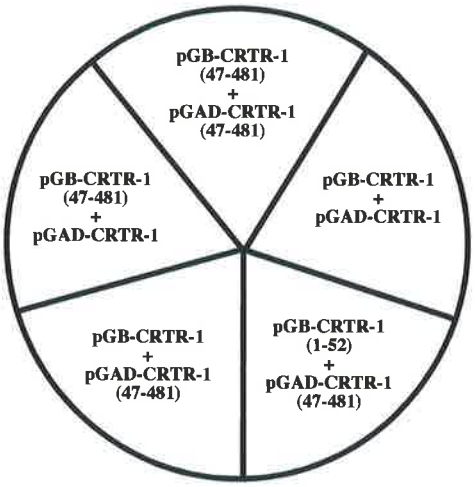
Yeast expression vectors for 2-hybrid analysis encoding full length CRTR-1 and amino acids 1-52 were generated by *Sal*I/*Sac*I digestion(2.3.1.1) of pGEM-T-CRTR-1 (3.3.1) and pGalO.CRTR-1(1-52) (4.2.1) respectively, producing fragments that were blunt ended (2.3.1.4) and cloned into pGBKT7 and pGADT7, digested with *Sma*I and treated with CIP (2.3.1.5), generating clones pGB-CRTR-1, pGB-CRTR-1(1-52) and pGAD-CRTR-1. Correct clones were determined by *Eco*RI digestion and automated BDT sequencing (2.3.1.14). pGB-CRTR-1, pGB-CRTR-1(1-52), pGB-CRTR-1(47-481), pGAD-CRTR-1, and pGAD-CRTR-1(47-481) were co-transformed (2.3.4.3) into AH109 yeast in various combinations. Yeast transformed with pGB and pGAD vectors were plated on -leu/-trp selection plates (2.2.12) and incubated at 30°C for 5 days. Colonies were then picked and streaked on -leu/-trp/-his and -leu/-trp/-his/-ade selection plates (2.2.12) and incubated at 30°C for 5 days.

Yeast co-transformed with pGB-CRTR-1(47-481) with pGAD-CRTR-1(47-481) showed growth at both levels of selection, confirming the ability of CRTR-1 to form homo-multimers (Figure 7.3). Yeast co-transformed with pGB-CRTR-1 and pGAD-CRTR-1, pGB-CRTR-1 and pGAD-CRTR-1(47-481) or pGB-CRTR-1(47-481) and pGAD-CRTR-1 showed little to no growth (Figure 7.3). Given that CRTR-1(47-481) did not have this

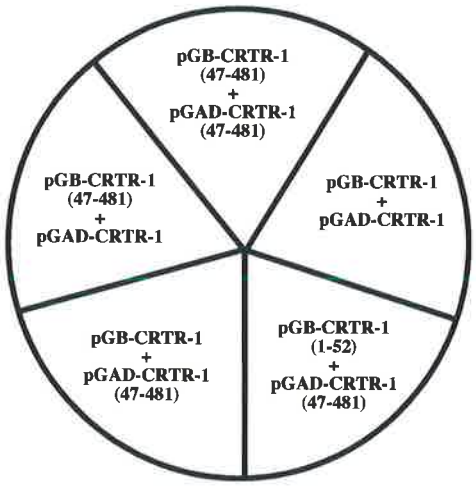
FIGURE 7.3

CRTR-1 represses Gal4-AD-mediated transcriptional activation.

AH109 yeast were co-transformed (2.3.4.3) with pGB-CRTR-1(47-481)/pGAD-CRTR-1(47-481), pGB-CRTR-1/pGAD-CRTR-1, pGB-CRTR-1(1-52)/pGAD-CRTR-1(47-481), pGB-CRTR-1/pGAD-CRTR-1(47-481) or pGB-CRTR-1(47-481)/pGAD-CRTR-1, plated on -leu/-trp selection plates and incubated at 30°C for 5 days. Colonies were then streaked onto -leu/-trp/-his **(A)** and -leu/-trp/-his/-ade **(B)** selection plates and incubated at 30°C for 5 days. Plates were scanned using a HP scanjet 7400c scanner.



-leu/-trp/-his



-leu/-trp/-his/-ade

effect, this observation suggests that the presence of the N-terminal 52 amino acids of CRTR-1 at Gal4 responsive promoters is inhibitory to yeast growth and is consistent with dominant repression of Gal4-AD mediated transcriptional activation of growth selection genes, *his3* and *ade2*, by the CRTR-1 repression domain.

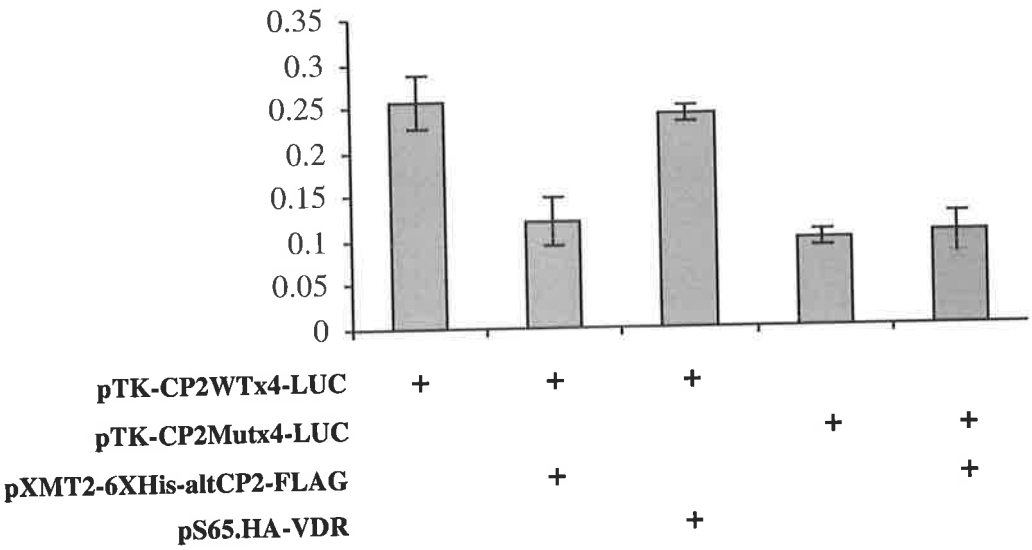
7.4 altCP2 CAN ACT AS A DOMINANT REPRESSOR OF CP2 MEDIATED ACTIVATION IN MAMMALIAN CELLS.

pTK-CP2WTx4-LUC and pTK-CP2Mutx4-LUC were transfected in combination with pXMT2-6XHis-altCP2-FLAG (5.4.1) and pSG5.HA-VDR (5.4.1) into COS-1 cells (2.4.5). Transfection of pTK-CP2WTx4-LUC alone demonstrated luciferase activity 2.5 fold greater than transfection of pTK-CP2Mutx4-LUC alone (Figure 7.4, columns 1 and 4), suggesting that endogenous CP2 family proteins present in COS-1 cells bind the CP2 responsive promoter region and activate transcription. Co-transfection of pXMT2-6XHis-altCP2-FLAG with pTK-CP2WTx4-LUC resulted in 1.2-2.5 fold reduction in luciferase activity (Figure 7.4, columns 2 and 1). This transcriptional repression was specific for pTK-CP2WTx4-LUC as expression of luciferase from pTK-CP2Mutx4-LUC was not altered by co-transfection with pXMT2-6XHis-altCP2-FLAG (Figure 7.4, columns 4 and 5). Furthermore, transfection of pSG5.HA-VDR did not alter luciferase levels from pTK-CP2WTx4-LUC compared with the reporter vector transfected alone (Figure 7.4, columns 3 and 1) . These results demonstrate that altCP2 is able to repress transcription from a promoter containing CP2 consensus response elements. The inability of altCP2 to bind the α-globin CP2 consensus response element suggests that the mechanism used by altCP2 to repress transcription is distinct from CRTR-1 and be mediated by the formation of non-DNA binding heteromeric protein complexes with endogenous CP2- like proteins.

FIGURE 7.4

Dominant transcriptional regulation of a CP2 responsive promoter by altCP2.

COS-1 cells were transfected (2.4.5) with expression vectors for 6xHis-altCP2-FLAG and HA-VDR and the reporter plasmids, pTK-CP2WTx4-LUC and pTK-CP2Mutx4-LUC, as indicated. Luciferase activity was assayed (2.4.9) using a TD-20/20 luminometer and normalised against expression of Renilla luciferase expressed from the co-transfected plasmid pRLTK. The mean and standard deviation of three independent experiments is represented.


0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
pTK-CP2WTx4-LUC + + +
pTK-CP2Mutx4-LUC + +
pXMT2-6XHis-altCP2-FLAG + +
pS65.HA-VDR +

7.5 DISCUSSION.

7.5.1 CRTR-1 Acts as a Dominant Repressor of Transcription

CRTR-1 has been demonstrated to act as a transcriptional repressor (4.2.2). Results presented in this chapter demonstrated the ability of CRTR-1 to act as a dominant repressor of transcriptional activation using two different eukaryotic systems, yeast and mammalian cells.

CRTR-1 has been shown to bind the α-globin CP2 response element (6.2) and form heteromeric protein/protein interactions with other CP2 family members in solution (5.3.2.1) and when bound to DNA (6.4). Consistent with these observations, expression of CRTR-1 repressed transcription 3.5-6 fold from a promoter containing four copies of the wild type (WT) α-globin CP2 response element, but not from a promoter containing mutant α-globin CP2 response elements (Figure 7.1b). The fact that repression was specific for the WT promoter demonstrates that DNA binding of CRTR-1 is required for transcriptional repression. Co expression of CRTR-1 with CP2, shown to activate transcription 1.4-5 fold by a mechanism dependent on DNA binding, reduced the level of CP2 mediated transcriptional activation by 1.3-3 fold (Figure 7.2). This indicates that CRTR-1 was able to act as a dominant repressor and negate CP2 mediated transcriptional activation. The mechanism required for dominant repression was specific for CRTR-1, as co-expression of VDR with CP2 did not affect levels of CP2 activation.

Some mechanistic insight was provided by investigation of CRTR-1 activity in yeast. Homomeric protein interactions of CRTR-1 in yeast within the context of the yeast-2-hybrid system position the Gal4-AD on responsive heterologous promoters adjacent to CRTR-1. Localisation of the Gal4-AD at these promoters should result in transcriptional activation of selection genes and thereby permit yeast growth. The observation that these yeast did not grow suggests that the presence of CRTR-1 inhibited transcriptional activation mediated by Gal-4-AD (Figure 7.3). The ability to repress yeast growth was localised to the N-terminal 52 amino acids of CRTR-1, as co-expression of Gal4-DBD-

CRTR-1(47-481) and Gal4-AD-CRTR-1(47-481) resulted in yeast growth (Figure 7.3) demonstrating that the CRTR-1 repression domain was able to act as a dominant repressor of Gal4-AD mediated transcriptional activation.

The Gal4-AD is an acidic amino acid rich transcriptional activation domain (Ma and Ptashne, 1987) that activates transcription by direct recruitment of TATA factor II D (TFIID) (Horikoshi *et al.*, 1988) and TATA binding protein (TBP (Melcher and Johnston, 1995), both components of the basal transcriptional machinery, to the promoter of target genes. The ability of CRTR-1 to act as a dominant repressor of Gal4-AD transcriptional activation suggests that the N-terminal 52 amino acids of CRTR-1 is able to recruit proteins that block recruitment of TFIID and TBP or act as co-repressors of transcription in a dominant fashion to TFIID and TBP activation.

Dominant repression of transcription mediated by CRTR-1 was observed in yeast and mammalian cells demonstrating that the mechanism employed by CRTR-1 is conserved. Mechanisms used to achieve dominant repression of transcription could include the recruitment of co-repressor proteins such as polycomb family members, histone deacetylases or multimeric protein complexes containing histone deacetylase activity to the promoter by CRTR-1 that mediate transcriptional repression and could prevent recruitment of co-activator complexes otherwise recruited by CP2. Alternatively, formation of CP2/CRTR-1 heteromeric complexes may prevent the stable interaction of co-activators otherwise recruited by CP2 homomeric complexes.

The mechanism employed by CRTR-1 to act as a dominant repressor of CP2 mediated transcriptional activation could be characterised further by experiments co-transfecting expression vectors for CP2 and CRTR-1(47-481) with pTK-CP2WTx4-LUC to confirm that the CRTR-1 repression domain is required. Alternatively, the requirement for the CRTR-1 repression domain could be demonstrated by experiments co-transfecting expression vectors for CP2 with CRTR-1(1-52)-CP2(40-501) fusion protein. Furthermore,

identification and mutation of the amino acids critical for CRTR-1 DNA binding could be carried out to determine if the mechanism of dominant repression requires DNA binding.

Temporally and spatially regulated expression of the dominant repressor of transcription, CRTR-1, during mammalian development provides a mechanism for developmental control of promoters regulated by ubiquitously expressed CP2 family transcriptional activators.

7.5.2 Dominant Negative Regulation May be a Feature of CP2 Family Mediated Transcriptional Activation.

Transfection of pTK-CP2WTx4-LUC produced a background level of luciferase activity that was as much as 2.5 fold greater than that observed for transfection of pTK-CP2Mutx4-LUC. This suggests that endogenous CP2-like factors present in COS-1 cells were able to bind the CP2 response elements and activate transcription. Expression of altCP2 in cells transfected with pTK-CP2WTx4-LUC resulted in reduction of luciferase activity to levels comparable with the mutant luciferase reporter construct (Figure 7.4). These observations suggest that altCP2, previously shown to be unable to bind the α-globin CP2 response element (6.4.1) and able to negate the DNA binding ability of CP2 (Figure 6.6), may be able to form heteromeric complexes with CP2-like factors present in COS-1 cells and prevent DNA binding and hence transcriptional activation. This defines altCP2 as a dominant negative isoform of CP2 that functions independent of DNA binding. This observation could be further characterised by experiments co-transfecting expression vectors for CP2 and altCP2 to test the ability of altCP2 to negate the transcriptional activation ability of exogenous CP2 from pTK-CP2WTx4-LUC confirming the specificity of altCP2 action to CP2 family transcription factors.

altCP2 has been shown to be expressed at low levels and in a regulated fashion (3.7.3). The ability of altCP2 to prevent the activation of CP2 regulated promoters by abrogating DNA binding provides a novel mechanism for the regulation of CP2 transcriptional activation which is distinct from the dominant repression ability of CRTR-1. Together, CRTR-1 and altCP2 provide two distinct and independent mechanisms for the developmentally regulated control of promoters activated by the ubiquitously expressed members of the CP2 transcription factor family.

CHAPTER 8

Interaction between CRTR-1 and cellular proteins.

8.1 INTRODUCTION.

Cellular binding partners identified for LBP-1c include YY1 (shown to bind the HIV-1 LTR promoter with LBP-1c) (Romerio *et al.*, 1997), NF-E4 (important for the regulation of γ-globin gene expression) (Zhou *et al.*, 2000), RING1 (shown to bind LBP-1c and repress transcriptional activation) (Tuckfield *et al.*, 2002b) and FE65 (associated with the APP at the cell membrane. The importance of the interaction between LBP-1c and FE65 is unknown) (Zambrano *et al.*, 1998). Identification of these binding proteins has implicated LBP-1c in certain signaling pathways and transcriptional networks providing insight into possible biological functions of LBP-1c.

The region of LBP-1c required for interaction with YY1 has not been determined. However, the regions of LBP-1c used in experiments identifying NFE4 and RING1 as binding partners has defined amino acids 260-502 of LBP-1c as required for interaction with these proteins (Tuckfield *et al.*, 2002b; Zhou *et al.*, 2000). Similarly, the region of LBP-1c required for interaction with FE65 is the C-terminal 195 amino acids (Zambrano *et al.*, 1998). These observations are consistent with the region required for homomeric and heteromeric interaction with other CP2 family members and support localization of the CP2 family protein interaction domain to the C-terminal 240 amino acids.

The ability of CRTR-1 to form heteromeric protein complexes with CP2 family members was investigated using the Match maker yeast-2-hybrid system (4.3). This analysis was extended to screening a yeast-2-hybrid cDNA library to identify non-CP2 related CRTR-1 binding proteins. Further analysis included testing the ability of CRTR-1 to interact with known CP2 binding proteins and other potential candidate binding proteins with the aim of uncovering signalling pathways and/or other transcription factors that may be regulators of CRTR-1 function.

8.2. AMPLIFICATION AND VALIDATION OF A YEAST-2-HYBRID cDNA LIBRARY.

As CRTR-1 was identified as a gene expressed in ES and EPL cells a pluripotent cell cDNA library would ideally be used to identify potential CRTR-1 binding partners. However, a validated ES or EPL cell yeast-2-hybrid cDNA library was not available. CRTR-1 is also expressed in the adult mouse testis (1.12.2), a site rich in stem cells and believed, as with ES cells, to have promiscuous expression of many genes (Dr. S.Wood, The University of Adelaide. pers com; (Ramalho-Santos *et al.*, 2002)). For this reason the Clontech adult mouse testis yeast-2-hybrid cDNA library, constructed from $(dT)_{15}$-*Xho*I primed cDNA generated using mRNA isolated from normal, whole testis pooled from 200 BALB/c male mice, aged between 8-12 weeks cloned into *Eco*RI/*Xho*I digested pACT2 (2.2.6.1) was used.

The library titre was determined to be $3x10^8$ cfu/ml by serial dilution and quantitation of colony numbers. For unbiased amplification, the library was spread, at a density of 40,000 colonies per plate, onto 250 150 mm diameter ampicillin bacterial selection plates which were cultured overnight at 37°C. Colonies from all 250 plates were pooled, mixed and separated between 6 large oakridge tubes. Four tubes were stored at –80°C while the other two were used for CsCl plasmid DNA preparation (2.3.2.7). 2.5 µg of the library was digested (2.3.1.1) with *Eco*RI/*Xho*I and separated by 1% TAE agarose gel electrophoresis (2.3.1.2) (Figure 8.1). This showed a band at approximately 8 Kb, consistent with the size of the pACT2 vector, and a smear that extended for the length of the gel and concentrated at approximately 2 kb, representative of the average insert size of the library (Clontech).

The mouse testis cDNA library was validated for use in isolation of novel CRTR-1 binding proteins by demonstration that transcripts encoding known CRTR-1 binding proteins were present in the library. Platinum Taq PCR (2.3.1.12) was carried out on 100 ng of the testis cDNA library using primers SR7/SR2, CP2SR3/CP2SR2 and

FIGURE 8.1

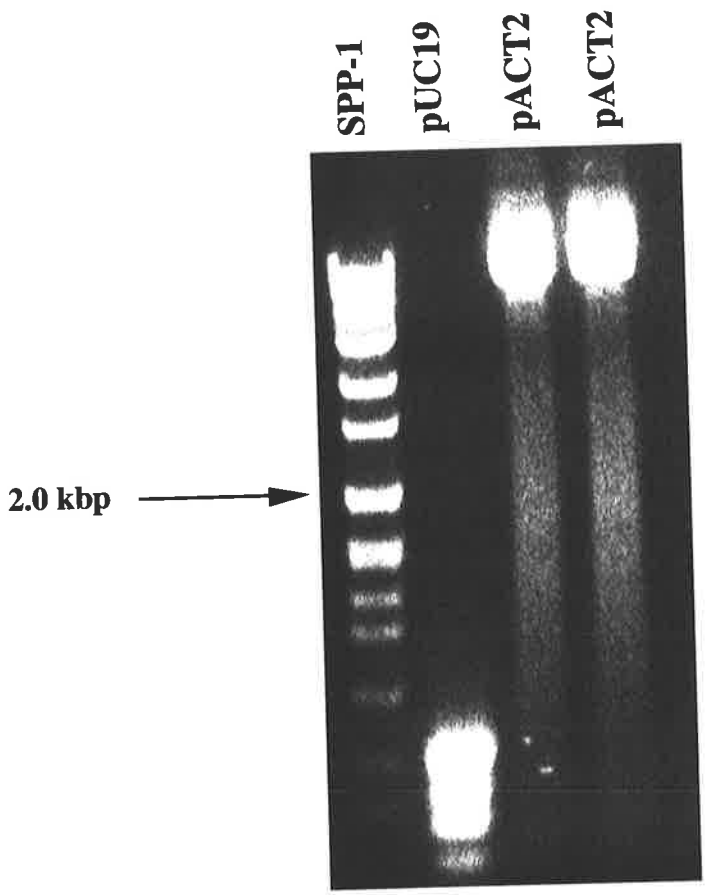
Amplification and validation of the yeast-2-hybrid mouse testis cDNA Library.

A) A yeast-2-hybrid library in the pACT2 vector (Clontech) constructed from $(dT)_{15}$-*Xho*I primed cDNA generated using mRNA isolated from normal, whole testes pooled from 200 BALB/c male mice, aged between 8-12 weeks, was amplified (2.3.4.1) and 2.5 µg was digested with *Eco*RI/*Xho*I. Duplicate samples (pACT2) of the reaction were separated on a 1% TAE agarose gel and visualised under UV light after staining with ethidium bromide. Spp-1; *Eco*RI digested Spp1 phage size markers (2.2.13); pUC19; *Hpa*II digested pUC19 DNA markers (2.2.13)

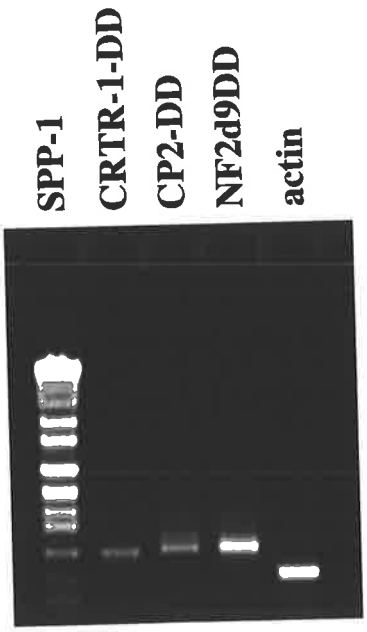
B) Platinum Taq PCR (2.3.1.12) using the primer sets SR7/SR2 (for CRTR-1-DD), CP2SR3/CP2SR2 (for CP2-DD) and NF2d9SR3/NF2d9SR2 (for NF2d9-DD) (2.2.7.2) was carried out on 100 µg of the yeast-2-hybrid library **(A)**. PCR reactions were separated on a 1% TAE agarose gel and visualised by exposure to UV light after ethidium bromide staining. Spp-1; 500 ng of *Eco*RI digested Spp1 phage DNA (2.2.13).

PCR Parameters:
Step 1: 94°C for 3 minutes
Step 2: 94°C for 30 seconds
Step 3: 45°C for 30 seconds
Step 4: 72°C for 2 minutes
Step 5: cycle back to step 2, 29 times.
Step 6: 72°C for 4 minutes
Step 7: 4°C
Step 8: End

A


SPP-1
pUC19
pACT2
pACT2
2.0 kbp

B


SPP-1
CRTR-1-DD
CP2-DD
NF2d9DD
actin

NF2d9SR3/NF2d9SR2 (2.2.7.2), specific for the dimerisation domain of CP2 family members. The presence of PCR products of expected sizes (3.3.1, 3.6) (Figure 8.1b) confirmed the presence of transcripts encoding the dimerisation domain equivalents (region of LBP-1c required for protein/protein interaction) of CRTR-1, CP2, altCP2, NF2d9 and altNF2d9PCR in the cDNA library.

8.3 YEAST-2-HYBRID SCREEN ONE.

150mls of YPDA medium (2.2.12) was inoculated with AH109 yeast (2.2.11) transformed with pGB-CRTR-1(47-481) (5.3.1) and cultured at 30°C overnight. 50mls of the overnight culture was sub-cultured into each of two 500 ml YPDA cultures and grown at 30°C to an OD_{600} of 0.5. Yeast were pelleted, transformed with 500 µg of pACT2-testis cDNA library (2.3.4.2), plated out at 200µl per plate onto 50 -leu/-trp/-his selection plates and incubated at 30°C for 5 days. Samples of the transformed yeast were also plated out on -leu/-trp plates to enable the determination of transformation efficiency.

890 colonies grew on the -leu/-trp/-his plates after 5 days. These were spotted onto -leu/-trp/-his/-ade plates for more stringent selection and grown at 30°C for 5 days. 15 viable colonies were grown up overnight for plasmid preparation and analysis (2.3.4.4). In this experiment the transformation efficiency was low and only $2.7x10^6$ library clones were screened in comparison with $3.5x10^6$ independent library clones present in the library (Clontech).

8.4 YEAST-2-HYBRID SCREEN TWO.

A second yeast-2-hybrid screen was carried out using co-transformation of the cDNA library with pGB-CRTR-1(47-481). Co-transformation of yeast plasmids is an alternative method to sequential transformation, used in 8.3, and has yielded higher transformation efficiencies in some experiments (Clontech).

AH109 yeast were cultured as described in 8.3, co-transformed with 1 μg of pGB-CRTR-1(47-481) and 500 μg of pACT2-testis cDNA library (2.3.4.2) and plated as described in 8.3. Determination of the transformation efficiency established that $9.5x10^6$ library clones were screened, 3 fold greater than the first attempt and 2 fold greater than the number of independent clones in the library. 462 colonies grew on the -leu/-trp/-his plates after 5 days. These colonies were inoculated onto -leu/-trp/-his/-ade plates and grown at 30°C for 5 days. 46 viable colonies were grown up for plasmid preparation and analysis (2.3.4.4).

8.5 ANALYSIS OF YEAST-2-HYBRID POSITIVE CLONES.

Clones were digested with *Eco*RI/*Xho*I to confirm the presence of a cDNA insert and sequenced using BDT automated sequencing (2.3.1.14) with the Gal4SR1 primer (2.2.7.2). The sequence of each clone was compared to nucleotide sequences present in the BlastN database (2.3.1.16). Nucleotide sequences were also translated in-frame with the Gal4-AD and compared to amino acid sequences present in the BlastP database (2.3.1.16).

Of the 15 yeast clones isolated for the first yeast-2-hybrid screen:

- plasmid DNA could not be isolated from 4 clones (A62, C33, C121 and D55)
- 8 clones shared no sequence identity with known amino acid sequences (A1, A39, C61, E62, F7, G23, G71 and G113).
- Clone D76 encoded a protein of 93 amino acids that shared 100% identity to a phosphodiesterase, 6D, clone MGC:11435 (direct submission, accession number: BC005636).
- Clone G56 encoded an amino acid sequence that showed 100% identity to the ubiquitin conjugating enzyme 9, Ubc9 (Gottlicher *et al.*, 1996).
- Clone G117 encoded an amino acid sequence that showed 100% identity to CRTR-1.

Clones G56 and G117 were selected for further investigation.

Of the 45 yeast clones isolated for the second yeast-2-hybrid screen:

- plasmid DNA could not be isolated from 2 clones (A7 and A23)
- 15 clones encoded short proteins less that showed no identity to known amino acid sequences (A1, A2, A9, A12, A16, A33, A44, A56, A62, A73, B5, B17, B27, B54 and B73).
- 10 clones encoded heat shock or elongation factor proteins that have been described as false positives frequently obtained from yeast-2-hybrid libraries. (A13, A14, A43, A49, A50, B77, C1, C2, C7 and C8).
(http://www.fccc.edu/research/labs/golemis/main_false.html)
- 8 clones encoded proteins that shared significant identity to hypothetical proteins identified as part of genome sequencing projects that have not been functionally investigated (A54, A57, A58, A66, B102, C3, C4 and C5).
- 5 clones encoded an amino acid sequence that showed 100% identity to T-complex testis specific protein 2 (TCTEX-2), a highly expressed sperm tail surface protein (Huw *et al.*, 1995) (B4, B8, B28, B40 and B101).
- 2 clones encoded an amino acid sequence that showed 100% identity to the protein, PIAS1, protein inhibitor of activated Stat1 (Liu *et al.*, 1998) (A68 and A59).
- one clone encoded an amino acid sequence that showed 100% identity to Ubc9 (Gottlicher *et al.*, 1996) (A11).
- one clone encoded an amino acid sequence that showed 100% identity to NF2d9 (Sueyoshi *et al.*, 1995) (A46).
- one clone encoded an amino acid sequence that showed 100% identity to the immunophillin, FK506 binding protein 4, FKBP4 (Schmitt *et al.*, 1993) (B87).

Clones A11, A46, A59, A68 and B87were selected for further investigation.

Sequence data for each clone isolated from both yeast-2-hybrid screens is presented in appendix 1. Clones chosen from both yeast-2-hybrid screens for further investigation were selected based on the information available for each protein from literature searches. Availability of the information provides greater insight to the function of these proteins and how they are likely to contribute to regulation of CRTR-1 activity and elucidation of CRTR-1 biological function.

Isolation of CRTR-1 (clone G117) and NF2d9 (clone A46), known CRTR-1 binding proteins, confirm that the yeast-2-hybrid system could be used to identify cellular CRTR-1 binding partners. However, the yeast-2-hybrid screen was not comprehensive as the complete spectrum of known CRTR-1 binding proteins, including CP2, altCP2 and altNF2d9, were not isolated in either screen indicating that the total repertoire of non-CP2 related CRTR-1 binding partners was unlikely to have been isolated.

8.5.1 Amino Acid Sequence Analysis.

Clones G56, G117, A11, A46, A59, A68 and B87 were sequenced in both directions using BDT automated sequencing (2.3.1.14) and primers Gal4SR1 and Gal4SR2 (2.2.7.2). Complied sequence data was then translated to determine the region of these proteins required for interaction with CRTR-1.

- Clones G56 and A11 and A59 and A68 encoded full length Ubc9 (158 amino acids) and PIAS1 (651 amino acids) proteins, respectively.

- Clone B87 encoded a 334 amino acid N-terminally truncated form of FKBP4 (458 amino acids) extending from amino acid 125 to the C-terminus.
- Clone A46 encoded a 336 amino acid N-terminally truncated form of NF2d9 (505 amino acids) extending from amino acid 137 to the C-terminus.
- Clone G117 encoded a 433 amino acid N-terminally truncated form of CRTR-1 (481 amino acids) extending from amino acid 49 to the C-terminus.

8.5.2 Confirmation of Putative CRTR-1 Binding Partners Identified by Yeast-2-Hybrid.

Plasmids G56, G117, A11, A68, A46 and B87 were transformed (2.3.4.3) into AH109 yeast (2.2.11) with pGB-CRTR-1(47-481) to validate the interaction between proteins encoded by these cDNAs and CRTR-1. Transformed yeast were plated on -leu/-trp selection plates (2.2.12) and incubated at 30°C for 5 days. Colonies for each co-transformation were then streaked in duplicate onto -leu/-trp/-his and -leu/-trp/-his/-ade selection plates (2.2.12) and incubated at 30°C for 5 days. Proteins encoded by all clones tested showed positive interaction with CRTR-1(47-481), indicated by strong yeast growth at both selection levels (Figure 8.2). AH109 yeast co-transformed with the cDNA clones and pGB-LAM-1 (2.2.6.1) (Clontech) showed no growth after 5 days culture at 30°C on -leu/-trp/-his selection plates (2.2.12) verifying the specificity of interaction between proteins encoded by the clones and CRTR-1(47-481) (data not shown). These results identify Ubc9 (G56 and A11), PIAS1 (A68) and FKBP4 (B87) as novel CRTR-1 binding proteins and confirm previous results demonstrating CRTR-1 (G117) and NF2d9 (A46) as CRTR-1 binding proteins (5.3.2).

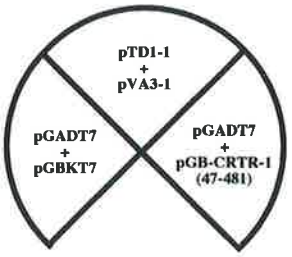
FIGURE 8.2

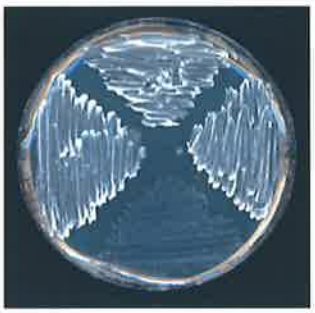
Yeast-2-hybrid analysis of interactions between CRTR-1(47-481) and Ubc9, PIAS1 and FKBP4.

AH109 yeast were co-transformed (2.3.4.3) with pGB-CRTR-1(47-481) and pACT2 based cDNA clones identified by yeast-2-hybrid screen 1 **(A)** and screen 2 **(B).** Transformed yeast were plated on -leu/-trp selection plates and incubated at 30°C for 5days. Colonies were then streaked onto -leu/-trp/his and -leu/-trp/-his/-ade selection plates as indicated and incubated at 30°C for 5 days. Plates were scanned using a HP scanjet 7400c scanner. AH109 yeast were co-transformed with pGADT7/pGBKT7, pTD1-1/pVA3-1 and pGADT7/pGB-CRTR-1(47-481) as growth controls described previously in figure 5.4

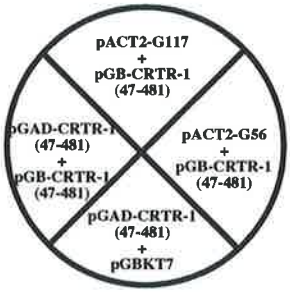
A

-leu/-trp/-his

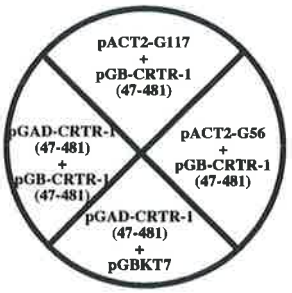




-leu/-trp/-his

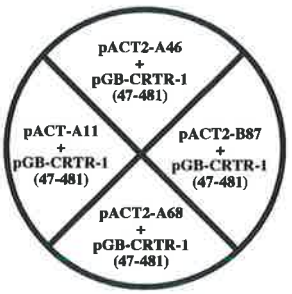


-leu/-trp/-his/-ade

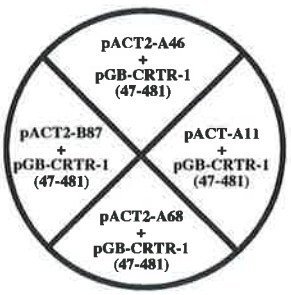


B

-leu/-trp/-his



-leu/-trp/-his/-ade



8.6 CO-PURIFICATION OF CRTR-1 WITH UBC9, PIAS1 AND FKBP4.

Interaction between CRTR-1 and the putative binding proteins Ubc9, PIAS1 and FKBP4 in mammalian cells was investigated by co-purification of proteins from transfected COS-1 cells.

8.6.1 Generation of Tagged Fusion Proteins for Purification and Identification.

To enable purification and identification Ubc9, PIAS1 and FKBP4 were fused to 6xHis and FLAG moieties expressed at the N- and C-terminus of ORFs respectively (5.4.1).

cDNAs encoding full length Ubc9 and PIAS1 fused to N-terminal 6xHis and C-terminal FLAG tags were generated by platinum Taq PCR (2.3.1.12) using primers Ubc9SR1/Ubc9SR2 and PIAS1SR1/PIAS1SR2 (2.2.7.2) on polyT primed cDNA (2.3.1.11) generated from total adult mouse kidney RNA (2.3.5.2). cDNAs encoding full length FKBP4 fused to N-terminal 6xHis and C-terminal FLAG tags were generated by platinum Taq PCR (2.3.1.12) using primers FKBP4SR1/FKBP4SR2 (2.2.7.2) on 100 ng of the yeast-two-hybrid cDNA testis library (2.2.6.1). PCR products, in each case a single band of the appropriate size, were isolated, purified (2.3.1.3) and cloned into pGEM-T easy, excised by *Eco*RI digestion (2.3.1.1) and cloned into pXMT2 digested with *Eco*RI and CIP treated (2.3.1.5) generating pXMT2-6xHis-Ubc9-FLAG, pXMT2-6xHis-PIAS1-FLAG and pXMT2-6xHis-FKBP4-FLAG. Correct clones were selected by directional digests and sequenced by BDT automated sequencing (2.3.1.14).

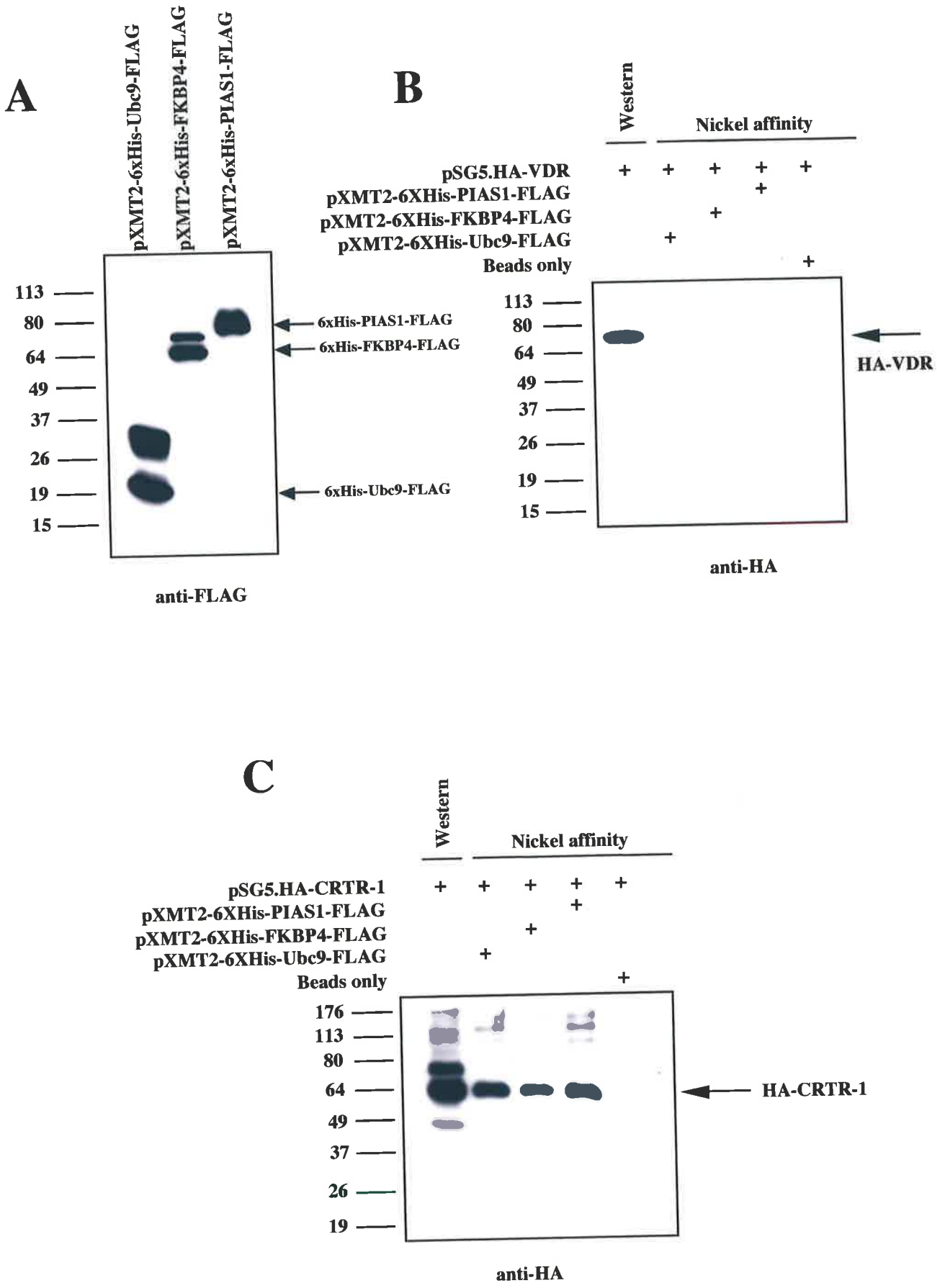
Expression of His and FLAG tagged proteins was tested in $3x10^6$ COS-1 cells transfected (2.4.5) with 2 μg of each vector. Whole cell protein extracts were made 24 hours post-transfection (2.3.6.2) and analysed by western blot (2.3.6.6) using the mouse anti-FLAG monoclonal antibody (2.2.8) for detection of FLAG-tagged fusion proteins (Figure 8.3a). Bands were observed at 20 and 30 kDa for 6xHis-Ubc9-FLAG (lane 1), 63 and 67 kDa for 6Xhis-FKBP4-FLAG (lane 2) and 72 kDa for 6Xhis-PIAS1-FLAG (lane 3),

FIGURE 8.3

Co-purification of CRTR-1 with Ubc9, PIAS1 and FKBP4.

A) $3x10^5$ COS-1 cells, grown in 60mm diameter tissue culture dishes, were transfected with 2µg of pXMT2-6xHis-Ubc9-FLAG, pXMT2-6xHis-FKBP4-FLAG or pXMT2-6xHis-PIAS1-FLAG (2.4.5). Whole cell extracts were produced 24hrs post transfection (2.3.6.2) and separated by 10% SDS-PAGE (2.3.6.4) prior to western analysis (2.3.6.6). The filter was probed with the monoclonal mouse anti-FLAG antibody prior to incubation with rabbit anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody (2.2.8) and visualised using chemiluminescence (2.3.6.6). Markers; Benchmark pre-stained protein ladder (2.2.14).

2µg of pSG5.HA-CRTR-1 **(B)** and pSG5.HA-VDR **(C)** were transfected in combination with 2 µg of pXMT2-6xHis-Ubc9-FLAG, pXMT2-6xHis-FKBP4-FLAG or pXMT2-6xHis-PIAS1-FLAG, into $3x10^5$ COS-1 cells grown in 60mm diameter tissue culture dishes (2.4.5). Whole cell extracts were produced 24hrs post transfection, nickel affinity purification of CP2 family members was carried out (2.3.6.3) and proteins were separated by 10% SDS-PAGE (2.3.6.4) prior to western analysis (2.3.6.6). Filters were probed with monoclonal mouse anti-HA prior to incubation with horseradish peroxidase (HRP) conjugated rabbit anti-mouse secondary antibody (2.2.8) and visualised using chemiluminescence (2.3.6.6). Extracts of COS-1 cells transfected with pSG5.HA-CRTR-1 or pSG5.HA-VDR alone were included in the analysis as controls. Markers; Benchmark pre-stained protein ladder (2.2.14).


A
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pXMT2-6xHis-FKBP4-FLAG
pXMT2-6xHis-PIAS1-FLAG
113
80
64
49
37
26
19
15
6xHis-PIAS1-FLAG
6xHis-FKBP4-FLAG
6xHis-Ubc9-FLAG
anti-FLAG
B
Western
Nickel affinity
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pXMT2-6XHis-PIAS1-FLAG
pXMT2-6XHis-FKBP4-FLAG
pXMT2-6XHis-Ubc9-FLAG
Beads only
113
80
64
49
37
26
19
15
HA-VDR
anti-HA
C
Western
Nickel affinity
pSG5.HA-CRTR-1
pXMT2-6XHis-PIAS1-FLAG
pXMT2-6XHis-FKBP4-FLAG
pXMT2-6XHis-Ubc9-FLAG
Beads only
176
113
80
64
49
37
26
19
HA-CRTR-1
anti-HA

confirming expression of tagged proteins. Multiple bands observed for 6xHis-Ubc9-FLAG and 6Xhis-FKBP4-FLAG are consistent multiple bands observed previously (Davies *et al.*, 2002; Johnson and Blobel, 1997).

8.6.2 CRTR-1 Co-purifies with Ubc9, PIAS1 and FKBP4.

pSG5.HA-CRTR-1 (5.4.1) was co-transfected (2.4.5) into $3x10^6$ COS-1 cells with either pXMT2-6xHis-Ubc9-FLAG, pXMT2-6xHis-PIAS1-FLAG or pXMT2-6xHis-FKBP4-FLAG. 24 hours post transfection, whole cell extracts were produced (2.3.6.3) and nickel affinity purification of 6xHis-Ubc9-FLAG, 6xHis-PIAS1-FLAG and 6xHis-FKBP4-FLAG (2.3.6.3) was carried out by addition of talon beads in the presence of 5 mM imidazole. Beads were washed several times and resuspended in 2xSDS load buffer (2.2.5) prior to separation of bound proteins by 10% SDS-PAGE (2.3.6.4) and analysis by western analysis (2.3.6.6).

Probing of Ni-affinity purified extracts from COS-1 cells co-transfected with pSG5.HA-CRTR-1 and pXMT2-6xHis-Ubc9-FLAG, pXMT2-6xHis-PIAS1-FLAG or pXMT2-6xHis-FKBP4-FLAG with mouse anti-HA monoclonal antibody (2.2.8) revealed a band at 64 kDa (Figure 8.3c, lanes 2-4) consistent with the size expected for HA-CRTR-1 (5.4.1). This demonstrates that HA-CRTR-1 was co-purified with 6xHis-Ubc9-FLAG, 6xHis-PIAS1-FLAG and 6xHis-FKBP4-FLAG and confirms the identification of Ubc9, PIAS1 and FKBP4 as CRTR-1 binding proteins. The specificity of these interactions was verified by failure to detect HA-CRTR-1 in Ni-affinity purified extracts from pSG5.HA-CRTR-1 transfected COS-1 cells (Figure 8.3c, lane 5). Further, HA-VDR, expressed from pSG5.HA-VDR (5.4.1), was not co-purified from COS-1 cells co-transfected with pXMT2-6xHis-Ubc9-FLAG, pXMT2-6xHis-PIAS1-FLAG or pXMT2-6xHis-FKBP4-FLAG (Figure 8.3b, lanes 2-4).

8.7 INTERACTIONS BETWEEN CP2 FAMILY MEMBERS AND UBC9, PIAS1 AND FKBP4.

The region of LBP-1c required for protein/protein interactions between CP2 family members and non-related cellular proteins is highly conserved throughout the CP2 family. It was therefore of interest to determine whether binding partners identified for CRTR-1 by yeast-two-hybrid analysis were specific for CRTR-1 or able to bind other members of the CP2 family.

pGB-CP2-DD, pGB-altCP2-DD, pGB-NF2d9-DD and pGB-altNF2d9-DD (5.3.1) were co-transformed (2.3.4.3) into AH109 yeast with G56 (Ubc9), A68 (PIASI) and B87 (FKBP4). Transformed yeast were plated and cultured as described in 8.5.2.

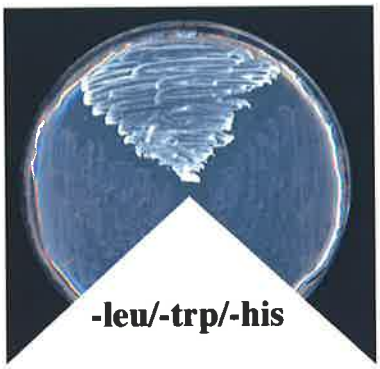
Yeast co-transformed with pGB-CP2-DD, pGB-altCP2-DD, pGB-NF2d9-DD or pGB-altNF2d9-DD and G56 or A68 showed growth at both levels of selection indicating a positive interaction between CP2, altCP2, NF2d9 and altNF2d9 with Ubc9 and PIAS1 (Figures 8.4 and 8.5). In contrast, yeast co-transformed with pGB-CP2-DD, pGB-altCP2-DD, pGB-NF2d9-DD or pGB-altNF2d9-DD and B87 showed limited growth on the -leu/-trp/-his selection (2.2.12) and no growth on the -leu/-trp/-his/-ade selection plate (2.2.12) indicating that CP2, altCP2, NF2d9 and altNF2d9 did not interact strongly with FKBP4 (Figures 8.4 and 8.5). CP2, altCP2, NF2d9 and altNF2d9 therefore interact with Ubc9 and PIAS1, consistent with CRTR-1, but fail to interact with FKBP4.

The region used for identification of CRTR-1 binding partners in yeast-2-hybrid analysis was amino acids 47-481 (5.3.1). This is more extensive than the dimerisation domain of CP2, altCP2, NF2d9 and altNF2d9 used in this analysis which failed to interact with FKBP4. The area required for interaction between CRTR-1 and FKBP4 may therefore be outside the dimerisation domain, in amino acids 47-232. To test this the dimerisation domain equivalent region of CRTR-1, amino acids 233-481, was tested for interaction with FKBP4 in yeast-2-hybrid analysis.

FIGURE 8.4

Interaction of CP2 and altCP2 with Ubc9, PIAS1 and FKBP4.

AH109 yeast were co-transformed (2.3.4.3) with plasmids as indicated. Yeast were grown and analysed as described in figure 8.2.



-leu/-trp/-his

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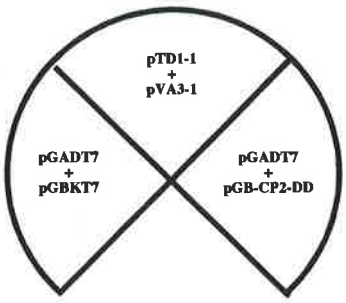

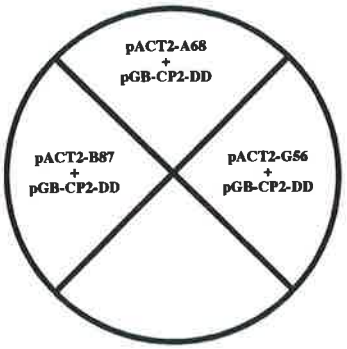

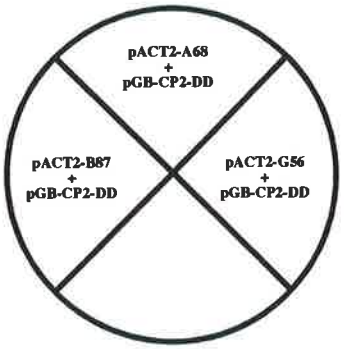

-leu/-trp/-his

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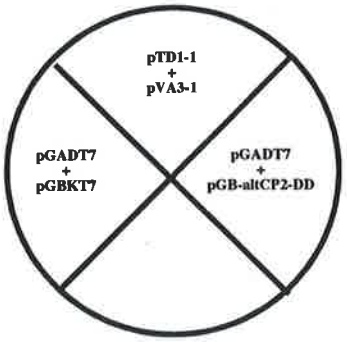
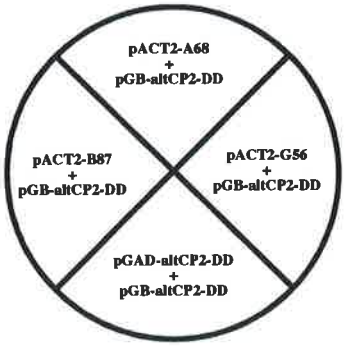




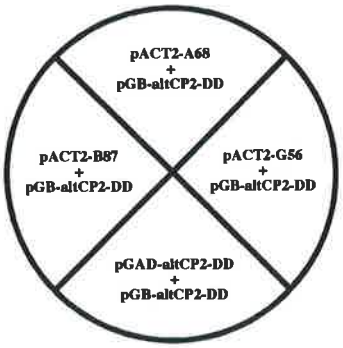
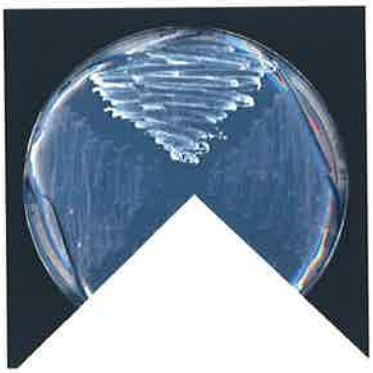


FIGURE 8.5

Interaction of NF2d9 and altNF2d9 with Ubc9, PIAS1 and FKBP4.

AH109 yeast were co-transformed (2.3.4.3) with plasmids as indicated. Yeast were grown and analysed as described in figure 8.2.



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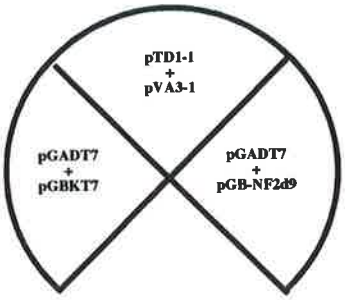

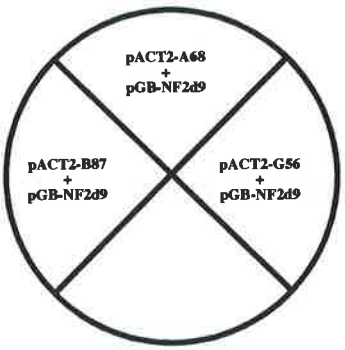

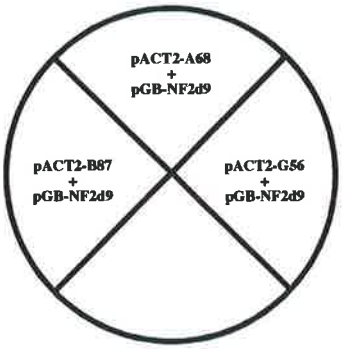

-leu/-trp/-his

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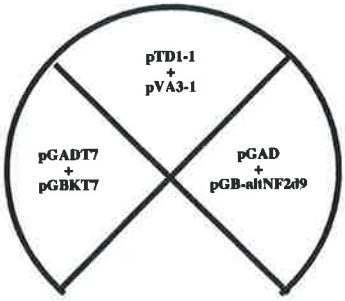

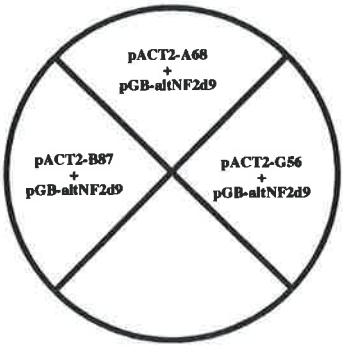

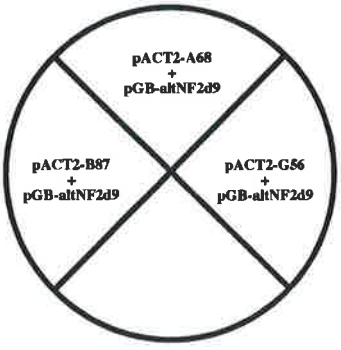

A cDNA fragment of CRTR-1 encoding amino acids 233-481 was isolated by Platinum Taq PCR (2.3.1.12) using primers SR7 and SR2 (2.2.7.2) on pGEM-T-CRTR-1 (3.3.1). The single PCR product was cloned into pGEM-T easy, excised by *Eco*RI digestion (2.3.1.1) and cloned into pGBKT7 digested with *Eco*RI and CIP treated (2.3.1.5), generating pGB-CRTR-1-DD that was confirmed by BDT automated (2.3.1.14) sequencing and IvTT (2.3.6.7) (data not shown). AH109 yeast co-transformed with pGB-CRTR-1-DD and B87, plated and cultured as described in 8.5.2, showed growth equivalent to yeast co-transformed with pGB-CRTR-1(47-481) and B87 at both levels of selection (data not shown). This demonstrated that amino acids 47-232 of CRTR-1 were not required for CRTR-1 to interact with FKBP4, and confirmed FKBP4 as a distinct binding partner for CRTR-1, distinct from other CP2 family members.

8.8 INTERACTION OF CRTR-1 WITH RING1 AND HDAC1, MODULATORS OF TRANSCRIPTIONAL REPRESSION.

Published reports have demonstrated LBP-1c to interact with several unrelated cellular proteins including RING1, a member of the Polycomb group of proteins (Tuckfield *et al.*, 2002b), NF-E4, a component of the stage selector protein important for regulation of γ-globin genes (Zhou *et al.*, 2000), FE65, a neural specific protein of undefined function (Zambrano *et al.*, 1998), and YY1, a zinc finger transcription factor shown to repress HIV-1 transcription (Romerio *et al.*, 1997). Other proteins demonstrated by others to bind LBP-1c include histone deacetylase-1 (HDAC1) and SUMO-1 (International Congress of Developmental Biologists, Kyoto, Japan, July 2001). It was of interest to test the ability of CRTR-1 and other CP2 family members to interact with these LBP-1c binding proteins to determine if they were unique to LBP-1c or capable of interacting with CRTR-1. Interaction between CRTR-1 and NFE4 or FE65 was not investigated as NFE4 has been shown to have tissue specific expression limited to fetal liver, cord blood and bone marrow

while FE65 shows neural specific expression, sites that do not express CRTR-1. Interaction between CRTR-1 and YY1 is investigated in 8.9.

RING1 and HDAC1 have been shown mediate transcriptional repression (Coull *et al.*, 2000; Tuckfield *et al.*, 2002b). CRTR-1 has been demonstrated to repress transcription from a CP2 responsive promoter and to act as a dominant repressor over CP2 and Gal4-AD mediated transcriptional activation (7.3). It was therefore of interest to investigate the ability of CRTR-1 to bind both proteins as a means to understand the possible mechanisms of CRTR-1 mediated transcriptional repression.

Platinum Taq PCR (2.3.1.12) was used to isolate cDNAs encoding full-length open reading frames for both RING1 and HDAC1. Specifically, reactions with primers RING1SR1/RING1SR2 and HDAC1SR1/HDAC1SR2 (2.2.7.2) were carried out using polyT primed cDNA (2.3.1.11) generated from ES cell and adult mouse kidney total RNA (2.3.5.2) respectively. Products were gel purified (2.3.1.3), cloned into pGEM-T easy, excised with *Eco*RI and cloned into pGADT7 digested with *Eco*RI, generating clones pGAD-RING1 and pGAD-HDAC1. Correct clones were selected by BDT automated sequencing (2.3.1.14) and IvTT (2.3.6.7) was used to confirm generation of proteins of the expected molecular weight (data not shown).

8.8.1 Yeast-2-Hybrid Analysis of Interaction Between CP2 Family Members and RING1 and HDAC1.

To test the ability of CP2 family members to bind RING1 and HDAC1, pGB-CRTR-1(47-481), pGB-CP2-DD, pGB-altCP2-DD, pGB-NF2d9-DD and pGB-altNF2d9-DD were co-transformed (2.3.4.3) into AH109 yeast (2.2.11) with pGAD-RING1 or pGAD-HDAC1. Transformed yeast were plated and cultured as described in 8.5.2.

Yeast co-transformed with pGB-CP2-DD or pGB-altCP2-DD and pGAD-RING1 or pGAD-HDAC1 showed growth at both levels of selection indicating a positive interaction

FIGURE 8.6

Interaction of CP2 and altCP2 with RING1 and HDAC1.

AH109 yeast were co-transformed (2.3.4.3) with plasmids as indicated. Yeast were grown and analysed as described in figure 8.2.

-leu/-trp/-his

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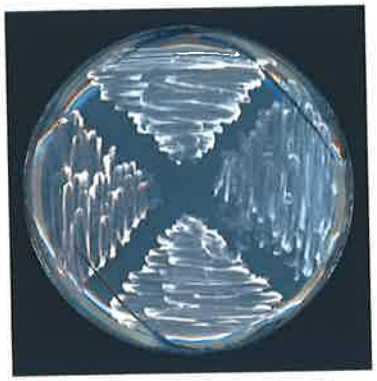

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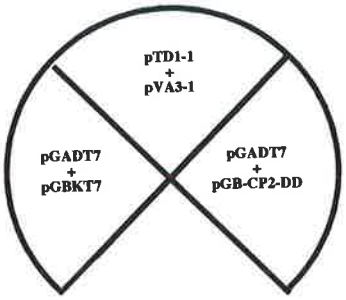

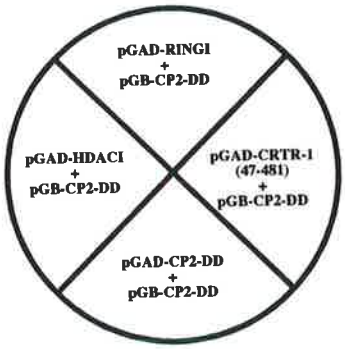

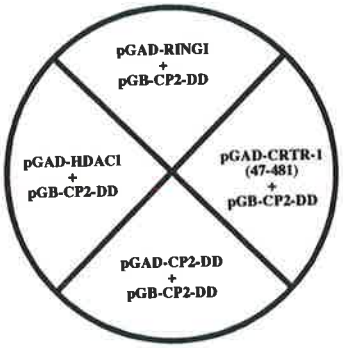

-leu/-trp/-his

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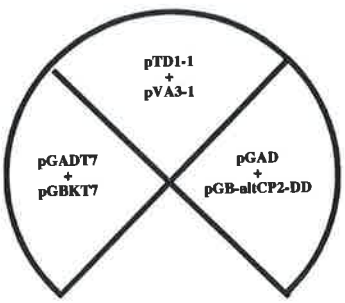
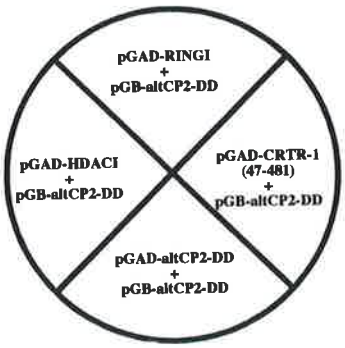
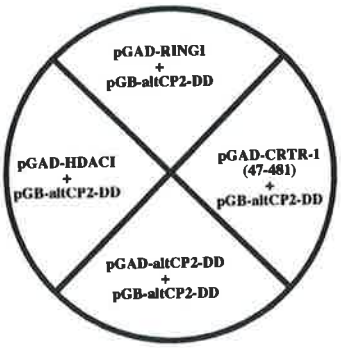






between CP2/altCP2 and RING1 and HDAC1 (Figure 8.6). In contrast, yeast co-transformed with pGB-NF2d9-DD, pGB-altNF2d9-DD or pBG-CRTR-1(47-481) and pGAD-RING1 or pGAD-HDAC1 showed no growth at the stringent selection level indicating that NF2d9, altNF2d9 and CRTR-1 do not interact strongly with RING1 or HDAC1 (Figures 8.7 and 8.8). Together, these results demonstrate that strong binding between CP2 family members and RING1 and HDAC1 is unique to CP2 and altCP2 binding proteins.

Results from 8.5, 8.7 and 8.8 are summarised in Figure 8.9.

8.9 INTERACTION OF CRTR-1 WITH YY1, REX1, PIAS3 AND SMRTα.

The ability of CRTR-1 to interact with several candidate proteins was investigated. Candidate binding proteins tested that were not identified by yeast-2-hybrid were selected on the basis of their relation to proteins identified as CRTR-1 binding proteins by yeast-2-hybrid, their ability to interact with other CP2 family members, expression pattern during development or function as a transcriptional co-repressors. These proteins were:

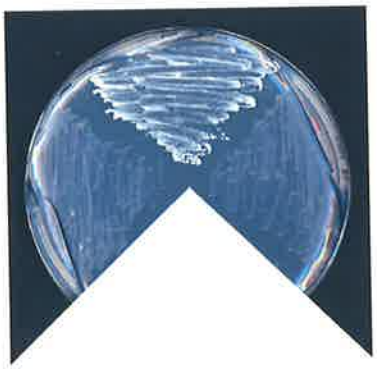
YY1; Interaction of YY1 with LBP-1c and amino acid sequence conservation between LBP-1c and CRTR-1 across the region required for LBP-1c protein interactions highlight YY1 as a possible CRTR-1 binding protein. Furthermore, interaction between CRTR-1 and YY1 could provide a possible mechanism for CRTR-1 transcriptional repression.

Rex1; a Zinc finger domain protein, expected to function as a transcription factor, is expressed in the pluripotent cells of the early mouse embryo and rapidly down regulated upon differentiation of the pluripotent cells (Ben-Shushan *et al.*, 1998) in a manner parallel to CRTR-1 (Pelton *et al.*, 2002). Rex1, like CRTR-1 is predicted to play a role in the

FIGURE 8.7

Interaction of NF2d9 and altNF2d9 and altCP2 with RING1 and HDAC1.

AH109 yeast were co-transformed (2.3.4.3) with plasmids as indicated. Yeast were grown and analysed as described in figure 8.2.



-leu/-trp/-his

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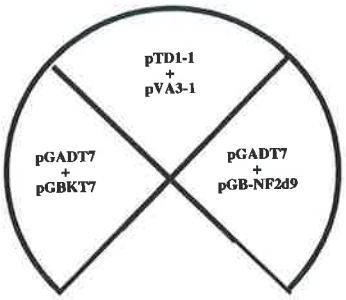

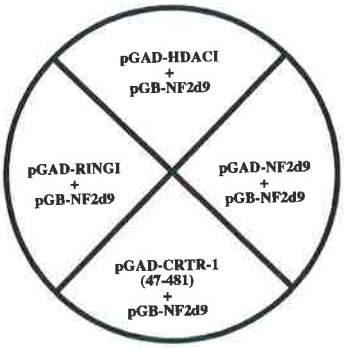

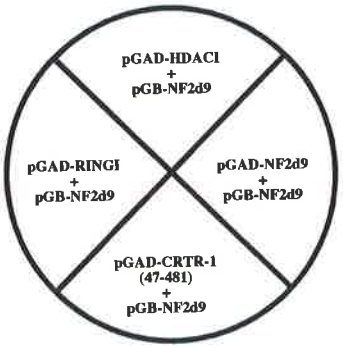

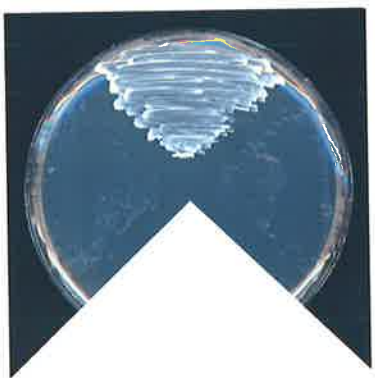

-leu/-trp/-his

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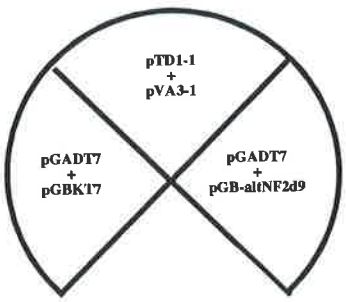
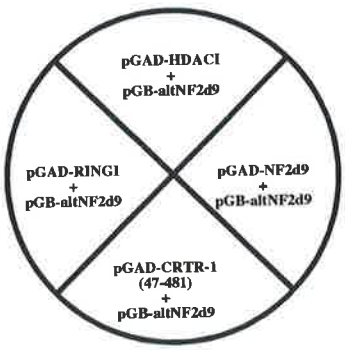




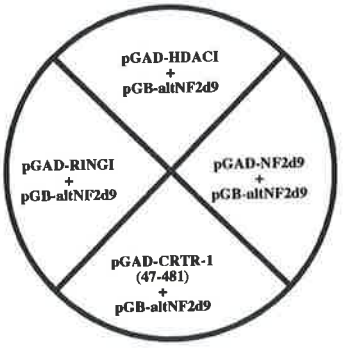
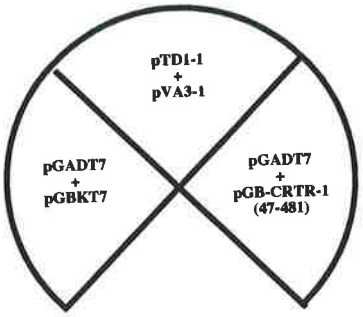


FIGURE 8.8

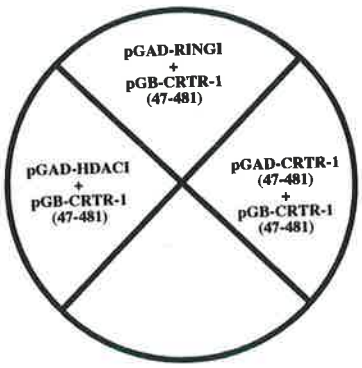
Interaction of CRTR-1 with RING1 and HDAC1.

AH109 yeast were co-transformed (2.3.4.3) with plasmids as indicated. Yeast were grown and analysed as described in figure 8.2.

-leu/-trp/-his



-leu/-trp/-his



-leu/-trp/-his/-ade

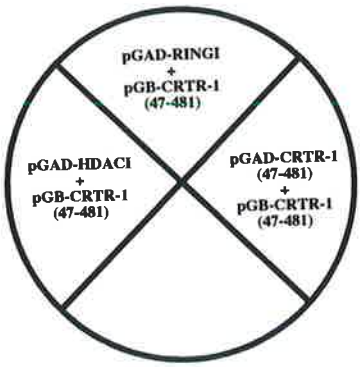


FIGURE 8.9

Summary of CP2 family homomeric and heteromeric protein interactions analysed by yeast-2-hybrid.

Comparative assessment of AH109 yeast growth co-transformed with pGB and pGAD based plasmids, as indicated (Figures 5.5-9). +++++; strong growth, +: poor growth.

A

	pACT2-A11	pACT2-A46	pACT2-G117
pGB-CRTR-1(47-481)	+++++	+++++	+++++

B

	pACT2-G56	pACT2-A68	pACT2-B87	pGAD-HDACI	pGAD-RINGI
pGB-CP2-DD	++++	+++	-	+++++	+++++
pGB-altCP2-DD	++++	++++	-	+++++	+++++
pGB-NF2d9-DD	+	+++	-	-	-
pGB-altNF2d9-DD	+	+++	-	-	-
pGB-CRTR-1(47-481)	++++	++++	+++++	-	-

regulation and maintenance of pluripotency during mammalian development. The expected function of Rex1 as a transcription factor, together with the equivalent expression pattern during early development, suggest that Rex1 may be a CRTR-1 binding protein.

PIAS3; protein inhibitor of activated Stat3, is an intracellular protein shown to regulate specifically the activity of Stat3 (Chung *et al.*, 1997), a secondary messenger for signalling from gp130 receptors activated by interleukin-6 (IL6) family members (Heinrich *et al.*, 1998). LIF, a member of the IL-6 family, can be used to maintain ES cells in a pluripotent state (Rathjen *et al.*, 1990a) and has been proposed to have a role in the induction of nephrogenesis in the developing kidney (Bard and Ross, 1991; Schedl and Hastie, 2000). Testing the ability of CRTR-1 to bind PIAS3 was of interest to test if specificity of interaction that exists between PIAS1 with Stat1 and PIAS3 with Stat3, where PIAS1 does not bind Stat3 and PIAS3 does not bind Stat1, exists with PIAS1 and CRTR-1. Furthermore, interaction of CRTR-1 with PIAS3 would provide evidence for a possible role of CRTR-1 in gp130 signalling, important for pluripotent cell maintenance and nephrogenesis, sites of CRTR-1 expression.

SMRTα; is a co-repressor protein recruited to transcription factors bound to DNA, which mediates transcriptional repression (Chen and Evans, 1995) by recruitment of other co-repressor proteins such as HDAC3 (Li *et al.*, 2000). CRTR-1 does not interact with HDAC1 or RING1 (8.8). As such testing the ability of CRTR-1 to interact with SMRTα was carried out as a means to providing insight into other possible mechanisms for CRTR-1 mediated transcriptional repression.

The ability of these proteins to interact with CRTR-1 was investigated with the aim of providing insight into the possible biological importance of CRTR-1, mechanisms

regulating CRTR-1 transcriptional repression and the interaction of CRTR-1 with various cellular signaling pathways.

8.9.1 Generation of Mammalian Expression Vectors for Co-precipitation.

A vector for expression in mammalian cells of PIAS3 tagged N-terminally with 6xHis and C-terminally with FLAG epitopes was generated by platinum Taq PCR (2.3.1.12) with primers PIAS3SR1 and PIAS3SR2 (2.2.7.2) on polyT primed cDNA (2.3.1.11) generated from ES cell total RNA (2.3.5.1). The PCR product was gel purified (2.3.1.3), cloned into pGEM-T easy, excised with *Xho*I and cloned into pXMT2 digested with *Xho*I, generating pXMT2-6XHis-PIAS3-FLAG. Correct clones for both constructs were identified by BDT automated sequencing (2.3.1.14). A mammalian expression vector for N-terminal 6xHis tagged CRTR-1 was generated by Platinum Taq PCR (2.3.1.12) with primers SR32 and SR31 (2.2.7.2) using pGEM-T-CRTR-1 (3.3.1) as a template. The PCR product was gel purified, cloned into pGEM-T-easy, excised with *Xho*I and cloned into pXMT2 digested with *Xho*I, generating pXMT2-6X-His-CRTR-1. Mammalian expression vectors pCEP4F-YY1, pEF-6Myc-Rex1 and pCMX-mSMRTα-FLAG, directing expression of tagged proteins YY1-FLAG, 6Myc-Rex1 and SMRTα-FLAG, respectively, were kind gifts form Drs. Ed Setto (H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, Tampa, Florida), Paul Bello (Department of Molecular Biosciences, The University of Adelaide, Australia) and Ronald Evans (HHMI, The Salk Institute for Biological Studies, San Diego)

To confirm expression of proteins, 2 μg of mammalian expression vectors were transfected into $3x10^6$ COS-1 cells (2.4.5). Whole cell extracts were generated (2.3.6.2) 24 hours post transfection and analysed by Western blot (2.3.6.6). Filters were probed with the appropriate mouse monoclonal anti-FLAG (2.2.8) or anti-Myc (2.2.8) or polyclonal rabbit anti-CRTR-1 (appendix 2) antibodies and revealed specific bands at the expected size for

each protein confirming the correct expression of tagged proteins (Figure 8.10). Sizes are approximately 66 kDa (6xHis-PIAS3-FLAG) (A), 64 kDa (6xHis-CRTR-1) (B), 60 kDa (YY1-FLAG) (C), 77 kDa (6Myc-Rex1) (D) and 90 kDa (SMRTα-FLAG) (E).

8.9.2 Specific Interaction of YY1, Rex1 and PIAS3 with CRTR-1.

pSG5.HA-CRTR-1 was co-transfected with pXMT2-6xHis-PIAS3-FLAG and pXMT2-6xHis-CRTR-1 was co-transfected with either pSG5.HA-CRTR-1, pCEP4F-YY1-FLAG, pEF-6Myc-Rex-1, pCMX-mSMRTα-FLAG or pSG5.HA-VDR into $3x10^6$ COS-1 cells (2.4.5). 24 hours post transfection, whole cell extracts were produced, 6xHis proteins nickel affinity purified as described (8.6.2 and 2.3.6.3) and analysed by Western blot (2.3.6.6).

Nickel affinity purified extracts of COS-1 cells transfected with pSG5.HA-CRTR-1 and pXMT2-6xHis-PIAS3-FLAG showed co-purification of HA-CRTR-1 (Figure 8.11b, lane 2), indicating that CRTR-1 interacts with PIAS3. The specificity of this interaction was evidenced by the fact that HA-CRTR-1 was also co-purified from Ni-purified extracts of cells transformed with pXMT2-6xHis-FKBP4-FLAG as a positive control (Figure 8.11a lane 2), but was not co-purified from pSGS.HA-CRTR-1 transfected cell extracts mixed with nickel affinity beads as a negative control (Figure 8.11 a and b, lane 3). Further, HA-VDR, expressed from pSG5.HA-VDR (ch4), was not co-purified from COS-1 cells co-transfected with pXMT2-6xHis-PIAS3-FLAG or pXMT2-6xHis-FKBP4-FLAG (Figure 8.11c, lanes 2 and 3).

Nickel affinity purified extracts of COS-1 cells co-transfected with pXMT2-6xHis-CRTR-1 and pSG5.HA-CRTR-1, pCEP4F-YY1 or pEF-6Myc-Rex1 showed co-purification of HA-CRTR-1, YY1-FLAG and 6Myc-Rex1 (Figure 8.12a, b and c, lane 2) confirming that CRTR-1 forms homomeric complexes and indicating that CRTR-1 interacts with YY1 and Rex-1. The specificity of this interaction was evidenced by the fact

FIGURE 8.10

Validation of expression plasmids for co-purification experiments.

$3x10^5$ COS-1 cells, grown in 60mm diameter tissue culture dishes, were transfected with 2μg of pXMT2-6xHis-PIAS3-FLAG **(A)**, pXMT2-6xHis-CRTR-1 **(B)**, pcDNA3-YY1-FLAG **(C)**, pEF-6Myc-Rex1 **(D)** or pCMX-mSMRTα-FLAG **(E)**. Whole cell extracts were produced 24 hrs post transfection (2.3.6.2) and separated by 10% SDS-PAGE (2.3.6.4) prior to western analysis (2.3.6.6). Filters were probed with mouse monoclonal anti-FLAG **(A, C and E)**, mouse monoclonal anti-Myc **(D)** or rabbit polyclonal anti-CRTR-1 (appendix 2), **(B)** antibodies prior to incubation with the appropriate horse radish peroxidase (HRP) conjugated secondary antibody (2.2.8) and visualised by chemiluminescence (2.3.6.6). Markers; Benchmark pre-stained protein ladder (2.2.14).

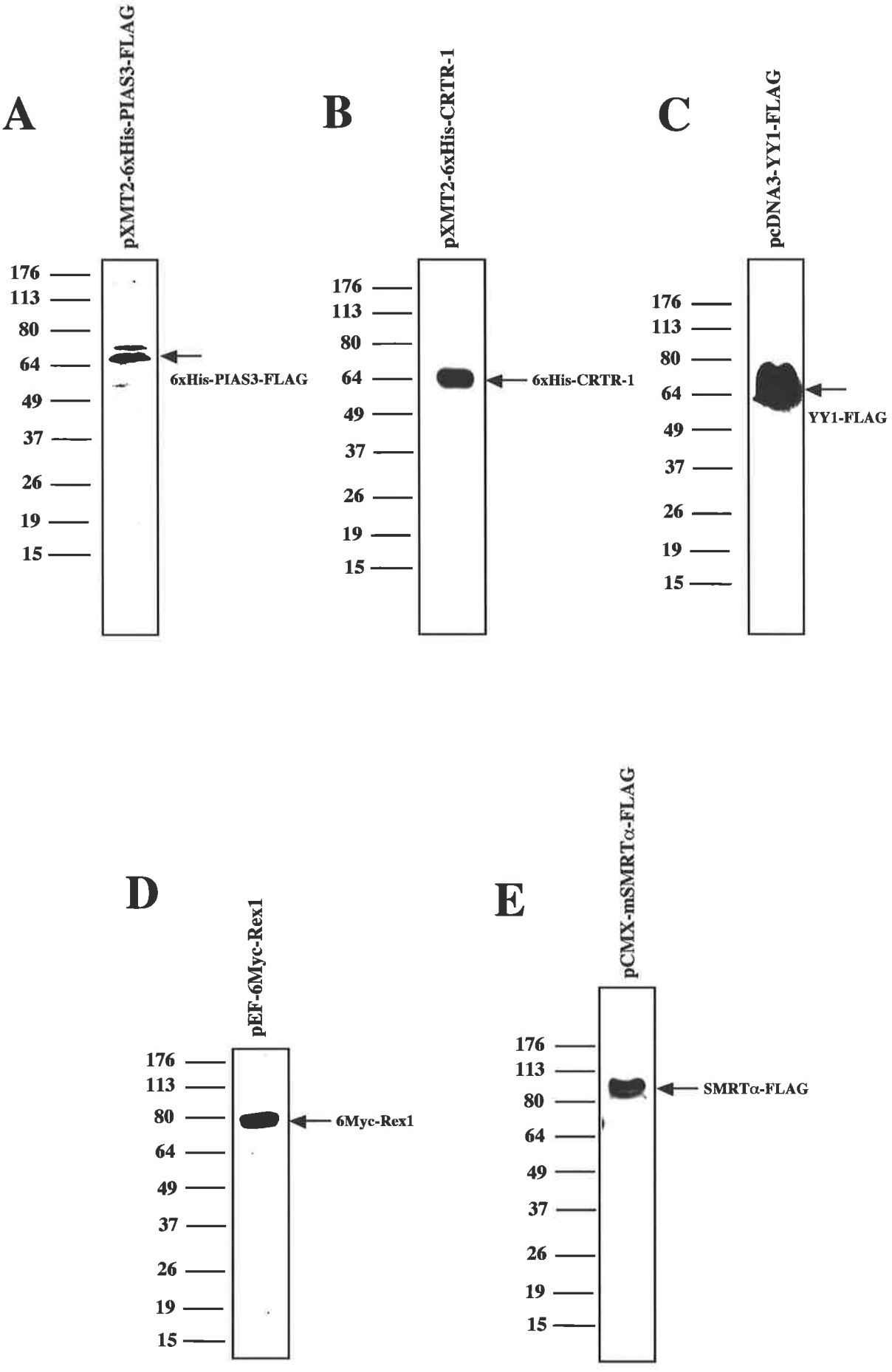

A
pXMT2-6xHis-PIAS3-FLAG
176
113
80
64
49
37
26
19
15
6xHis-PIAS3-FLAG
B
pXMT2-6xHis-CRTR-1
176
113
80
64
49
37
26
19
15
6xHis-CRTR-1
C
pcDNA3-YY1-FLAG
176
113
80
64
49
37
26
19
15
YY1-FLAG
D
pEF-6Myc-Rex1
176
113
80
64
49
37
26
19
15
6Myc-Rex1
E
pCMX-mSMRTα-FLAG
176
113
80
64
49
37
26
19
15
SMRTα-FLAG

FIGURE 8.11

CRTR-1 co-purifies with PIAS3.

2μg of pSG5.HA-CRTR-1 **(A and B)** and pSG5.HA-VDR **(C)** were transfected in combination with 2 μg of pXMT2-6xHis-FKBP4-FLAG or pXMT2-6xHis-PIAS3-FLAG, into $3x10^5$ COS-1 cells grown in 60 mm diameter tissue culture dishes (2.4.5). Whole cell extracts were produced 24 hrs post transfection, nickel affinity purification of 6xHis tagged proteins was carried out (2.3.6.3) and proteins were separated by 10% SDS-PAGE (2.3.6.4) prior to western analysis (2.3.6.6). Filters were probed with monoclonal mouse anti-FLAG prior to incubation with horseradish peroxidase (HRP) conjugated rabbit anti-mouse secondary antibody (2.2.8) and visualised by chemiluminescence (2.3.6.6). Extracts of COS-1 cells transfected with pSG5.HA-CRTR-1 or pSG5.HA-VDR alone were included in the analysis as controls. Markers; Benchmark pre-stained protein ladder (2.2.14).

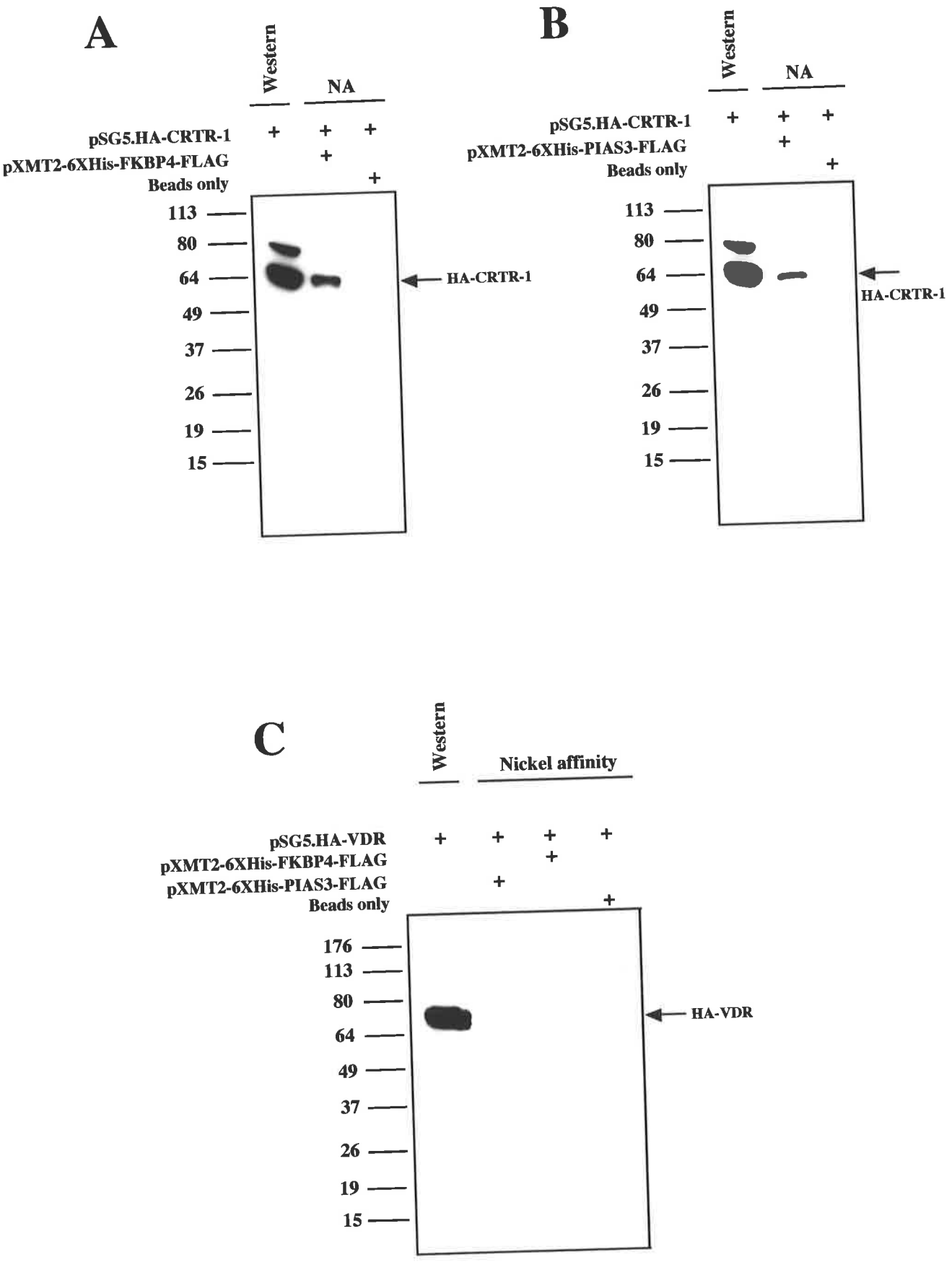
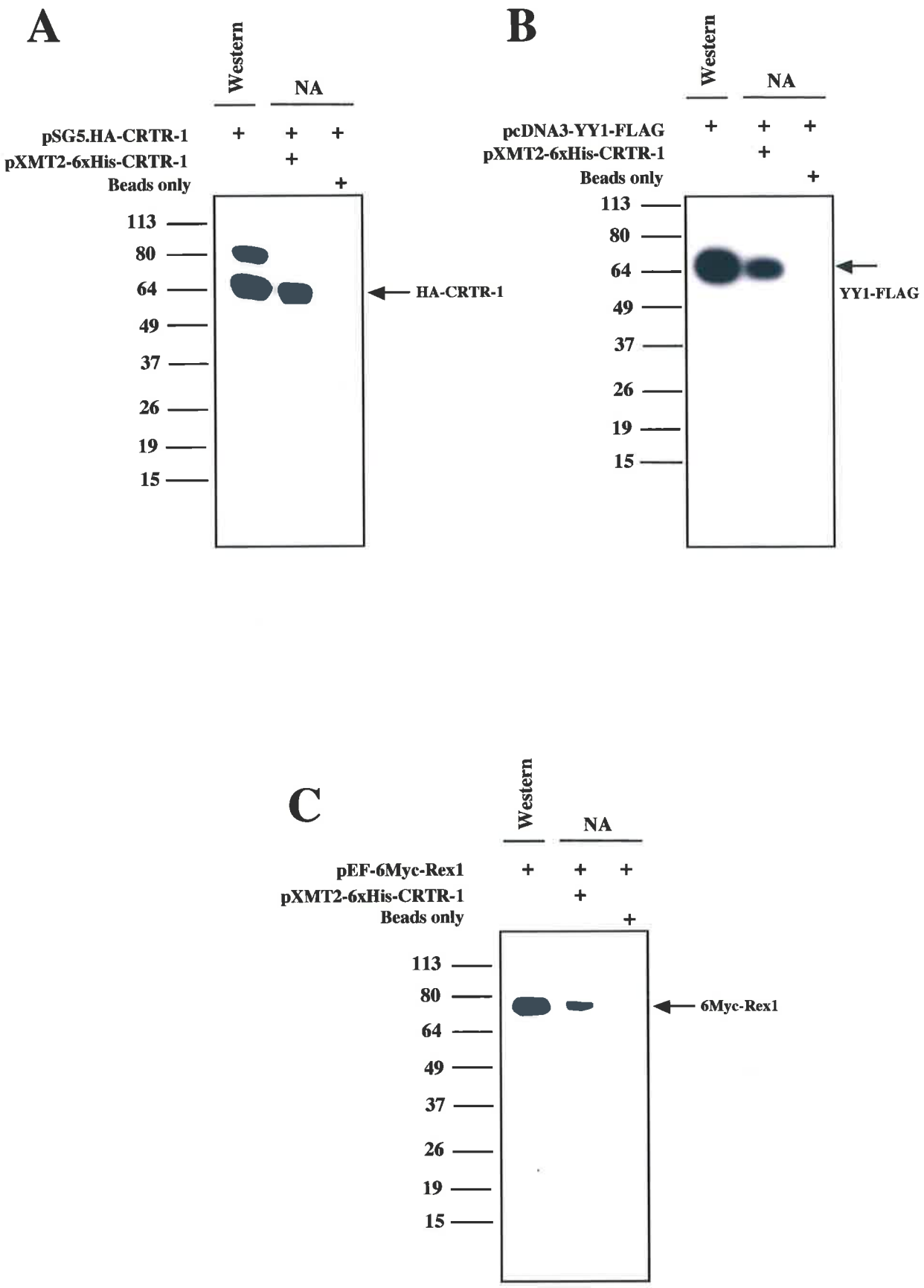

A
Western
NA
pSG5.HA-CRTR-1
pXMT2-6XHis-FKBP4-FLAG
Beads only
113
80
64
49
37
26
19
15
HA-CRTR-1
B
Western
NA
pSG5.HA-CRTR-1
pXMT2-6XHis-PIAS3-FLAG
Beads only
113
80
64
49
37
26
19
15
HA-CRTR-1
C
Western
Nickel affinity
pSG5.HA-VDR
pXMT2-6XHis-FKBP4-FLAG
pXMT2-6XHis-PIAS3-FLAG
Beads only
176
113
80
64
49
37
26
19
15
HA-VDR

FIGURE 8.12

Co-purification of CRTR-1 with YY1, and Rex1.

2 µg of pXMT2-6xHis-CRTR-1 was transfected in combination with 2 µg of pSG5.HA-CRTR-1, pcDNA3-YY1-FLAG or pEF-6Myc-Rex1, into $3x10^5$ COS-1 cells grown in 60 mm diameter tissue culture dishes (2.4.5). Whole cell extracts were produced 24hrs post transfection, nickel affinity purification of 6xHis tagged proteins was carried out (2.3.6.3) and proteins were separated by 10% SDS-PAGE (2.3.6.4) along with extracts of cells transfected with pSG5.HA-CRTR-1, pcDNA3-YY1-FLAG or pEF-6Myc-Rex1 alone prior to western analysis (2.3.6.6). Filters were probed with monoclonal mouse antibodies specific for HA **(A)**, FLAG **(B)** or 6Myc **(C)** epitopes prior to incubation with horse radish peroxidase (HRP) conjugated rabbit anti-mouse secondary antibody (2.2.8) and visualised using chemiluminescence (2.3.6.6). Markers; Benchmark pre-stained protein ladder (2.2.14).


A
Western
NA
pSG5.HA-CRTR-1
pXMT2-6xHis-CRTR-1
Beads only
113
80
64
49
37
26
19
15
HA-CRTR-1
B
Western
NA
pcDNA3-YY1-FLAG
pXMT2-6xHis-CRTR-1
Beads only
113
80
64
49
37
26
19
15
YY1-FLAG
C
Western
NA
pEF-6Myc-Rex1
pXMT2-6xHis-CRTR-1
Beads only
113
80
64
49
37
26
19
15
6Myc-Rex1

that 6xHis-CRTR-1 did not co-purify SMRTα-FLAG (Figure 8.13a lane 2) or HA-VDR (Figure 8.13b, lane 2) and that HA-CRTR-1, YY1-FLAG or Rex-1-Myc were not purified from pSG5.HA-CRTR-1, pcDNA3-YY1-FLAG or pcDNA3-Rex1-Myc transfected cell extracts mixed with the nickel affinity beads (Figure 8.12a, b and c, lane 3).

Together, these results identify PIAS3, YY1 and Rex1, but not SMRTα, as CRTR-1 binding proteins that could regulate the biological activity and promoter specificity of CRTR-1.

8.10 DISCUSSION.

8.10.1 Identification of CRTR-1 Binding Partners.

Yeast-2-hybrid analysis and co-purification from transfected COS-1 cells were used to identify CRTR-1 binding proteins Ubc9, PIAS1 FKBP4, PIAS3, YY1, Rex1. Proteins tested and shown not to interact with CRTR-1 were HDAC1, RING1 and SMRTα. Properties of the interacting proteins are described below.

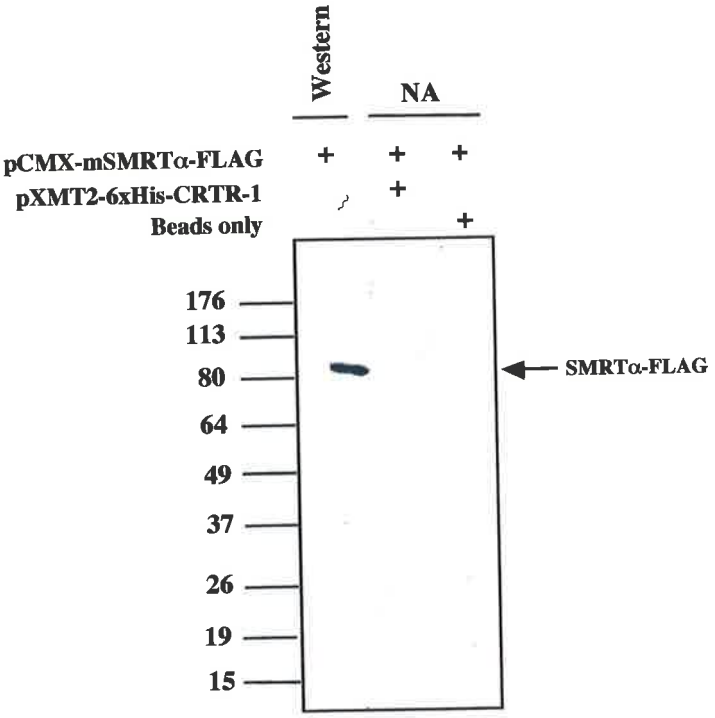
• **Ubc9** (ubiquitin conjugating enzyme 9) was identified based on sequence similarity to the E2 ubiquitin conjugating enzymes (Gottlicher *et al.*, 1996), although it does not function as a ubiquitin conjugating enzyme. Ubc9 is a conjugating enzyme for the ubiquitin-like protein, SUMO-1 (small ubiquitin-like modifier-1) (Johnson and Blobel, 1997), a 101 amino acid precursor protein that is activated by cleavage of 4 amino acids from the C-terminus (Rodriguez *et al.*, 2001). Ubc9 catalyses the formation of an isopeptide bond between the C-terminus of SUMO-1 and the lysine residue present in a consensus sumoylation sequence (ψKXE, where ψ represents a large hydrophobic amino acid) present in target proteins (Rodriguez *et al.*, 2001; Sampson *et al.*, 2001). CRTR-1 contains two consensus sequence sumoylation motifs, FKQE, amino acids 29-32, and EKME, amino

FIGURE 8.13

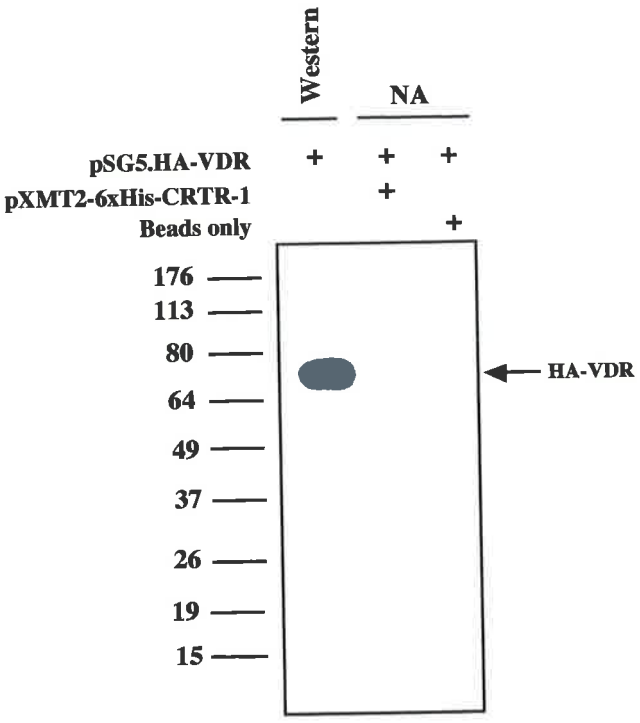
CRTR-1 does not co-purify with SMRTα or HA-VDR.

2 μg of pXMT2-6xHis-CRTR-1 was transfected in combination with 2 μg of, pCMX-mSMRTα-FLAG or pSG5.HA-VDR, as indicated into $3x10^5$ COS-1 cells grown in 60 mm diameter tissue culture dishes (2.4.5). Whole cell extracts were produced 24hrs post transfection, nickel affinity purification of 6xHis tagged proteins was carried out (2.3.6.3) and proteins were separated by 10% SDS-PAGE (2.3.6.4) prior to western analysis (2.3.6.6). Filters were probed with monoclonal mouse antibodies specific for FLAG **(A)** or HA **(B)** epitopes prior to incubation with horseradish peroxidase (HRP) conjugated rabbit anti-mouse secondary antibody (2.2.8) and visualised using chemiluminescence (2.3.6.6). Extracts of cells transfected with pCMX-mSMRTα-FLAG or pSG5.HA-VDR alone were included in the analysis as controls. Markers; Benchmark pre-stained protein ladder (2.2.14).

A


Western
NA
pCMX-mSMRTα-FLAG + + +
pXMT2-6xHis-CRTR-1 +
Beads only +
176
113
80
64
49
37
26
19
15
SMRTα-FLAG

B


Western
NA
pSG5.HA-VDR + + +
pXMT2-6xHis-CRTR-1 +
Beads only +
176
113
80
64
49
37
26
19
15
HA-VDR

acids 234-237, which are 100% conserved throughout the CP2 family and are present in LBP-1c at amino acids 49-52 and 252-255 respectively.

The specific effects of SUMO-1 modification appear to be substrate dependent and distinct from the role of ubiquitin modification. SUMO-1 modification has been shown to regulate the sub-cellular distribution of various target proteins. For example, RanGAP1 is re-directed to the nuclear pore complex from the cytoplasm by sumoylation (Mahajan *et al.*, 1998; Matunis *et al.*, 1998), while sumoylation of promyelocytic leukemia protein (PML) targets it from the nucleoplasm to nuclear bodies (Kamitani *et al.*, 1998; Muller *et al.*, 1998). Sumoylation has also been shown to act as a negative regulator for ubiquitination. IkBα and MDM2 are SUMO-modified on a lysine residue that also functions as a site for ubiquitination, thereby blocking the targeting of these proteins for the proteosome and extending their half life (Desterro *et al.*, 1998). Other examples of SUMO-1 function include enhanced proteosome degradation of p73 (Minty *et al.*, 2000), increase in p53 transcriptional activation ability (Gostissa *et al.*, 1999; Rodriguez *et al.*, 1999), negative regulation of c-Jun transcriptional regulation (Muller *et al.*, 2000) and repression of Sp3 transcriptional activation (Ross *et al.*, 2002). Interestingly, a recent study has demonstrated the ability of Ubc9 to interact with a nuclear localisation signal of the homeobox protein Vsx-1 and regulate its nuclear localisation independent of SUMO-1 modification (Kurtzman and Schechter, 2001).

SUMO-2 and SUMO-3 share 96% amino acid sequence identity and 50% amino acid sequence identity to SUMO-1 (Saitoh and Hinchey, 2000). Both can be ligated to lysine residues in target proteins by Ubc9 which is not able to discriminate between SUMO-1, -2 or -3 (Tatham *et al.*, 2001). Distinct from SUMO-1, however, SUMO-2 and -3 are able to form poly-chains (Tatham *et al.*, 2001). While studies have suggested distinct

biological roles for SUMO-2 and -3 in comparison to SUMO-1, no clear function of SUMO-2 and SUMO-3 has been demonstrated.

The first conserved sumoylation site in CRTR-1 (FKQE, amino acids 29-32) is present in the transcriptional regulatory domain of CP2 family members, suggesting that sumoylation at this site could play a role in the control of transcriptional regulation. The second conserved sumoylation site in CRTR-1 (EKME, amino acids 234-237) is contained within the region important for nuclear localisation of LBP-1c, amino acids 245-257 (Dr. S Jane Pers. Comm.). Sumoylation at this site could regulate the distribution of CP2 family members between the nucleus and cytoplasm thereby regulating their activity.

• **PIAS1** (protein inhibitor of activated Stat1) was originally identified as a protein able to block the DNA binding ability of activated Stat1 (signal transducer and activator of transcription 1) in response to interferon stimulation of cells (Liu *et al.*, 1998; Liu and Shuai, 2001). Stat1 is a latent cytoplasmic transcription factor that is activated by tyrosine phosphorylation in response to cytokine stimulation, in particular interferon stimulation (Liu and Shuai, 2001). PIAS1 interaction with Stat family proteins is specific for Stat1 as demonstrated by the ability of PIAS1 to co-immunoprecipitate Stat1 but not Stat2 or Stat3 (Liu *et al.*, 1998).

PIAS1 has been shown to associate with and be functionally important for other transcription factors such as the androgen receptor where interaction leads to a 3-5 fold increase in transcription activation (Tan *et al.*, 2002). Similar observations have been reported for interaction between PIAS1 and the glucocorticoid receptor, while interaction of PIAS1 with the progesterone receptor repressed activation of transcription (Tan *et al.*, 2002).

Interestingly, recent studies have identified a role for PIAS1 as an enhancer of SUMO ligation (Kotaja *et al.*, 2002). Specifically, PIAS1 was identified by yeast-2-hybrid analysis as a protein that binds SUMO-1, Ubc9 and p53 (Kahyo *et al.*, 2001). *In vitro* and *in vivo* experiments demonstrated the ability of PIAS1 to greatly enhance the sumoylation of p53 in the presence of Ubc9 in comparison to a cysteine to an alanine or serine (C350A or C350S, present in the RING finger domain of PIAS1) mutant PIAS1 unable to bind Ubc9 but still able to bind SUMO-1 (Kahyo *et al.*, 2001). Interaction between PIAS proteins, SUMO-1 and Ubc-9 is hypothesised to be a mechanism for modulation of transcription factor action (Kotaja *et al.*, 2002).

PIAS1 and Ubc9 were also shown to interact with CP2, altCP2, NF2d9 and altNF2d9. These interactions provide potential pathways for the regulation of CP2 family activity by sumoylation at the level of transcriptional regulation and cellular localization.

• **PIAS3** (Protein Inhibitor of Activated Stat3) has been shown to specifically block Stat3 mediated transcriptional activation by preventing DNA binding of activated Stat3 in response to IL-6 family cytokine signal transduction activation (Chung *et al.*, 1997) in a manner analogous to PIAS1 negative regulation of Stat1. Stat3 plays a role in the maintenance of mouse ES cells in culture, acting as a secondary messenger for gp130 signalling activated by the cytokine LIF (Heinrich *et al.*, 1998; Yoshida *et al.*, 1994), used in culture for maintenance of mouse ES cells in a pluripotent state (Rathjen *et al.*, 1990a). A recent study has demonstrated the ability of PIAS3 to function as a SUMO-1 ligase enhancer for Interferon regulatory factor 1 (IRF-1). Consistent with PIAS1, this activity was dependent on the function of the RING finger domain of PIAS3 (Levy *et al.*, 2002), consistent with PIAS1. IRF-1 was also demonstrated to bind Ubc-9 and be sumoylated resulting in repression of IRF-1 transcriptional activity (Nakagawa and Yokosawa, 2002), an alternative potential mechanism for modulation of CRTR-1 activity by PIAS3.

Given that down regulation of CRTR-1 expression coincides with differentiation of ES cells and the down regulation of gp130 signalling, the possibility exists that CRTR-1 may be responsive to gp130 signalling or a factor regulated by phosphorylation cascades controlling STAT3 or PIAS3. Further, LIF signalling via activation of STAT3 has been shown to induce nephrogenesis during kidney development (Bard and Ross, 1991; Schedl and Hastie, 2000). Expression of CRTR-1 in the developing kidney at the time of nephrogenesis is consistent with the possibility that CRTR-1 is responsive to activation of gp130 signalling pathway and/or other signalling pathways that result in modulation of PIAS3 activity.

- **Rex1**, a Zn finger domain protein proposed to function as a transcription factor, is expressed in mouse ES cells and rapidly down regulated upon ES cell differentiation (Ben-Shushan *et al.*, 1998) in a fashion that parallels CRTR-1 (Pelton *et al.*, 2002). Rex1 binding partners have not been previously described and studies have not provided information regarding possible Rex1 target genes. The observation that CRTR-1 and Rex1 interact suggests that the biological function of these proteins may be co-regulated and that it is the formation of CRTR-1/Rex1 complexes that could be important for the maintenance and regulation of pluripotence during early development. Formation of CRTR-1/Rex1 complexes would be expected to determine the specificity of target promoters that are bound by CRTR-1 and the ability and specificity of interactions with other protein complexes important for the regulatory function of CRTR-1/Rex1 complexes.

- **FKBP4** (FK506 binding protein 4; also known as FKBP52 and FKBP59) is an immunophilin protein identified by its ability to bind the immunosuppressant drug FK506. FKBP4 contains peptidyl-prolylcis-trans isomerase (PPIase) activity that is inhibited by interaction with FK506 (Schmitt *et al.*, 1993), and has been found to associate with Hsp90

and latent steroid receptor complexes such as androgen, estradiol, glucocorticoid and progesterone receptors (Peattie *et al.*, 1992). Recent studies have demonstrated a role for FKBP4 in translocation of the glucocorticoid receptor (GR), which is able to bind both FKBP51 and FKBP4, from the cytoplasm to the nucleus (Galigniana *et al.*, 2001). Hormonal activation of the GR results in exchange of FKBP51 for FKBP4 in the cytoplasm (Davies *et al.*, 2002). This complex concomitantly recruits the transport protein dynein and permits translocation into the nucleus (Davies *et al.*, 2002). The identification of FKBP4 as a CRTR-1 binding protein suggests a similar mechanism might regulate the translocation of CRTR-1 or a CRTR-1 containing protein complex to the nucleus.

The FKBP4 mediated mechanism regulating CRTR-1 cellular localisation could be distinct from sumoylation mediated by Ubc9 and/or PIAS1 providing multiple levels of regulating CRTR-1 cellular localisation from various signalling pathways. While there is no experimental evidence linking the regulatory abilities of FKBP proteins and SUMO-ligases, the GR is also sumoylated by Ubc9 (Gottlicher *et al.*, 1996; Tian *et al.*, 2002; Kaul *et al.*, 2002), which is enhanced by activation of the GR with ligand (Tian *et al.*, 2002). The biological result of GR-sumoylation has not been elucidated but is coincidental with the exchange of FKBP51 for FKBP4 in the activated GR complex (Davies *et al.*, 2002).

It is of particular interest that FKBP4 was demonstrated to be a specific binding partner for CRTR-1 and not able to interact with other CP2 family members (Figures 8.4 and 8.5). These observations suggest that regulation of CRTR-1 by FKBP4 is specific for CRTR-1 and that perhaps other FKBP proteins show specificity in their ability to interact with other CP2 family members. This would provide a mechanism for specific regulation of CP2 family members through various signalling pathways regulating FKBP function and CP2 transcription factor family activity.

• **YY1** is a ubiquitous zinc finger containing transcription factor shown to interact with LBP-1c and regulate transcription from the HIV-1 LTR promoter (Coull *et al.*, 2000; Shrivastava and Calame, 1994). YY1 interacts with a wide range of proteins including proteins of the basal transcriptional machinery TATA binding protein (Austen *et al.*, 1997) and TFIIB (Usheva and Shenk, 1994), sequence specific DNA binding proteins Sp1 and cMyc, transcriptional co-activators p300 and CREB-binding protein and transcriptional co-repressors HDAC1, 2 and 3 (Yao *et al.*, 2001). The ability of CRTR-1 to interact with YY1 suggest that CRTR-1 may be able to bind and co-regulate promoters regulated by YY1 and that mechanisms employed by YY1 to repress transcription could mediate CRTR-1 transcriptional repression. Examples of cellular promoters regulated by YY1 include ε-globin (Raich *et al.*, 1995), γ-interferon (Ye *et al.*, 1996) cardiac α-actin (Chen and Schwartz, 1997) and potentially Hoxb4 (Gilthorpe *et al.*, 2002).

Of particular interest is the observation that YY1 has been shown to interact with immunophilins FKBP12 and FKBP25. FKBP12 has been shown to abrogate YY1 mediated transcriptional repression (Yang *et al.*, 1995). In contrast, interaction of FKBP25 with YY1 has been shown to enhance YY1 DNA binding in a dose dependent manner resulting in a greater level of transcriptional repression (Yang *et al.*, 2001). While an interaction between YY1 and FKBP4 has not been demonstrated, the ability of YY1 and CRTR-1 to interact with each other and with specific FKBP proteins suggests that the regulatory role of FKBP proteins may be a common feature controlling the transcriptional regulatory ability of CP2 family members and their binding partners.

8.10.2 Regulation of CRTR-1 Activity Through Binding Partner Interaction.

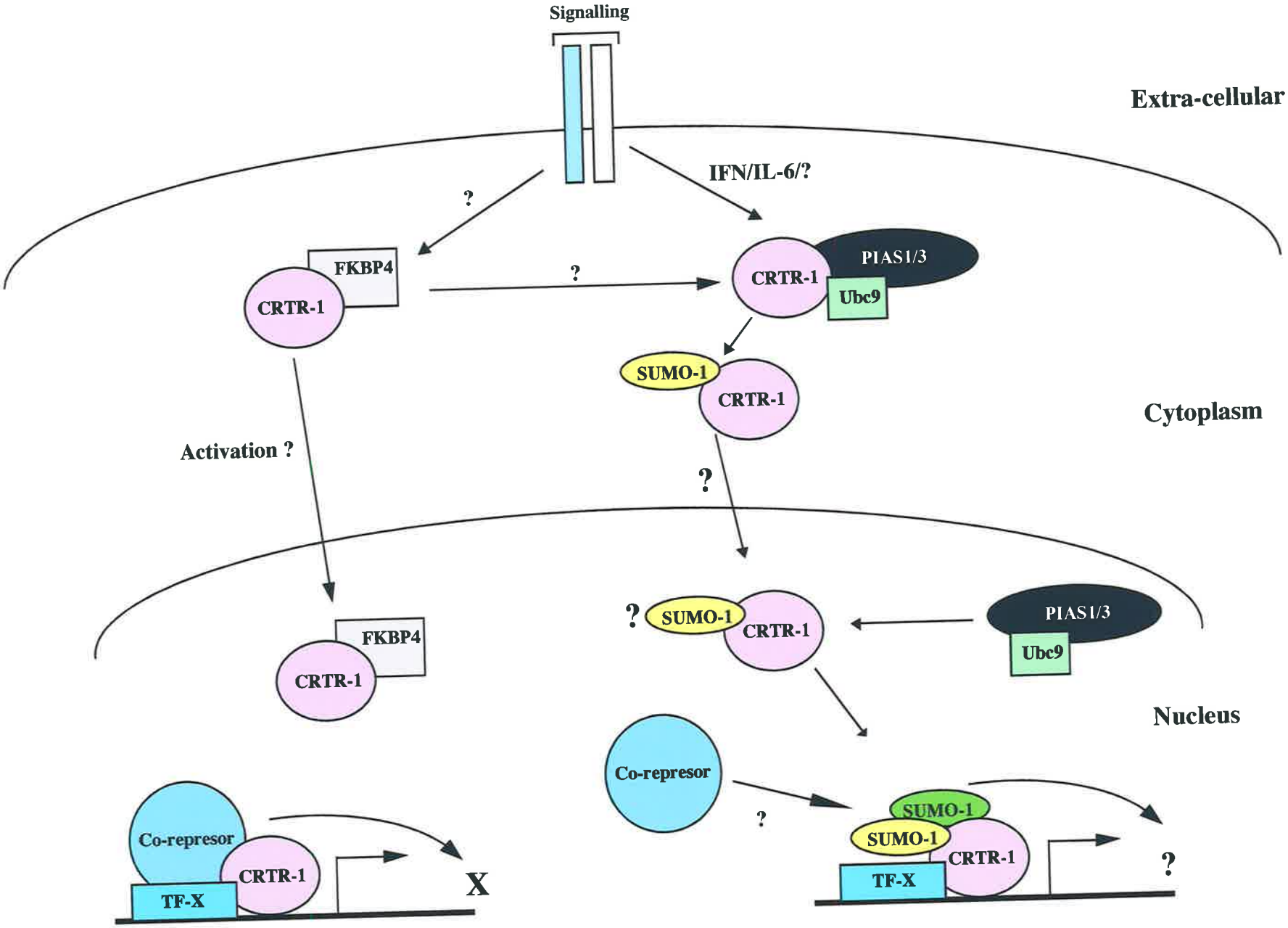
Analysis of CRTR-1 binding proteins identified several potential mechanisms for the regulation of CRTR-1 cellular localization and transcriptional regulatory ability, presented schematically in figure 8.14.

FIGURE 8.14

Schematic representation potential regulation of CRTR-1 function by interaction with cellular binding proteins.

Localisation of CRTR-1 to the cytoplasm could be mediated by interaction with Ubc9 and/or FKBP4. This inert complex could be activated by signaling cascades which directly target CRTR-1, FKBP4 or PIAS1/3 and result in translocation of CRTR-1 to the nucleus. Migration from the cytoplasm to the nucleus could be regulated by sumoylation from Ubc9 sumoylating CRTR-1 at the second consensus sumoylation site (EKME, amino acids 234-237), consistent with the region required for nuclear localisation of LBP-1c. Sumoylation could be enhanced by interaction of CRTR-1 with PIAS1 and/or PIAS3 providing a mechanism for specific regulation of CRTR-1 cellular localisation by signaling pathways influencing PIAS1/3. Alternatively, translocation of CRTR-1 to the nucleus could be accompanied by FKBP4 whose activity would be regulated by as yet un-determined signaling cascade(s).

In the nucleus, CRTR-1 may associate with CP2, altCP2, NF2d9, altNF2d9 or other non-related transcription factors such as YY1 or Rex1 (denoted as TF-X), and bind DNA to repress transcription. Sumoylation and/or the ability of the transcription factor complex to recruit co-repressor proteins could modulate transcriptional regulation. Regulation of transcriptional activity by sumoylation could be achieved by Ubc9 mediated sumoylation of CRTR-1 at the first consensus sumoylation site (FKQE, amino acids 29-32), consistent with the region required CRTR-1 mediated transcriptional repression. The net effect of this sumoylation event could be enhanced or abrogated transcriptional regulatory activity.


Signalling
Extra-cellular
IFN/IL-6/?
?
FKBP4
CRTR-1
?
CRTR-1
PIAS1/3
Ubc9
SUMO-1
CRTR-1
Cytoplasm
Activation ?
?
FKBP4
CRTR-1
?
SUMO-1
CRTR-1
PIAS1/3
Ubc9
Nucleus
Co-represor
CRTR-1
TF-X
X
Co-represor
?
SUMO-1
SUMO-1
CRTR-1
TF-X
?

Interaction of CRTR-1 with Ubc9 and/or FKBP4 suggests that CRTR-1 could be localised to the cytoplasm as a component of an inert complex. Release of CRTR-1 in the complex would allow CRTR-1 to translocate to the nucleus. Migration from the cytoplasm to the nucleus could be regulated by sumoylation from Ubc9 at the second consensus sumoylation site (EKME, amino acids 234-237), consistent with the region required for nuclear localisation of LBP-1c. Sumoylation may play a positive role for regulation of CRTR-1 activity permitting it to translocate to the nucleus, or a negative role where sumoylation of CRTR-1 would inhibit its nuclear translocation. Sumoylation could be enhanced by interaction of CRTR-1 with PIAS1 and/or PIAS3 and would provide a mechanism for specific regulation of CRTR-1 cellular localisation by regulatory pathways influencing PIAS1 and PIAS3 activity such as Interferon (Liu *et al.*, 1998; Liu and Shuai, 2001) and IL-6 (Chung *et al.*, 1997) signaling respectively. Alternatively, translocation of CRTR-1 to the nucleus would be accompanied by FKBP4 whose activity would be regulated by as yet un-determined signaling cascade(s).

In the nucleus, CRTR-1 may associate with CP2, altCP2, NF2d9, altNF2d9 or other non-related transcription factors such as YY1 or Rex1, and bind DNA to repress transcription. Specificity of DNA binding would be modulated by the transcription factor partners constituting the complex as determined by their expression pattern and presence in the nucleus. The ability to modulate transcriptional regulation could be determined by sumoylation and/or the ability of the transcription factor complex to recruit co-repressor proteins, specificity of which would be dictated by the components of the complex, providing mechanisms for CRTR-1 mediated transcriptional repression, known not to be mediated by interaction of CRTR-1 with HDAC1, RING1 or SMRTα. Regulation of transcriptional activity by sumoylation could be achieved by Ubc9 mediated sumoylation of CRTR-1 at the first consensus sumoylation site (FKQE, amino acids 29-32), consistent with the region required CRTR-1 mediated transcriptional repression. Sumoylation of

CRTR-1 at this site may regulate the ability of CRTR-1 to repress transcription. Alternatively, sumoylation may function to modulate CRTR-1 transcriptional regulatory ability between repression and activation.

The possibility that CRTR-1 may interact with three SUMO ligases could imply that regulation of CRTR-1 cellular localisation, DNA binding and transcriptional regulation could be achievable from multiple regulatory pathways perhaps distinct from other CP2 family members.

8.10.3 CP2 Family Members Bind Distinct Protein Partners.

Searches of the mouse genome using CP2 family member nucleotide sequence indicates that members of the CP2 family are limited to CP2, NF2d9 and CRTR-1. While these proteins have common DNA binding specificity, it is possible that distinct cellular functions could be achieved and regulated through interaction with alternative binding partners.

HDAC1 and RING1 were shown not to interact with CRTR-1, NF2d9 or altNF2d9 demonstrating that the transcriptional regulatory role of these proteins is CP2 and altCP2 specific. Inability of CRTR-1 to interact with these proteins suggests that the mechanism employed to repress transcription may use other co-repressor proteins such as HDAC2, or N-CoR. The ability of CRTR-1 to interact with HDAC3 could also be investigated, however, HDAC3 is typically recruited to transcriptional repressor protein complexes by association with SMRTα (Li *et al.*, 2000). As CRTR-1 did not interact with SMRTα, an interaction between CRTR-1 and HDAC3 is unlikely.

Ubc9 was shown to interact with CP2 , altCP2 and CRTR-1 but poorly with NF2d9, while PIAS1 was shown to interact with all CP2 family members. Therefore, it is likely that NF2d9 is sumoylated but by regulatory pathways independent of Ubc9.

Interaction with FKBP4 was specific for CRTR-1 and not CP2, altCP2, NF2d9 or altNF2d9. This suggests a mechanism for distinct regulation of CRTR-1 activity by sequestration in the cytoplasm in protein complexes with FKBP4. Other CP2 family members may interact with other FKBP proteins providing an opportunity for differential regulation by linking transcription factor activity to alternate signaling pathways. Taken together, regulation of CP2 family activity could be achieved by modulation of binding partner proteins, some of which are common to all members while others are unique. This mechanism would permit the specific regulation of CP2 family members at multiple levels from cellular localisation to the specificity of DNA binding and transcriptional regulation.

Overall, investigation into the identity and function of CRTR-1 binding proteins has provided insight to the possible regulation of CRTR-1 function. Interaction of CRTR-1 with Ubc9 and FKBP4 provides two potentially distinct methods for controlling CRTR-1 cellular distribution, through sumoylation and FKBP4 mediated translocation. Identification of YY1 and Rex1 as CRTR-1 binding proteins demonstrates that CRTR-1 is able to interact with non-CP2 related transcription factors, consistent with CP2, and provides a method for promoter specificity of CRTR-1 DNA binding. Combined with the interaction between CRTR-1 and PIAS1 and PIAS3, these observations have provided insight to the possible signalling pathways regulating the biological function of CRTR-1 which have significance to the sites of CRTR-1 expression in the mouse such the pluripotent cells during early mouse development and in kidney nephrogenesis.

CHAPTER 9

Toward the generation of *CRTR-1* null mice.

9.1 INTRODUCTION.

Published examples of studies investigating the functional importance of CP2 family members by gene knockout are limited to CP2 null mice. Mice null for CP2 were born in typical Mendelian distribution with no obvious defects in growth, behaviour, fertility or development (Ramamurthy *et al.*, 2001). Analysis was focused on the effect of CP2 loss of function on the regulation of hemotopoietic development, globin gene expression and T- and B-cell mitogen stimulated immunological response, for which no phenotype was observed after 18 months of observation. The biochemical basis for the lack of phenotype was shown to be compensation by the ubiquitously expressed CP2 family member, NF2d9. This was demonstrated by NF2d9 binding to CP2 promoter response elements and known protein interactors of CP2 in CP2 null mice (Ramamurthy *et al.*, 2001). Gene targeting experiments describing the phenotype of NF2d9 null mice have not been published, however, NF2d9 appears to have non-redundant roles important for development of wild type mice as the NF2d9 null mutation is embryonic lethal (S.M. Jane and J. M. Cunningham, pers comm).

In humans the importance of LBP-1c, the CP2 homologue, for mammalian biological processes is evidenced by the association of disease states with mutations in the LBP-1c mRNA and LBP-1c binding sites of promoters regulated by LBP-1c. Linkage of LBP-1c polymorphisms with Alzheimers disease (AD) has been described (Lambert *et al.*, 2000) (Taylor *et al.*, 2001) (1.7). Further, congenital erythropoietic porphyria results from deficiency in the activity of uropophyrinogen III synthase (URO-synthase), which is predicted to be a consequence of a mutation in a putative CP2 binding site present in the URO-synthase promoter (Solis *et al.*, 2001). Association of LBP-1c with AD and congenital erythropoietic porphyria suggests that LBP-1c plays biological roles that are not compensated for by LBP-1a, the human homologue of NF2d9. However, the presence of

AD or congenital erythropoietic porphyria-like phenotypes were not investigated in CP2 null mice.

Investigation into CRTR-1 biological function is of interest for three reasons. Firstly, CRTR-1 is the only identified CP2 family member that displays temporally and spatially regulated expression during development and in the adult mouse. Down regulated expression of CRTR-1 in the pluripotent cells of the early stage mouse embryo at 3.5-4.75 d.p.c. suggests that CRTR-1 could play a role in the regulation of pluripotent cell maintenance and/or differentiation, while specific expression of CRTR-1 in the epithelial cells lining the distal convoluted tubules (DCTs) of the developing and adult mouse kidney suggests that CRTR-1 could play a role in the development and physiology of the kidney. Secondly, CRTR-1 is the only CP2 family member shown to act as a dominant repressor of transcription from CP2 responsive promoters. Thirdly, CRTR-1 has been demonstrated to bind a unique repertoire of cellular proteins. These features suggest that CRTR-1 is likely to show a unique role within the animal that cannot be compensated for CP2 or NF2d9, the other CP2 family members in mice. Functional importance of CRTR-1 was investigated here in two ways, further mapping of CRTR-1 expression during embryonic kidney development and generation of CRTR-1 mutant mice.

9.2 EXPRESSION OF CRTR-1 DURING EARLY KIDNEY DEVELOPMENT.

Specific expression of CRTR-1 in the epithelial cells lining the DCTs of the 16.5 d.p.c embryonic and adult mouse kidney suggests that CRTR-1 may play a role in the development of the DCTs. To examine this possibility further, expression of CRTR-1 during earlier stage kidney development was investigated in association with Assoc. Prof. Melissa Little (Institute for Molecular Bioscience, The University of Queensland) by whole mount *in situ* hybridisation

Whole mount *in situ* hybridisation analysis (2.3.5.6.6) was carried out on mouse embryo metanephroi dissected at 12.5 d.p.c and grown culture (2.3.5.6.6) for three days using DIG-labelled sense and anti-sense CRTR-1 specific riboprobes (2.3.5.6.1-2). Specific CRTR-1 expression was detected by anti-sense riboprobes in the developing ureteric bud throughout the developing metanephori (Figure 9.1a). CRTR-1 expression was also detected at the tip of the ureteric buds, consistent with the location of the developing distal convoluted tubules (Figure 9.1b and c). Expression of CRTR-1 during the earliest stages of DCT formation suggests that CRTR-1 may play an important role in the differentiation and/or formation of the DCTs in addition to a physiological role in the adult kidney. CRTR-1 expression in the ureteric bud, which go on to form the collecting ducts of the kidney, but not in the collecting ducts at later stages of kidney development or in the adult suggest that expression of CRTR-1 becomes restricted later during development of the kidney. Broad CRTR-1 expression suggests that CRTR-1 may have a role in development of the ureteric bud and tubule system which then becomes restricted to the specification and function of the DCTs. The importance of CRTR-1 function for kidney development and/or function can be determined by generation of CRTR-1 mutant mice.

9.3 STRUCTURE AND SEQUENCE OF THE *CRTR-1* LOCUS.

Mutation of genes by knockout requires detailed knowledge of the genomic structure and orgainisation of the gene of interest. The 9405 bp CRTR-1 cDNA sequence was compared to available sequences in the MGSCV3 mouse genomic database (2.3.1.15) by BlastN (2.3.1.15). Sequence comparisons returned a single region of 97% sequence alignment spanning the full length of the CRTR-1 cDNA sequence localised to the central region, 1E2+3, of mouse chromosome 1. Several single nucleotide discrepancies between the genomic sequence and the CRTR-1 cDNA sequence (3.4) are considered to be errors in

FIGURE 9.1

Expression of CRTR-1 during early kidney development by whole mount *in situ* hybridisation.

Mouse embryo metanephroi dissected at 12.5 d.p.c were grown culture (2.3.5.6.6) for three days. Whole mount *in situ* hybridisation analysis (2.3.5.6.6) was carried out using DIG-labelled anti-sense *CRTR-1* specific riboprobes (2.3.5.6.1-2). Probed samples were analysed as described in Piper *et al.*, 2000. CRTR-1 expressing Cells are identified by purple staining.

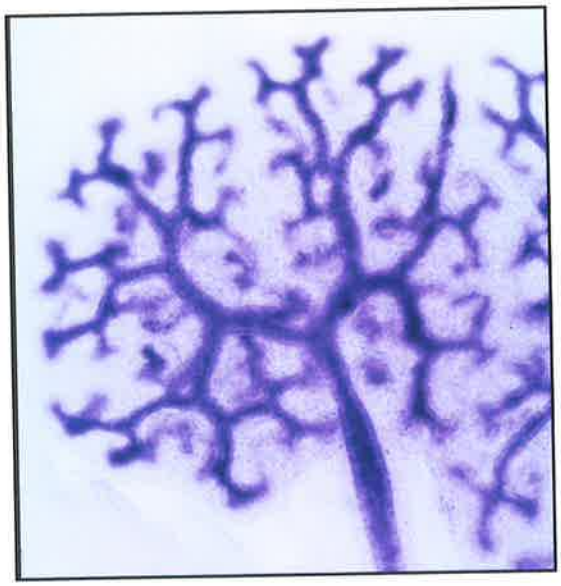
Magnifications, **(A)** 40X, **(B)** 100X **(C)**, 200X.

mm, metanephric mesenchyme

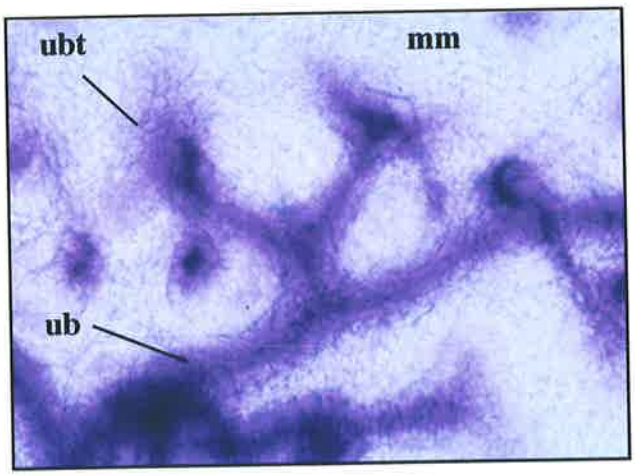
ub, ureteric bud

ubt, ureteric bud tip

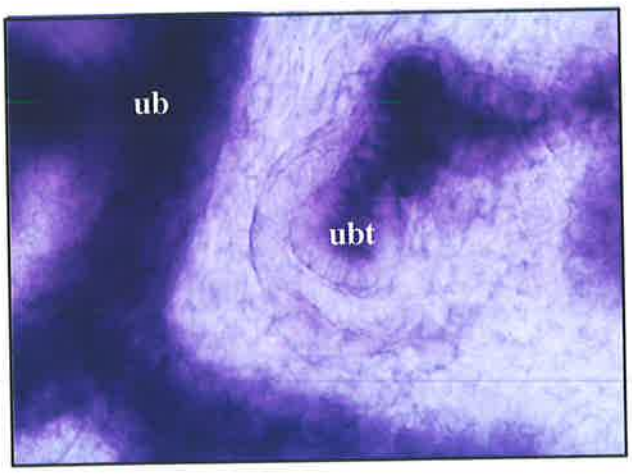
A



B



C



the MGSCV3 database as CRTR-1 has been isolated independently by the RIKEN group and shows 100% alignment with the CRTR-1 cDNA sequence (2.3.1.16).

Using DNasis, the genomic sequence was compared by alignment with regions of the CRTR-1 cDNA sequence, enabling the determination of intron/exon boundaries. The genomic sequence for CRTR-1 was divided between 15 exons and spread over a region of approximately 60 kb. Schematic representaion of the CRTR-1 genomic structure and organisation is presented in Figure 9.2a, together with the relative of intron/exon boundaries throughout the CRTR-1 amino acid sequence (Figure 9.2b).

Analysis of the genomic sequence upstream of the cDNA sequence revealed a consensus 'TATA' box sequence, TATAAAA, 33 nucleotides upstream of the 5' end of the longest cDNA clones (3.4). This spacing is consistent with the usual distance between the TATA box and the initiation of transcription and suggests that the CRTR-1 sequence represented in this thesis is full length.

The CRTR-1 cDNA sequence was also compared to sequence data available from the human genome at NCBI (2.3.1.15). Sequence comparisons revealed a single region of 94% sequence alignment localised to chromosome 2 region q14. Analysis of this human genomic sequence revealed that the gene mapping to this region was LBP-9. Human-mouse synteny maps generated by NCBI have demonstrated that mouse 1E2.3 is syntenic with human 2q14, providing further evidence that LBP-9 is the human homologue of CRTR-1.

A region of 33bp spanning nucleotides 4326-4358 (TTTATTTTTATTTTATGTGCATTGGTATTTTGC) of the CRTR-1 cDNA demonstrated 100% sequence alignment with 28 regions located on different mouse chromosomes. Analysis of this region by BlastN (2.3.1.15) did not provide further information about the possible functional importance of this conserved sequence.

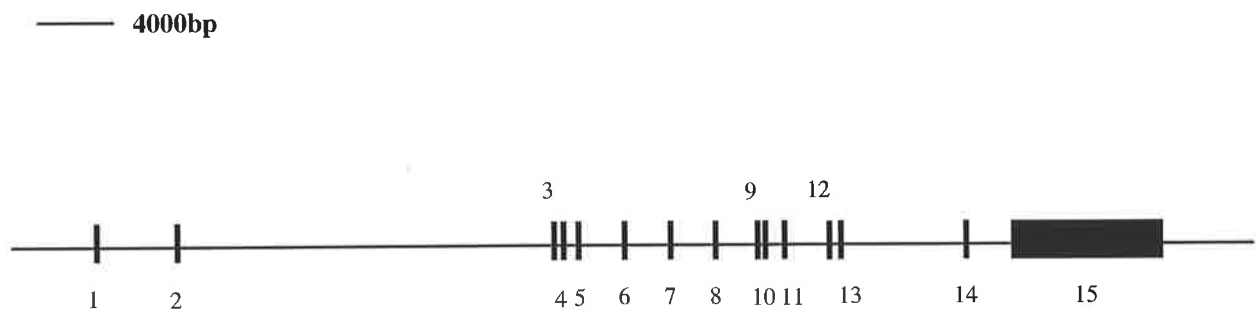
FIGURE 9.2

Structure of the mouse gene encoding *CRTR-1*.

A) Genomic structure of CRTR-1. Exon numbers are indicated.

B) The location of exon boundaries mapped onto the CRTR-1 amino acid sequence.

A



B

```
                                           1/2
1    M L F W H T Q P E H Y N Q H N S G S Y L R|D V L A L P I F K Q E E P Q L S P E N   40
                                                                       2/3
41   G A R L P P L Q Y V L C A A T S P A V R L H E E T L T Y L N Q|G Q S Y E I R L L   80
                                    3/4
81   E N R K L G D F Q D L N T K Y V K|S I I R V V F H D R R L Q Y T E Y Q Q L E G W   120
                           4/5
121  R W S R P G D R I L D I|D I P L S V G I L D P R A S P T Q L N A V E F L W D P S   160
                     5/6
161  K R A S A F I Q V|H C I S T E F T P R K H G G E K G V P F R V Q I D A F K Q N E   200
                                           6/7
201  K W G L L G A S T L L P A C Q I K G F K|A Q G E L I G N K K T D R E K M E K R T   240
                                       7/8
241  A Q E K E K Y Q P S Y E T T I L T E|C S P W P D V P Y Q A N T P P S P S Y N G S   280
                         8/9                                 9/10
281  P N S F G L R E G T|S S P I H P V E P L P L G S D|H L L P S A S I Q D A Q Q W L   320
                                    10/11
321  H R N R F S Q F C W L F A S F S G|A D L L K M S R D D L V Q V C G P A D G I R L   360
                     11/12
361  F N A I K G R|N V R P K M T I Y V C Q E L E Q N Q L P L P Q K Q D D S G D N S L   400
        12/13
401  C V|Y H A I F L E E L T T L E L T E K I A S L Y S I P P Q H I H R V Y R Q G P A   440
                       13/14                                14/15
441  G I H V V V S N E|M V Q N F Q D E S C F I L S T L K|A E S S D G Y H I I L K C G   480

481  L *
```

9.4 IDENTIFICATION AND CHARACTERISATION OF THE GENE-TRAP MOUSE ES CELL LINE W064C03.

Gene-trap is a method for the identification and analysis of genes by insertional mutagenesis based on random integration of the gene trap vector into the genome (reviewed (Cecconi and Meyer, 2000)). Multiple projects are on-going world-wide using differently engineered gene trap vectors. Gene trapping in mouse ES cells enables the functional importance of genes mutated by insertion to be analysed *in vivo* by the generation of chimeric mice following blastocyst injection of the gene-trapped ES cell line.

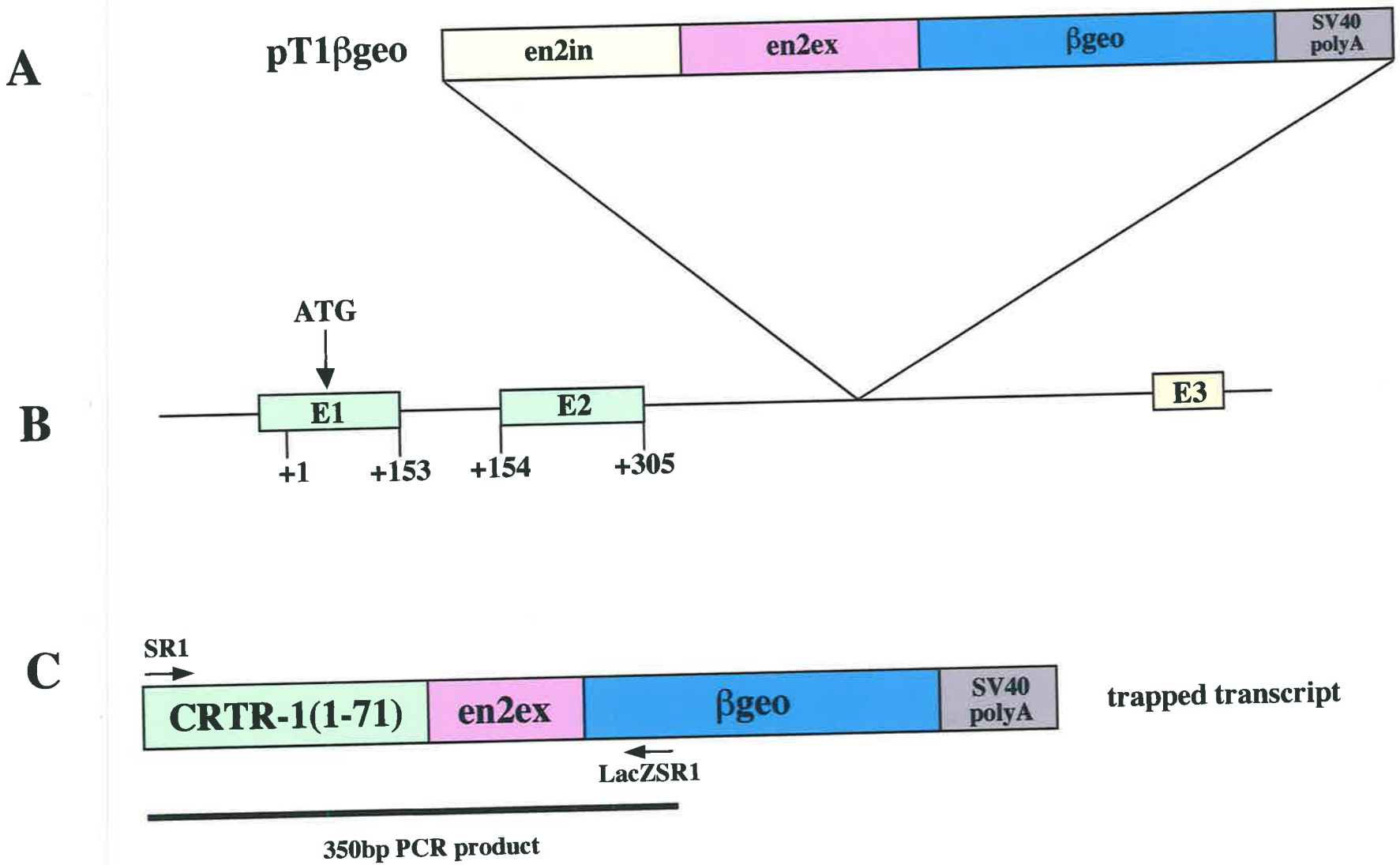
The German Gene-Trap Consortium (GGTC) was established to generate a reference library of gene trap sequence tags from insertional mutations generated in mouse embryonic stem (ES) cells. 12,000 targeted ES cell clones were generated and characterised by Southern analysis and 5'RACE-PCR (Wiles *et al.*, 2000). The pT1βgeo gene trap vector, used by the GGTC, contains an *engrailed* splice acceptor site immediately upstream of a promoterless β-galactosidase/neomycin resistance (βgeo) reporter gene and SV40 polyA sequence (Figure 9.3a). Integration of the vector into an intron of a gene places the reporter under the transcriptional control of the "trapped" gene promoter, resulting in expression of a fusion transcript between the upstream exons and the reporter gene (Figure 9.3c). Trapping of an expressed gene enables elucidation of the expression pattern *in vitro* and *in vivo* through generation of heterozygous mice. In cases where the fusion transcript disrupts gene function by generating a truncated mRNA, the functional importance of the gene can be investigated by creation of mice bearing homologous mutations following blastocyst injection. Cloning and sequencing of RACE products has enabled the establishment of a database (http://genetrap.gsf.de) that can be searched using BlastN for genes of interest that have been trapped.

Two gene trap ES cell lines, W008C03 and W064C03, are specifically trapped for CRTR-1. W008C03 is an E14 ES cell line that has the pT1βgeo vector inserted between exons 1 and 2. This cell line has been previously shown to carry a mycoplasm infection and

FIGURE 9.3

Schematic representation of the gene trapped *CRTR-1* locus in W064C03 ES cells.

pT1βgeo gene trap vector contains a region encompassing an engrailed intron (en2in) and exon (en2ex) boundary immediately upstream of a promoterless β-galactosidase/neomycin (βgeo) resistance reporter gene and SV40 polyA sequence **(A)**. W064C03 ES cells contain pT1bgeo inserted into the CRTR-1 genomic locus between exons 2 and 3 **(B)**. Expression of CRTR-1 from this locus results in transcription of a truncated mRNA encoding amino acids 1-71 of CRTR-1 fused to βgeo **(C)**. The relative location of sequences for primers SR1 and LacZSR1 (2.2.7.2) are indicated.


A
pT1βgeo
en2in
en2ex
βgeo
SV40
polyA
B
ATG
E1
E2
E3
+1
+153
+154
+305
C
SR1
CRTR-1(1-71)
en2ex
βgeo
SV40
polyA
trapped transcript
LacZSR1
350bp PCR product

was not considered. W064C03 is a TBV-2 ES cell line that has the pT1βgeo vector inserted in the intronic region between exons 2 and 3 (Figure 9.3b) and was obtained from the GGTC for characterisation and blastocyst injection.

9.4.1 Sequence and RT-PCR Analysis of ES Cell Line W064C03.

RACE PCR sequence data from the GGTC was 100% identical to the CRTR-1 cDNA sequence over the regions encoded by exons 1 and 2 (data not shown), and was in agreement with the intron/exon boundary determined for exon 2 (8.2). Insertion of the gene trap vector at this position suggests that mRNA sequence would encode the β-galactosidase reporter protein as an in-frame fusion with the first 71 amino acids of CRTR-1 (Figure 9.3c). This region of CRTR-1 contains the transcriptional repression domain (4.2.3) but would not bind CP2 family members (5.3.2), cellular binding proteins (8.8) or DNA (6.3).

Splicing of the β-galactosidase coding region into the CRTR-1 mRNA was confirmed by platinum Taq PCR (2.3.1.12) using polyT primed cDNA (2.3.1.11) generated from total RNA isolated from $2x10^5$ W064C03 cells grown on a gelatinised 10cm diameter tissue culture dish for 3 days (2.3.5.1). As a negative control, PCR was also performed using cDNA generated from total RNA isolated from D3 ES cells. PCR was performed using PCR primers (2.2.7.2) SR1/SR2 (specific for the CRTR-1 ORF) and SR1/LacZSR1 (specific for the β-galactosidase coding region) (Figure 9.3b). 5 μL samples of each PCR reaction were separated by 1% TAE agarose gel electrophoresis and visualised under UV light after staining with ethidium bromide (2.3.1.2). Reactions primed with SR1 and SR2 generated a band of approximately 1500 bp for cDNA derived from both D3 and W064C03 ES cells (Figure 9.4a and b, lane 2). Reactions primed with SR1 and LacZSR1 generated a band of approximately 460 bp for cDNA from W064C03 (Figure 9.4b lane 3) but no product from D3 ES cells (Figure 9.4a, lane 3). This result confirmed the expected heterozygosity of the CRTR-1 locus in W064C03 but not D3 ES cells.

FIGURE 9.4

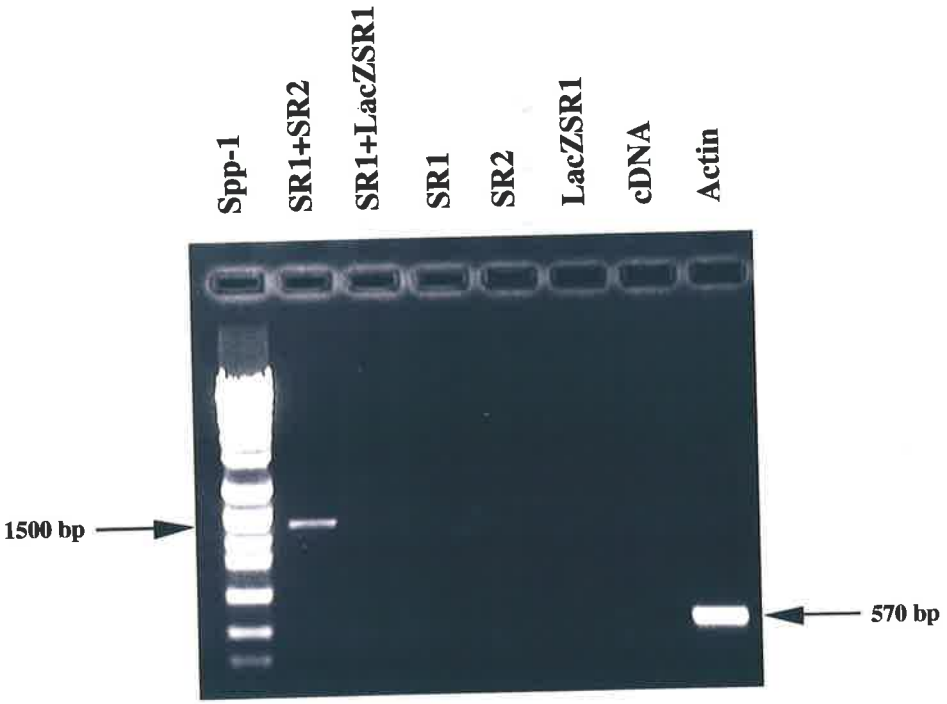
Confirmation of CRTR-1(1-71)/β-geo fusion transcript expression in W064C03 cell by RT-PCR.

Platinum Taq PCR (2.3.1.12) was carried out on polyT primed cDNA (2.3.1.11) generated from D3 **(A)** or W064C03 **(B)** ES cell total RNA (2.3.5.1) with primers SR1/SR2 (lane 2) and SR1/LacZSR1 (lane 3) (2.2.7.2). 5 μL samples of PCR reactions, together with 500ng of *Eco*RI digested Spp1 phage DNA markers (2.2.13), were separated on a 1% TAE agarose gel and visualised by exposure to UV light after ethidium staining. SR1, SR2 and lacZSR1, PCR reactions containing individual primers with cDNA (lanes 4-6); cDNA, PCR containing cDNA only (lane 7); actin, PCR using actin primers actinA and actinB (2.2.7.2) (lane 8). Sizes of bands are indicated.

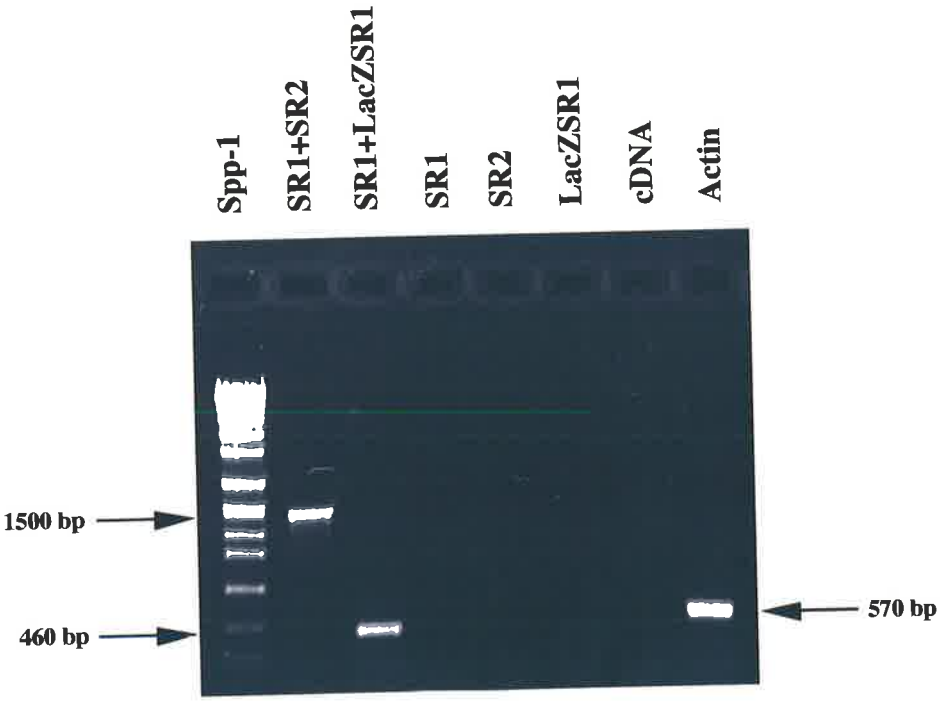
PCR Parameters:

Step 1: 94°C for 3 minutes
Step 2: 94°C for 30 seconds
Step 3: 50°C for 30 seconds
Step 4: 72°C for 2 minutes
Step 5: cycle back to step 2, 24 times.
Step 6: 72°C for 4 minutes
Step 7: 4°C
Step 8: End

A


Spp-1
SR1+SR2
SR1+LacZSR1
SR1
SR2
LacZSR1
cDNA
Actin
1500 bp
570 bp

B


Spp-1
SR1+SR2
SR1+LacZSR1
SR1
SR2
LacZSR1
cDNA
Actin
1500 bp
460 bp
570 bp

9.4.2 Culture of Clone W064C03 and Confirmation of Pluripotence.

W064C03 TBV-2 ES cells were cultured on a gelatinised 15 mm diameter tissue culture dish in culture media prescribed by the GGTC (2.4.3-4). Medium was changed each day for 5 days at which time cells were passaged and re-seeded onto gelatinised 60 mm diameter tissue culture dishes. Cells were cultured for a further passage into gelatinised 10 cm diameter dishes at a density of $2x10^5$cells/dish. 1ml aliquots of cells containing $1x10^6$ cells were then frozen down for storage in liquid nitrogen (2.4.6).

Consistent with previously described ES cell lines (Evans and Kaufman, 1981; Martin, 1981), W064C03 cells grew as tight domed colonies and contained few differentiated cells (Figure 9.5 a and b). $2x10^5$ W064C03 cells were cultured on a gelatinised 10cm tissue culture dish for 3 days, formaldehyde/methanol fixed and assayed for alkaline phosphatase (AP) activity (2.4.10), a marker for pluripotency in mouse ES cells (Hahnel *et al.*, 1990; Pease *et al.*, 1990). The AP assay demonstrated that W064C03 ES cell colonies (Figure 9.5c) but not differentiated cells (Figure 9.5d) expressed AP. These results demonstrate that W064C03 cells grow with the correct morphology, are able to differentiate and expression of AP was specific for the ES cell colonies which, taken together, confirming them to be pluripotent.

9.4.3 W064C03 Cells Contain a Full Complement of Chromosomes.

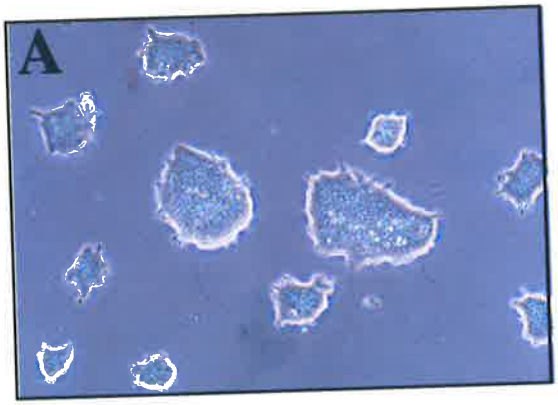
Mice contain 19 pairs of autosomes and one pair of sex chromosomes. Full contribution of ES cells to the development of a mouse following blastocyst injection requires ES cells to contain a full complement of chromosomes. $2x10^6$ W064C03 cells were grown on a gelatinised 10cm tissue culture dish overnight prior to replacement of culture media with fresh media containing 10 μg/ml of colchicine (2.4.11). Chromosome spreads were prepared (2.4.11) and viewed at 100x magnification under oil immersion on a

FIGURE 9.5

Alkaline phosphatase expression in W064C03 ES and differentiated cells.

W064C03 EC cells were cultured (2.4.4) in 10 cm gelatinised tissue culture dishes for 3 days and photographed on a Nikon Eclipse TE300 inverted microscope under phase contrast at 100x **(A)** and 200x **(B)** magnification. Cultures were assayed for alkaline phosphatase activity (2.4.10). The 10 cm dish assayed **(C)** was scanned using a HP scanjet 7400c. Colonies **(D)** were viewed and photographed under phase contrast at 200x magnification. Cells expressing alkaline phosphatase are identified by purple staining.

d; differentiated cells.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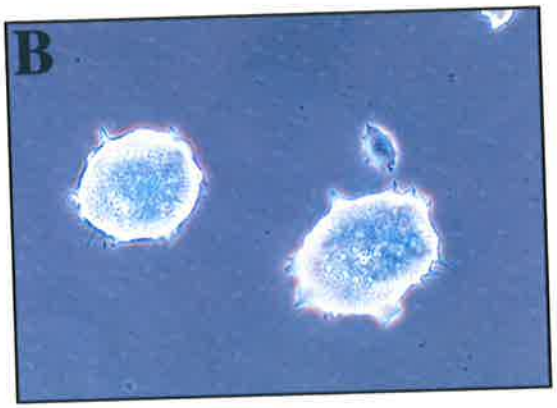
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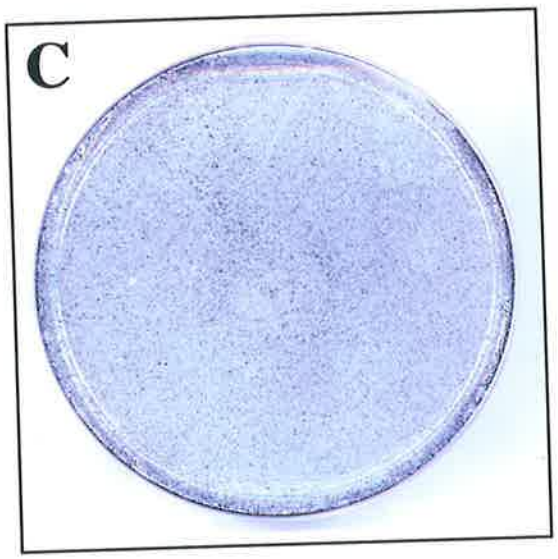
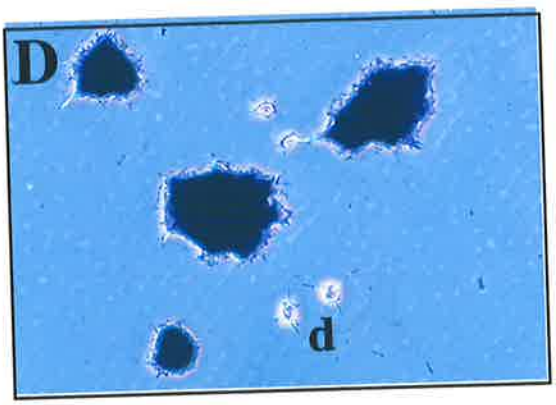

C


D
d

Zeiss axioplan microscope. 20 metaphase chromosome spreads were analysed and all contained 40 chromosomes. Three examples, representative of those counted, are presented in Figure 9.6. This result suggests that the ES cell line TBV-2 clone W064C03 contains a full complement of chromosomes.

9.4.4 W064C03 Cells Express *LacZ* and *CRTR-1* in a Regulated Fashion.

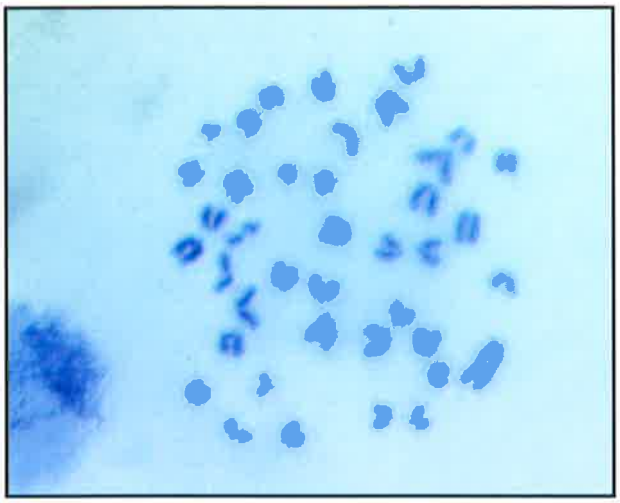
W064C03 cells are heterologous for the β-galactosidase insertion, suggesting that full length CRTR-1 would still be expressed from the second copy of chromosome 1. $7x10^4$ W064C03 ES cells were plated into 15 mm gelatinised tissue culture wells and cultured for 3 days prior to *in situ* hybridisation analysis (2.3.5.6.3) using DIG labelled sense and anti-sense 3' CRTR-1 specific riboprobes (2.3.5.6.1-2). Sense riboprobes did not display specific staining above background levels (Figure 9.7a). Anti-sense riboprobes readily detected CRTR-1 expression in undifferentiated W064C03 ES cell colonies (Figure 9.7b). CRTR-1 expression was not detected in differentiated cells present in the culture (Figure 9.7b), consistent with the expression pattern of CRTR-1 described previously (1.10).

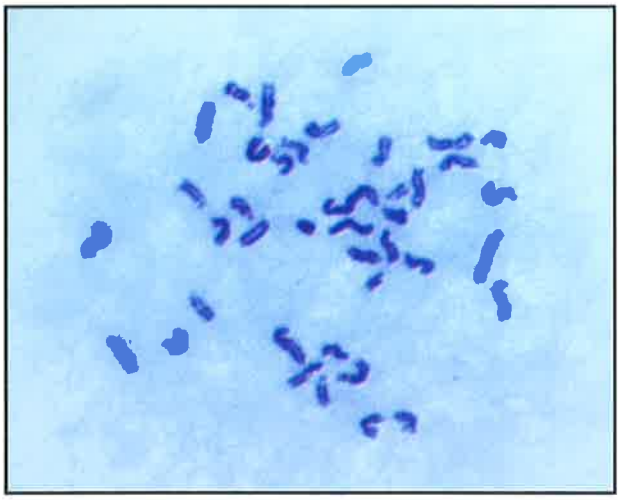
The W064C03 cell line contains the pT1βgeo gene-trap vector inserted into the CRTR-1 locus in such a manner that β-galactosidase expression is controlled by the CRTR-1 promoter which. $2x10^5$ W064C03 cells were plated on a gelatinised 10cm tissue culture dish and cultured for 3 days for analysis by staining for β-galactosidase activity (2.4.8). Stained cells were viewed using a Nikon inverted microscope. Staining demonstrated that undifferentiated W064C03 cells expressed β-galactosidease (Figure 9.7 c, d and e). β-galactosidase activity was not observed in differentiated cells present in the culture confirming that β-galactosidase expression is consistent with that expected for CRTR-1 (Figure 9.7e).

FIGURE 9.6

W064C03 ES cells contain a full complement of chromosomes.

W064C03 ES cells were cultured (2.4.4) in a 10 cm gelatinised tissue culture dish overnight. Cells were blocked at metaphase by culture in media containing 10μg/ml colchicine, harvested and metaphase chromosome spreads prepared (2.4.11) and viewed at 100x magnification under oil immersion on a Zeiss axioplan microscope.





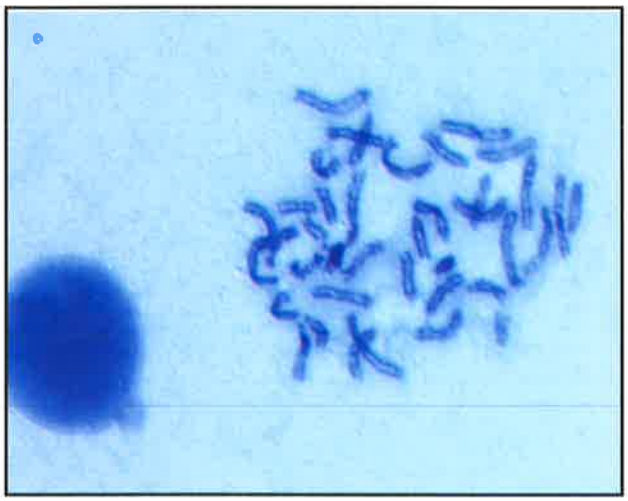


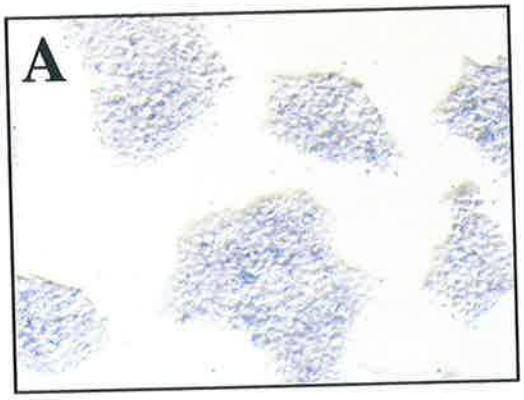
FIGURE 9.7

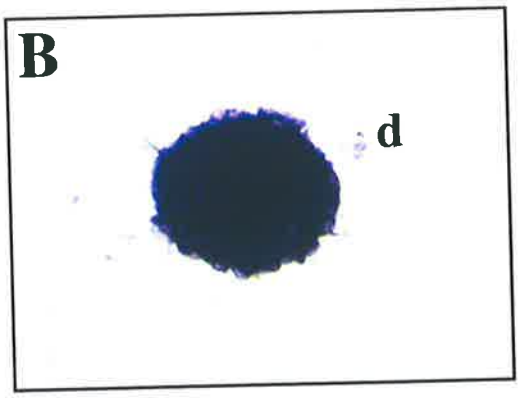
***CRTR-1* and β-galactosidase expression in W064C03 ES and differentiated cells.**

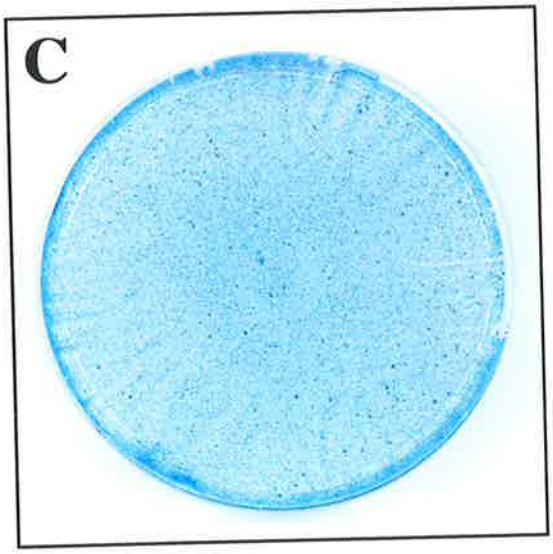
W064C03 cells cultured in 15mm diameter gelatinised tissue culture wells for 3 days prior to *in situ* hybridisation analysis (2.3.5.6) using a *CRTR-1* specific 736bp DIG labelled sense **(A)** or anti-sense **(B)** riboprobe. Colonies were photographed on a Nikon Eclipse TE300 inverted microscope using the Hoffman modulation contrast system at 100x **(A)** and 200x **(B)** magnification. CRTR-1 expressing cells are identified by purple staining.

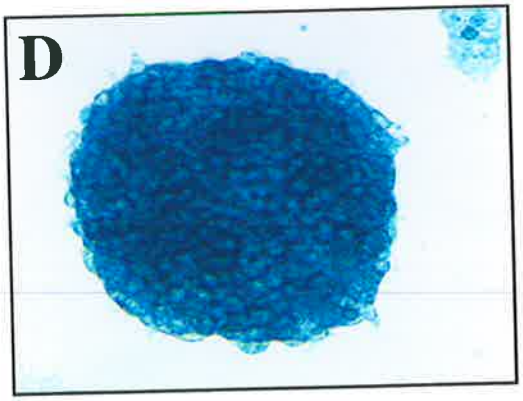
W064C03 EC cells were cultured (2.4.4) in 10 cm gelatinised tissue culture dishes for 3 days prior staining for β-galactosidase activity (2.4.8). Cultures were scanned using a HP scanjet 7400c scanner **(C)** and photographed on a Nikon Eclipse TE300 inverted microscope using the Hoffman modulation contrast system at 200x **(D)** and 100x **(E)** magnification. Cells expressing β-galactosidase are identified by dark green staining.

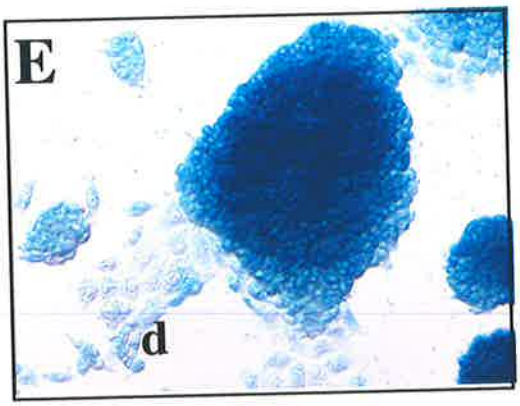
d; differentiated cells.


A


B
d


C


D


E
d

9.4.5 Injection of W064C03 ES Cells into Mouse Blastocysts.

Properties of the CRTR-1 gene-trap ES cell line W064C03 are consistent with those expected for pluripotent ES cells. Accordingly, cells were injected into mouse blastocysts (GenSA) for the generation of chimeric mice this is a prerequisite for the breeding of mice heterozygous and homozygous for the mutated CRTR-1 allele thereby enabling investigation into the precise expression of CRTR-1 in heterozygous mice, and elucidation of functional role of CRTR-1 in homozygotes. UPDATE

9.5 DISCUSSION

9.5.1 CRTR-1 Expression During Kidney Development.

CRTR-1 expression in 16.5d.p.c embryonic and adult mouse kidneys has been shown to be specific for the epithelial cells lining the DCTs (1.12.3). Analysis of CRTR-1 expression during kidney development demonstrated broader specific expression of CRTR-1, in the branching ureteric bud and in the cells present at the tips of the ureteric bud which are precursors that contribute to formation of the DCTs.

The ureteric bud is an epithelial tube that extends from the mesonephric duct (Wolffian duct) into a dense mass of about 5000 cells, called the metanephros (Figure 9.8). It is these cells that will give rise to the definitive kidney. The metanephric cells begin to form by 11 d.p.c (Davies and Bard, 1998). Invasion of the ureteric bud into the metanephros results in a reciprocal interaction between the two different cell types (Herzlinger, 1995). Firstly, a signal originating from the ureteric bud acts on the metanephros and directs these cells to differentiate into a population of stem cells present in the periphery of the kidney by 11.5 d.p.c. These stem cells ultimately go on to produce several cell types including the nephron and the medullary stroma. A reciprocal signal from the metanephros acts on the ureteric bud inducing branching of the ureteric bud into the metanephros (Davies and Bard, 1998) (Figure 9.8).

By 12.5 d.p.c stem cells have taken up residency in the cortex of the developing kidney. It is at this time that the ureteric bud branches while the stem cells proliferate rapidly. The stem cells divide asymmetrically, maintaining a pool of stem cells and providing cells to differentiate into mesenchymal condensations that form at the tip of the branching ureteric buds (Potter, 1972). Initiation of condensation marks the irreversible entrance into nephrogenesis. It is these condensations that will form the functional nephrons (Horster *et al.*, 1997).

The formation of nephrons is characterised by differentiation of the mesenchymal cells condensed at the tip of the ureteric bud to epithelial cells in a mesenchymal-to-epithelial transition (Figure 9.8). This is characterised by polarisation of the mesenchyme cells producing the classical epithelial phenotype (Horster *et al.*, 1997). It is these cells that will generate the distal and proximal convoluted tubules of the nephron (Orellana and Avner, 1998).

By approximately 15.5 d.p.c, the developing collecting duct fuses with the developing distal convoluted tubule at the same time that the proximal convoluted tubule is formed and the renal capsule differentiates, thereby producing a near complete but not yet functioning nephron (Davies and Bard, 1998).

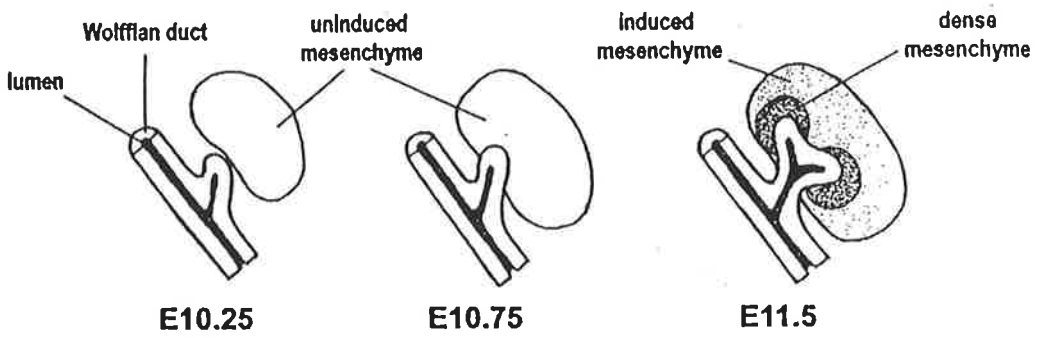
CRTR-1 expression at 15.5 d.p.c. during kidney development was specific for the branching ureteric bud and the cells present at the tips of the ureteric bud which are precursors for nephron differentiation. These are cell types derived from the mesonephros and metanephros. CRTR-1 expression during later kidney development and in the adult was restricted to the epithelial cells lining the DCTs (1.12.3). This could imply that CRTR-1 function may be divided into two roles, broad expression of CRTR-1 throughout the ureteric bud and developing tubule system suggest that CRTR-1 may be important for the development of these structures, while restriction of CRTR-1 expression to the DCTs later during development and in the adult suggest that CRTR-1 function may be important for

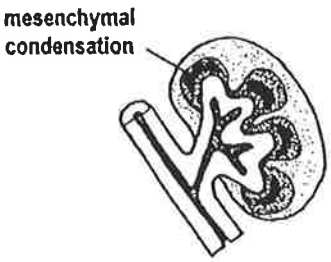
FIGURE 9.8

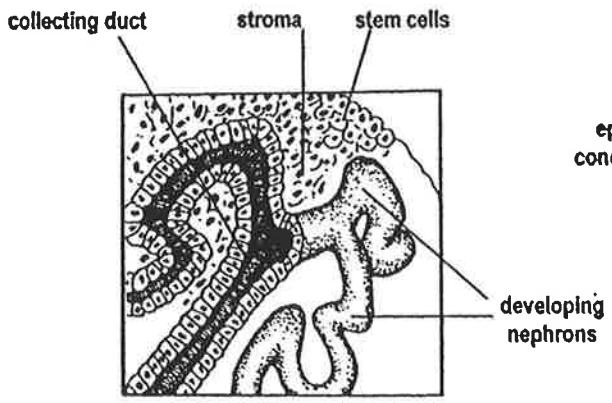
Schematic representation of the major steps of early kidney development.

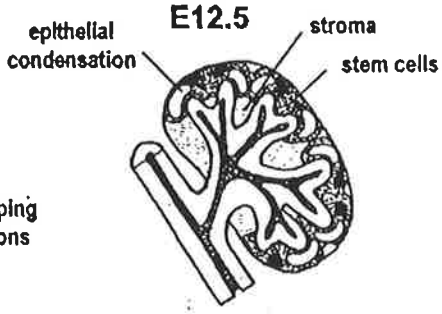
At about 10.5 d.p.c. the ureteric bud forms from the metanephric duct (wolffian duct) and protrudes into the developing metanephros (induced mesenchyme) by 11.5 d.p.c.. A reciprocal interaction between the two cell types results in branching of the ureteric bud and condensation of metanephric stem cells to the tip of ureteric buds differentiating to produce mesenchymal condensations (12.5 d.p.c. through to 13.5 d.p.c). By 15.5 d.p.c the developing collecting ducts fuse with the developing tubules such that by 16.5 d.p.c. epithelialised nephrons constitute the final collecting-duct/nephron system.

Figure taken from Davies and Bard, 1998.


Wolffian duct
lumen
uninduced mesenchyme
induced mesenchyme
dense mesenchyme
E10.25
E10.75
E11.5


mesenchymal condensation
E12.5


collecting duct
stroma
stem cells
developing nephrons
E16


epithelial condensation
stroma
stem cells
E13.5

DCT specification and function. Factors regulating the condensation and differentiation of the stem cell pool at the tips of the ureteric bud may be regulators of CRTR-1 expression and function which could ultimately result in specification of DCT formation. Together, these observations support the hypothesis that CRTR-1 function is important for the differentiation and/or formation of the DCTs and/or DCT physiology. Elucidation of the importance of CRTR-1 function in the kidney requires the generation of CRTR-1 null mice.

Functional analysis of CRTR-1 will enable elucidation of the importance of CRTR-1 for maintenance and differentiation of the pluripotent cells early during development. Should CRTR-$1^{-/-}$ mice be embryonic lethal, the exact timing of CRTR-1 effect on development of the embryo would be investigated by histological and morphological analysis. Survival of embryos past 4.75 d.p.c will be investigated using marker gene expression for various cell lineages to determine if CRTR-1 plays a role in the specification of cell differentiation to a particular germ layer or lineage.

Should the CRTR-$1^{-/-}$ genotype be viable past day 4.75 d.p.c the role of CRTR-1 during later development and in adult mice may be able to be investigated. Specifically, determination of correctly formed kidneys would be determined by histological analysis of the developing, adult tissues and marker gene expression. These techniques would also be employed if kidneys are formed incorrectly resulting in embryonic lethality at a later stage of development. Correct function of the kidney would be investigated by analysis of blood pH and ion levels.

CHAPTER 10

Final discussion.

10.1 INTRODUCTION.

This thesis describes the isolation and functional characterisation of CRTR-1, a novel member of the CP2 family of transcription factors. CRTR-1 is distinct from other family members in two respects, CRTR-1 expression is spatially and temporally regulated and CRTR-1 acts as a dominant transcriptional repressor of CP2 family mediated transcriptional activation, with the activity of CRTR-1 mediated transcriptional repression localized to an N-terminal 52 amino acid novel repression domain. Furthermore, this thesis describes the identification, isolation and functional characterisation of an alternatively spliced isoform of CP2, altCP2. Similar to CRTR-1, altCP2 appears to be differentially expressed and is capable of acting as a dominant repressor of CP2 family mediated transcriptional activation by inhibiting DNA binding of CP2 family protein complexes, thereby abrogating the ability to activate transcription. Together, altCP2 and CRTR-1 provide mechanisms to achieve spatially and temporally regulated activity of ubiquitously expressed CP2 family transcriptional activators.

10.2 CRTR-1 IS A NOVEL MEMBER OF THE CP2 FAMILY OF TRANSCRIPTION FACTORS.

The CRTR-1 cDNA encodes a 481 amino acid protein that shows significant amino acid sequence alignment with members of the CP2 family of transcription factors (Figure 3.6 and Table 3.1). CRTR-1 shared amino acid sequence similarity over regions of functional importance for protein/protein interactions and DNA binding (Figure 3.7) but not over the N-terminal 40 amino acids known to encode the CP2 transcriptional activation domain (Ramamurthy *et al.*, 2001; Tuckfiled, 2002a). Conservation in amino acid sequence suggests that CRTR-1 would be able to participate in homomeric and heteromeric protein complex formation and bind CP2 consensus response elements in a fashion analogous to CP2. This hypothesis was confirmed by GST-pull down, yeast-2-hybrid and co-purification from transfected COS-1 cells and by EMSA demonstrating the ability of CRTR-1 to form

homomeric and heteromeric complexes with other CP2 family members and bind the α-globin CP2 DNA binding response element. Together, these observations define CRTR-1 as a novel member of the CP2 family of transcription factors.

The CP2 family member with greatest amino acid identity with to CRTR-1, including the N-terminal 52 amino acids, was LBP-9 (88%). This together with the synteric relationship between the chromosome localization of each gene, mouse 1E2+3 for CRTR-1 and human 2q14 for LBP-9, the cell-type regulated expression and transcriptional repression activity of LBP-9 indicate that LBP-9 is the human homologue of CRTR-1.

10.3 REGULATED EXPRESSION OF *CRTR-1*.

CRTR-1 was identified as a cDNA differentially expressed between ES and EPL cells using ddPCR (Pelton *et al.*, 2002). Analysis of CRTR-1 expression has demonstrated CRTR-1 to have spatially and temporally regulated expression throughout development. CRTR-1 expression was specifically observed in the pluripotent cells during early mouse development and in the 16.5 d.p.c. and adult kidney DCTs. These sites of expression suggest that CRTR-1 could play two separate biological roles, firstly in the proliferation and maintenance of pluripotent cells during early mouse development and in the development and/or function of the kidney DCTs. Investigation into expression in the developing kidney at 15.5 d.p.c. demonstrated CRTR-1 to be expressed by the branching ureteric bud and at lower levels by the condensing metanephric cells at the tips of the ureteric buds which go on to produce the functioning nephron including the DCTs. These observations demonstrate that CRTR-1 is expressed broadly during early kidney development and suggest that CRTR-1 may be important for the correct branching and development of the ureteric bud system prior to being restricted to the DCTs.

10.4 CRTR-1 IS A DOMINANT REPRESSOR OF TRANSCRIPTION.

CRTR-1 was shown to act as a transcriptional repressor from a heterologous promoter when fused to the Gal4-DBD. Further, CRTR-1 mediated repression of transcription was observed from a CP2 responsive promoter in the presence of CP2. CRTR-1 was therefore able to act as a dominant repressor of transcription mediated by CP2. The mechanism by which CP2 family members regulate transcription is unknown with the exception that DNA binding is required as mutations in a CP2 family response element inhibits promoter regulation by CP2 family members (Lim *et al.*, 1993).

10.5 DOMINANT NEGATIVE FUNCTION OF altCP2.

altCP2 was identified as a novel isoform of CP2 expressed in mouse testes containing an additional 132 bp insert. Expression of altCP2 appears to be regulated with expression observed in mouse testis and 16.5 d.p.c. embryonic brain but not in mouse ES cells or adult heart. Characterisation of altCP2 revealed that the 132 bp insert encoded an in frame stop codon truncating CP2 at amino acid 384.

Despite lacking the C-terminal 117 amino acids of CP2, altCP2 was shown to interact with all CP2 family members and cellular binding proteins in a fashion consistent with CP2. However, deletion of these amino acids prevented altCP2 from binding the α-globin CP2 response element. Furthermore, inclusion of altCP2 in EMSA with CP2 abrogated CP2 DNA binding. Together these observations suggested that altCP2 may function a dominant negative CP2 isoform.

In vivo experiments using a CP2 responsive promoter demonstrated that expression of altCP2 repressed activation from this promoter. The inability of altCP2 to bind a CP2 DNA response element *in vitro* suggested that the mechanism of altCP2 mediated repression was interaction with CP2 family members *in vivo*, thereby abrogating their ability to bind DNA.

CP2 family members are generally described to act as transcriptional activators and to be expressed ubiquitously. The mechanisms by which CP2 family members activate transcription or the processes that regulate their activity have not been delineated. The identification of CRTR-1 and altCP2 provide possible mechanisms for the selective regulation of CP2 family members by two independent mechanisms, dominant repression of transcription mediated by CRTR-1 and inhibition of DNA binding mediated by altCP2. However, their functional roles may overlap as CP2 null mice do not show an obvious phenotype suggesting that the negative regulatory roles of altCP2 could be compensated for by CRTR-1.

10.6 FUNCTIONAL STRUCTURE OF THE CP2 FAMILY.

Truncation studies attempting to define the functionally important regions of LBP-1c have determined that the minimum region for DNA binding is between amino acids 65-383, however DNA binding equivalent to the full length protein was only observed with amino acids 65-502 (Shirra and Hansen, 1998). Similarly, the minimum region of LBP-1c required for protein interaction is amino acids 266-403, however protein binding equivalent to the full length protein was only observed with LBP-1c 266-502 (Shirra and Hansen, 1998). The overlapping of functionally important regions suggests that the ability to bind DNA may not be separable from the ability to interact with other proteins.

Identification and functional characterisation of altCP2 have, in part, redefined the regions of sequence required for DNA binding and protein interaction (Figure 10.1). The observation that the C-terminal 117 amino acids of CP2 are not required for protein interaction with other CP2 family members or unrelated cellular proteins but are essential for DNA binding indicates that the DNA binding and protein interaction functions can in fact be separated and that they exist in distinct regions of the amino acid sequence. It is this characteristic that enables altCP2 to act as a dominant negative CP2 isoform.

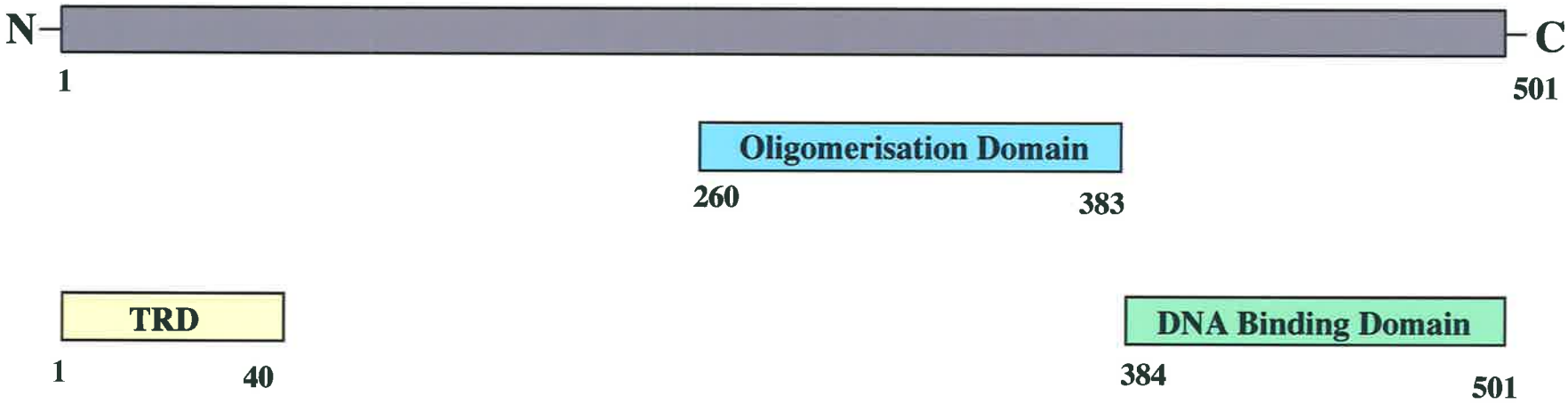
FIGURE 10.1

Schematic representation of CP2 functional domain structure.

The region in CP2 required for transcriptional repression is localised to the N-terminal 40 amino acids (TRD) (Ramamurthy *et al.*, 2001; Tuckfield, 2002). This region is consistent with the localisation of the CRTR-1 transcriptional repression domain.

Identification of altCP2 has defined the C-terminal 117 amino acids of CP2 as the DNA binding domain as altCP2 is unable to bind DNA (6.4.1). Despite this truncation, altCP2 is able to interact with all CP2 family members (5.3.2.2) and unrelated related cellular binding proteins (8.7 and 8.8.1) equivalent to CP2. This indicates that amino acids 260-383 of the dimerisation domain equivalent region (amino acids 260-501) used for yeast-2-hybrid analysis (Zhou *et al.*, 2000; Tuckfield *et al.*, 2002) is required for protein interactions, defining this region as the oligomerisation domain.

CP2 amino acid sequence



Furthermore, the use of dimerisation domain equivalent clones in yeast-2-hybrid experiments expressing amino acids 260-501 that are able to form homomeric complexes and heteromeric complexes with other family members as well as heteromeric complexes with other unrelated cellular proteins demonstrate that amino acids 1-259 are not required for protein interaction (Tuckfield *et al.*, 2002b; Zhou *et al.*, 2000). Finally, CP2 family members contain a defined transcriptional regulatory domain located at their N-terminus. This domain enables CP2 (Ramamurthy *et al.*, 2001; Tuckfiled, 2002a) to function as transcriptional activator and CRTR-1 to function as a transcriptional repressor. Experiments presented in this thesis (4.2) demonstrate that the N-terminal repression domain of CRTR-1 can function independent of the rest of the amino acid sequence.

10.7 PROTEIN/PROTEIN AND PROTEIN/DNA INTERACTIONS BETWEEN CP2 FAMILY MEMBERS

Yeast-2-hybrid (Chapter 5) and EMSA experiments (Chapter 6) presented in this thesis demonstrate that the level of amino acid sequence conservation of protein interaction and DNA binding regions between the family members is functionally important as all CP2 family members were shown to interact with each other. Furthermore, heteromeric protein complexes between family members were competent to bind a CP2 DNA response element and formed preferentially suggesting that heteromeric protein complexes have a higher DNA binding affinity than homomeric complexes.

These observations suggest that promoters described as being CP2 regulated (Table 1.1) could in fact be CP2 family regulated and that the members of the CP2 family constituting the DNA binding complex will dictate the nature of transcriptional regulation of the target gene. The mechanism of specificity for this mode of regulation of CP2 family members would be determined by their expression pattern and interaction with unrelated cellular proteins regulating cellular localisation and transcriptional regulation.

10.8 REGULATION OF CP2 FAMILY ACTIVITY THROUGH INTERACTION WITH CELLULAR BINDING PROTEINS.

10.8.1 Regulation of CP2 Family Cellular Localisation

Yeast-2-hybrid analysis identified Ubc9, PIAS1 and FKBP4 as CRTR-1 binding proteins (8.5.2). Ubc9 ligates SUMO to target proteins, which is known to control the translocation of proteins to the nucleus (Mahajan *et al.*, 1998; Matunis *et al.*, 1998). CRTR-1 contains two putative sumoylation consensus sequences, which are conserved across the CP2 family, in the N-terminal transcriptional repression domain and in a region known to be important for CP2 nuclear localisation (Dr. S.Jane, pers comm). Sumoylation of CRTR-1 by Ubc9 at the second consensus sumoylation site could regulate the localisation of CRTR-1 between the nucleus and cytoplasm. PIAS1 activity is regulated by signaling pathways such as interferon (Liu *et al.*, 1998; Liu and Shuai, 2001) and acts an enhancer of Ubc9 mediated sumoylation (Kahyo *et al.*, 2001; Kotaja *et al.*, 2002). Interaction of CRTR-1 with Ubc9 and PIAS1 therefore provides a mechanism for the controlled localisation of CRTR-1 in the cell by sumoylation and cell signaling pathways (Figure 8.14).

SUMO modification of transcription factors is also able to modulate their transcriptional regulatory ability (Gostissa *et al.*, 1999; Muller *et al.*, 2000; Rodriguez *et al.*, 1999; Ross *et al.*, 2002). Sumoylation of the N-terminal consensus sumoylation site could regulate the ability of CRTR-1 to repress transcription either through control of DNA binding or modulation of CRTR-1 interaction with other transcriptional regulatory proteins.

FKBP4 is a member of the FKBP group, which function to regulate transcription factor cellular localisation (Galigniana *et al.*, 2001) and transcriptional regulatory activity (Davies *et al.*, 2002; Yang *et al.*, 1995). Localistaion of CRTR-1/FKBP4 complexes to the cytoplasm would enable the regulation of CRTR-1 activity by nuclear exclusion. Migration of this complex to the nucleus could be regulated by signalling pathways specific for FKBP4 (Figure 8.14). Once in the nucleus, the transcriptional repression function of

CRTR-1 could be controlled by FKBP4 through the regulation of DNA binding and/or interaction with other transcriptional regulatory proteins (Figure 8.14).

CP2 and altCP2 interact with Ubc9 and PIAS1 (8.7) suggesting that the possible regulatory roles of these two binding proteins described for CRTR-1 may control CP2 function. Ubc9 however does not interact with NF2d9 or altNF2d9 suggesting that the regulatory mechanisms of Ubc9 are unique for CRTR-1, CP2 and altCP2 and that mechanism used by PIAS1 to regulate NF2d9 and altNF2d9 activity may be unique for these proteins.

FKBP4 was determined to be an exclusive binding protein for CRTR-1, failing to interact with other CP2 family members (8.7). The ability of CRTR-1 to interact with FKBP4 suggests that CRTR-1 activity could be uniquely regulated by FKBP4 modulating the cellular localisation and/or transcriptional repression function, providing a mechanism for the regulation of CRTR-1 independently of other CP2 family members.

Differential binding of CP2 family members with Ubc9, PIAS1 and FKBP4 permits the specific regulation of CP2 family member cellular localisation and, together with differential expression of CP2 family members CRTR-1 and altCP2, facilitates the selective formation of transcription factor complexes in the nucleus competent to regulate target gene expression.

10.8.2 Control of CP2 Family Transcriptional Regulation.

CP2 family members are generally described as transcriptional activators (Bing *et al.*, 1999; Huang and Miller, 2000; Kim *et al.*, 1990; Kim *et al.*, 1987; Lim *et al.*, 1993; Lim *et al.*, 1992; Murata *et al.*, 1998), with the exception of CRTR-1. The mechanism used by CP2 family members to regulate transcription is unknown but requires DNA binding as mutations in the DNA response element prevent DNA binding and thereby negate transcriptional regulation.

CRTR-1 was not able to interact with co-repressor proteins HDAC1, RING1 and SMRTα, tested as a means to determine the mechanism for CRTR-1 mediated transcriptional repression. This suggests that CRTR-1 may represses transcription directly, hindering the association of transcription factors with the promoter, or through the recruitment of other co-repressor complexes such as NcoR. However, CP2 has been shown to interact with HDAC1 (International Congress of Developmental Biologists, Kyoto, Japan, July 2001) and RING1 (Tuckfield *et al.*, 2002b) (8.8.1). These proteins were shown to be unique binding proteins for CP2 and altCP2 suggesting that HDAC1 and RING1 are capable of specifically regulating their activity and that CP2 may have a role in transcriptional repression for some cellular promoters.

Differential binding of CP2 family members with transcriptional co-factors permits the specific regulation of CP2 family member activity at the promoter and compliments expression and cellular localisation as mechanisms for controlling CP2 family function and target gene expression. Activation of these various regulatory pathways also provides a mechanism for the activity of CP2 family members to be integrated into signaling pathways enabling CP2 family activity to be responsive to cues in the extra-cellular environment.

10.9 A ROLE FOR CRTR-1 IN PLURIPOTENCY AND KIDNEY DEVELOPMENT.

CRTR-1 was shown to interact with Rex1 and PIAS3 (8.9.2). Rex1 is a Zn finger protein predicted to function as a transcription factor expressed in the pluripotent epiblast during early development (Ben-Shushan *et al.*, 1998; Rogers *et al.*, 1991). Rex1 expression is rapidly down regulated upon differentiation of the pluripotent cells in a fashion that parallels the expression of CRTR-1 (Pelton *et al.*, 2002). Regulated expression across this time of pluripotent cell differentiation suggests that Rex1 and CRTR-1 are responsive to factors regulating pluripotent cell differentiation and that these proteins may play a role in the maintenance and regulation of pluripotency.

Consistent with this possibility, CRTR-1 has been shown to interact with PIAS3. Maintenance of pluripotent cells *in vitro* and *in vivo* requires activation of Stat3, through gp130 signalling (Heinrich *et al.*, 1998). Stat3 functions as a transcription factor whose activity is regulated by PIAS3 (Chung *et al.*, 1997). As such, PIAS3 has a role in the regulation of Stat3 activity in response to gp130 signalling. The observation that CRTR-1 interacts with PIAS3 suggests that CRTR-1 activity may also be responsive to gp130 signalling via regulation of CRTR-1 activity through PIAS3.

Activation of Stat3 through gp130 signaling is also required during kidney development for the induction of nephogenesis, a site of CRTR-1 expression. The observation that CRTR-1 interacts with PIAS3 suggests that CRTR-1 activity may also be regulated by PIAS3 during this process and be required for kidney nephrogenesis.

Like PIAS1, PIAS3 has been shown to function as an enhancer of sumoylation (Nakagawa and Yokosawa, 2002). As such, PIAS3 could control CRTR-1 activity through sumoylation regulating the cellular localisation of CRTR-1 and/or the transcriptional regulatory ability of CRTR-1 in manner consistent with the proposed model for PIAS1 regulation of CRTR-1 activity (Figure 8.14).

Taken together, the identification of CRTR-1 binding proteins has provided insight into the possible mechanisms regulating CRTR-1 cellular localisation and transcriptional regulation ability and overall provided information into the possible biological function of CRTR-1.

10.10 FUTURE WORK

10.10.1 Is CRTR-1 Functionally Important for Pluripotency and/or Kidney Development and Function?

The biological importance of CRTR-1 function for the maintenance and regulation of pluripotency in early mouse development and in the development and function of the

kidney is being investigated by the generation of *CRTR-1* mutant mice from the gene trap cell line W064C03.

Mice heterozygous for the *CRTR-1* mutation will be used for detailed investigation of the cellular expression of *CRTR-1* by detection of β-galactosidase activity. This would permit elucidation of the expression of *CRTR-1* throughout all stages of development of the animal and in the adult tissues known to express *CRTR-1* but not previously investigated at the cellular level including the stomach, small intestine, placenta and testis.

Heterozygotes will be bred to homozygosity and the success of the gene trap in generating a null *CRTR-1* mutation will be determined by examination of developing embryos using Southern analysis to demonstrate homozygosity PCR and ribonuclease protection analysis to demonstrate that the *CRTR-1* transcript 3' of the genetrap vector is not expressed. In the event that the *CRTR-1* null mutation leads to embryonic lethality embryos harvested from inbred heterozygotes will be analysed to determine the stage at which death occurs by correlation with genotype using PCR. Morphological and histological analysis, together with the expression mapping of cell type specific marker genes, would be carried out to determine cellular and morphological defects and the presence and differentiation of embryonic and extra-embryonic cell populations. It would be of particular interest to establish whether the embryonic lethal phenotype is due to problems with the maintenance and/or regulation of the pluripotent cells early in embryogenesis. Similar techniques would be used to establish the cellular defects should embryos die at a later stage of development with particular emphasis on development of the kidney and other sites of early *CRTR-1* expression. If mice survive to birth then the effect of *CRTR-1* mutation on kidney physiology will be analysed by morphological and histological criteria as well as testing the composition of urine and blood. This analysis would enable investigation into the role of CRTR-1 and CRTR-1 expressing cells in

excretion, regulation of blood pH and ion concentrations, with potential implications for human disease

Early embryonic lethality resulting from *CRTR-1* null mutation would prevent investigation into subsequent roles of CRTR-1 including kidney development and in the adult kidney. Investigation of this could be achieved by generation of conditional *CRTR-1* null mice using the CRE recombinase system (Sauer, 1998). The first two exons of *CRTR-1*, encompassing the TATA box, ATG codon and repression domain, would be flanked by loxP sites introduced by homologous recombination. Analysis of the genomic *CRTR-1* sequence (9.3) indicates that deletion of these exons does not produce an alternative TATA box, transcriptional start site or significant ORF. Mice bearing the altered locus in homozygous form can then be bred to a mouse strain carrying the CRE recombinase gene under the control of an inducible promoter. An example is the ecdysone inducible system (No *et al.*, 1996) (Invitrogen) where expression of CRE is induced by administration of ecdysone (Sawicki *et al.*, 1998) in drinking water inactivating the *CRTR-1* locus, thereby enabling investigation of CRTR-1 function in kidney physiology. Administration of ecdysone to pregnant mice at various stages of embryonic development could be used for investigation of CRTR-1 function during kidney development.

10.10.2 Does CRTR-1 Repress Transcription *in vivo*?

CRTR-1 has been demonstrated to act as a dominant repressor of CP2 mediated transcriptional activation. To determine if CRTR-1 is able to act as a transcriptional repressor *in vivo*, microarray analysis could be carried out. If *CRTR-1*$^{-/-}$ ES cells, generated by isolation of ES cells from *CRTR-1* null embryos or targeting of the second allele from W064C03 cells, are viable the *CRTR-1* ORF can be reintroduced under the control of an inducible promoter such as the Tet-on system (Clontech). Microarray analysis comparing gene expression in *CRTR-1*$^{-/-}$ ES cells induced to express *CRTR-1*, CRTR-1$^{-/-}$ ES cells un-

induced and wild type ES cells could be carried out to determine target genes that are induced or repressed by CRTR-1. In the event that *CRTR-1*$^{-/-}$ ES cells are not viable, similar microarray analysis could be carried out in a cultured mouse cell line such as NIH3T3 in which both *CRTR-1* alleles have been targeted. Use of an inducible promoter system to direct *CRTR-1* expression in *CRTR-1* null cells has the advantage of being able to control the expression of *CRTR-1* such that direct gene targets of CRTR-1 activity are more likely to be identified, rather than downstream changes in gene expression as a result of prolonged CRTR-1 activity. This analysis would also establish if it is possible for CRTR-1 to activate transcription depending on the specific promoter context and the composition of associated binding partners.

Specific demonstration of CRTR-1 mediated transcriptional repression *in vivo* could be achieved through generation of a transgenic mouse carrying a green fluorescent protein (GFP) reporter gene under the transcriptional control of a *CRTR-1* responsive promoter. An equivalent mouse would also be generated containing mutations in the CRTR-1 binding site of the promoter, abrogating CRTR-1 regulation. Analysis of GFP expression by visualization using immunofluorescence, and quantitatively using ribonuclease protection analysis, in the pluripotent cells during development, the kidney and other sites of CRTR-1 expression from both mice would enable determination of changes in GFP expression in CRTR-1 expressing cells, confirming the repression role of CRTR-1 *in vivo*.

Transgenic mice could also be bred onto the genetic background of mice heterozygous for null mutations in genes encoding proteins important for various signaling pathways such as gp130. Mice null for *gp130* are viable up to 12.5 d.p.c. (Yoshida *et al.*, 1996). *gp130* null and heterozygous mice carrying the CRTR-1 responsive GFP reporter construct could be compared for GFP expression with the aim of determining if CRTR-1 activity is responsive to gp130 activated signaling. Similar experiments could be conducted

using mice heterozygous for null mutations in genes encoding proteins important for various aspects of development including kidney development.

Together, this series of experiments would enable identification of CRTR-1 responsive genes as well as information regarding the regulatory cascades operating upstream and downstream of CRTR-1 function, providing insight to the biological role of CRTR-1 during development and in the adult mouse.

10.10.3 What Factors Regulate *CRTR-1* Expression and Activity?

10.10.3.1 Identification of factors regulating CRTR-1 expression.

Expression of *CRTR-1* is spatially and temporally regulated suggesting that transcription from the CRTR-1 promoter is tightly regulated. Determination of factors that regulate expression of *CRTR-1* would be achieved by analysis of the *CRTR-1* promoter for identification of potential transcription factor binding sites. The ability of potential regulators to control *CRTR-1* expression can be investigated by generation and co-transfection of a luciferase reporter construct under the control of regions from the *CRTR-1* promoter encompassing predicted binding site with mammalian expression vectors for each putative transcription factor into COS-1 cells and the luciferase activity measured to demonstrate the ability of potential regulators to activate or repress transcription. The significance of these identified regulatory regions could then be investigated by determining the nucleotide sequence required for DNA binding using foot printing experiments prior to mutation of binding sequences in reporter constructs for analysis.

Identified factors and binding sites can be confirmed *in vivo* by generation of transgenic mice containing a reporter gene, such as GFP, under the control of promoter regions containing mutated binding sites for each factor and subsequent analysis determining marker gene expression throughout development and in the adult mouse. It

would be of particular interest to identify and compare factors regulating *CRTR-1* expression in the pluripotent cells and kidney DCTs.

Identification of transcription factors that activate or repress transcription would give insight to the possible regulatory and signaling systems controlling *CRTR-1* expression and in conjunction with the *CRTR-1* mutant, perhaps knowledge of the systems regulating pluripotent cell and/or kidney DCT biology.

10.4.10.2 Modulation of CRTR-1 cellular localization and transcriptional activity.

• **Sumoylation:**

Factors identified as CRTR-1 binding proteins include Ubc9, a SUMO ligase, and PIAS1 and PIAS3, enhancers of sumoylation (Kahyo *et al.*, 2001; Nakagawa and Yokosawa, 2002). Sumoylation of other transcription factors has been shown to be important for the regulation of cellular localisation and transcriptional regulatory activity (Gostissa *et al.*, 1999; Kamitani *et al.*, 1998; Mahajan *et al.*, 1998; Matunis *et al.*, 1998; Muller *et al.*, 2000; Rodriguez *et al.*, 1999). The ability of these binding proteins to direct sumoylation of CRTR-1 can be determined by *in vitro* sumoylation experiments (Johnson and Blobel, 1997; Rodriguez *et al.*, 1999) and the *in vivo* relevance tested by co-purification of 6xHis-tagged SUMO-1 with HA-tagged CRTR-1 from transfected COS-1 cells. Mutation of the lysine residue in each of the putative sumoylation consensus sequences identified in CRTR-1 to an alanine residue, shown to prevent sumoylation of proteins (Sampson *et al.*, 2001), could be carried out to determine the site of CRTR-1 sumoylation in *in vitro* assays.

The biological role of sumoylation sites can be investigated by transfection of constructs directing the expression of HA-tagged CRTR-1 and HA-tagged CRTR-1 with the lysine to alanine mutation for each identified sumoylation site into HeLa (Rodriguez *et al.*, 2001; Tian *et al.*, 2002) cells with Ubc9 and SUMO-1 expression constructs

(Rodriguez *et al.*, 2001). Immunohistochemistry could then be used to visualize the cellular localization of mutant HA-CRTR-1 compared with wild type HA-CRTR-1 and comparison of nuclear and cytoplasmic distribution conducted.

The effects of sumoylation on CRTR-1 transcriptional regulation of cellular promoters could be determined by co-transfection of CRTR-1 sumoylation mutants with pTK-CP2WTx4-LUC into COS-1 cells with the luciferase activity being measured and compared. This series of experiments would be extended, including co-transfection of pXMT2-6xHis-CP2-FLAG (5.4.1), investigating the possible roles of CRTR-1 sumoylation on dominant repression. Sumoylation could potentially enhance or abrogate CRTR-1 transcriptional regulation via modulation of DNA binding. This could be investigated using EMSA, testing the ability of IvTT CRTR-1 sumoylated *in vitro* to bind the CP2 α-globin DNA response element.

• **Interaction with FKBP4:**

FKBP4 and other members of the FK506 binding protein group have been shown to regulate the cellular localization and transcriptional regulatory ability (Davies *et al.*, 2002; Galigniana *et al.*, 2001) of transcription factors. This suggests two possible mechanisms by which FKBP4 might be able to modulate CRTR-1 transcriptional activity. The effect FKBP4 has on CRTR-1 cellular localization could be investigated using immunohistochemistry to compare the cellular localisation of CRTR-1 in COS-1 cells co-transfected with expression constructs for HA-CRTR-1 and FKBP4 (Yang *et al.*, 1995; Yang *et al.*, 2001) and in cells transfected with HA-CRTR-1 alone. The effect of FKBP4 on CRTR-1 mediated transcriptional control could be tested by co-expression of CRTR-1, FKBP4 and CRTR-1 responsive luciferase reporter constructs with luciferase expression being compared to expression of CRTR-1 alone with the reporter constructs. The ability of FKBP4 to modulate CRTR-1 DNA binding as a mechanism for regulating CRTR-1 activity

could be determined using EMSA comparing the ability of IvTT CRTR-1 to bind DNA in the presence and absence of IvTT FKBP4.

10.4.3.3 CRTR-1 promoter specificity

Interaction with the transcription factors Rex1 and YY1 could affect CRTR-1 mediated transcriptional repression at the level of DNA binding. This could be investigated by EMSA to determine if the ability of CRTR-1 to bind the α-globin CP2 response element and/or promoter regions of genes regulated by CRTR-1 identified by microarray is altered in the presence of YY1 or Rex1. Changes in DNA binding could be further characterized by investigating the effect on luciferase expression from CRTR-1 responsive luciferase reporter constructs in transfected COS-1 cells expressing CRTR-1 in the presence or absence of Rex1 or YY1.

10.4.3.4 Mechanisms of CRTR-1 mediated transcriptional repression

CRTR-1 represses transcription by an unknown mechanism. Proteins that interact with the CRTR-1 N-terminal repression domain could not be identified by yeast-2-hybrid due to the dominant nature of CRTR-1 mediated transcriptional repression over Gal4-AD transcriptional activation of growth selection genes. Proteins that interact with the CRTR-1 repression domain could be identified by co-purification of binding proteins with the CRTR-1 repression domain expressed in transfected 293T cells fused to an N-terminal 6xHis tag. Proteins co-purifying with the repression domain could be separated by PAGE, isolated and sequenced by mass spectrometry. Determination of proteins able to interact with the CRTR-1 repression domain would provide insight into the mechanism employed by CRTR-1 to repress transcription and the possible regulatory and signaling pathways that regulate CRTR-1 activity.

Together, elucidation of factors which control expression and activity of CRTR-1 would provide valuable information into the possible biological roles of CRTR-1 for regulation and maintenance of pluripotency during early mouse embryogenesis and in the development and function of the kidney.

APPENDIX 1

APPENDIX 1: SEQUENCE OF CLONES ISOLATED BY YEAST-2-HYBRID.

Sequence data from pACT2 cDNA library clones isolated form two yeast-2-hybrid screens using CRTR-1(47-481) as bait are presented. Each sequence begins with CGA ATT CGN, which follows in frame from the Gal4 activation domain encoded by pACT2. Amino acid sequence conservation with proteins from BlastP (2.3.1.16) are indicated.

A1.1 Yeast-2-Hybrid Screen 1:

Clone A1, nil amino acid sequence identity.

>CGAATTCGCGGCCGCGTCGACTGTGGGAGAGCAAATGACAGGAAGACTGGACCTGATACA
GCTGCCATCACCTGGGAACAACAGACTCCAGTACCAGGAAGCCTCCAGACCTTCAGCCTCA
GACTAGGGCTGCATCATTGGTCCAGCCTATAGGAAGTTGGTGAGGCTGGTGTGAGGAATTT
CATCAGTGTGTGATGCTTCCAGCTACTGGGACCTACAGCATGCAGATGGTCACTGTGGGAC
TCTTCTGTCTGCAGTTTTGTAAACCAATCCAATGAATGTTTCCATAATCATTTTATAATTC
CATATATAACAATATGTGGTGTGTATTACCACA

Clone A39, nil amino acid sequence identity.

>CGAATTCGCGGCCGCGTCGACGCAACTCCAGCCACAGTCACATCCCAAGTTCCAGCAACC
GCCACCGCTGAGCTGTCACCGCCAATGCCCCAACCCAAGACTCGTACACGCAGGAACAAAG
GTCCCCGAGCGGCCCGGGGTGTTATCCGTGAAGAAGGGACTTCTGGAGACGGCCCTCGAGA
ACCGAATACGGCTCCGGTGACTGACAGCAGCAGTGGAGGGGGTGGCGGTGGTAGCAACGGA
ACCTCCACAGCTGGGGCCTCTAACAAGGGAACAGCAAGGCACTGGCCGCCTTTCGAGGTGC
TTAATTCCTGTCCCTGCAAGTGCTACTGTCGCCATCAACGTCGTCACCGTCGGCTGCCACG
CAACGTGTCTGCCTGGCTGAGCACACCTACCAACCACCTGAGCGAGCCACCGTGGGTGGCC
ACTGTCAAGCTGGCTGGCTCCCTAGTAGCGGGCCTGGAGCACTACGACTTGCAGGCCACCC
ATTCTACCTGAGTGCGACTGCCCAACCCCCTCGGGCTCACCCTNCTCGCCAATAAAGTACC
CAGTCTACAAGTTAAAAAAAAAAAAAAAAA

Clone C61, nil amino acid sequence identity.

>CGAATTCGCGGCCGCGTCGACGAGAACACCACACTGAATGTGATAATAGAGCTAGAGGAA
AGGGAAGATGGATACATTTCACCTCCAGCACCCAGAAAGGTAGAGTTTGAATCAGGACTCC
ATGCATGCTAGCCAAGAGCTCTGAATGGAGTATGTCCCTAGCATTTAAGGGGTATTTTTAA
AGACACAGAGAGAGGTGAAGTCAGATGGCATGAGTGGAGAGTCAGAAGTAGTTTGAACAAG
GACAAAAGATGTCAAGAGCTGAAGTAGTATAGAGAAGTCAAAAGGGCCCAAGAACACCTGG
GCAATGGTGGTGCACTATTCATCCTAGCACTCAGGAGGCAGAGGCAGGTGGACCTCTTGAG
TTCACCTGCACAGGCCTTTGTGGCACAGGTGAGTGTCTGANGTANGGTGAGCCTTCTCACT
CTGTTCCCATGCCTGTCCTCTTGAGTGACATCAGTAGCTGTTCAGAATAGTCACTTCTAGA
AGACCTGTCATTAAAAAGTATCTCTTCCTACATAAACTCCTTTCCATCAGCATTCANGATA
CATCTGCTCAAATAGCCCTGACATATNCATCATTCCCTTTACCATATAAGCCCCAAACATC
TTAACAAAACAACTCTGCACTGGCTTCTTCCAAGCTGCCCTNTTGGGTAATAACATCCATC
TCATGACTTNACTGNGAATCCTTTTGNACTCAAAAGGTTCAAGAACCTAACTANGAAAGTT
AAGCCACCTAGTCTATGAGTGATTTCTCGATATCAGGACTCACAAAAAACCCTGTCTNAAA
AAAAAAAAAA

Clone D76, encodes 93 amino acids that shared 100% identity to phosphodiesterase 6D clone MGC:11435, direct submission. Accession number: BC005636.

>CGAATTCGCGGCCGCGTCGACCTGGCCGCCACCGCTGTTCACCTGAGGTGTCCGGCCGGG
CTGCGGCTGTCCTCGGTTCCTGGGTACCGTCTGCGAGGCCCCGCGGAGCCAGCGTGGGAGG
GCCGCGGGCGGCAGGCGCCGCATCATGTCAGCCAAGGACGAGCGGGCCAGGGATATCCTGA
GAGGCTTCAAACTAAATTGGATGAACCTTCGGGATGCCGAAACAGGGAAGATACTCTGGCA
AGGAACAGAAGACCTGTCTGTCCCTGGTGTGGAACATGAAGCCCGTGTGCCCAAGAAAATC
CTCAAGTGCAAGGCAGTGTCTCGAGAATTGAACTTTTCTTCAGCAGAACAAATGGAAAAAT
TCCGCCTGGAACAAAAAGTTTACTTCAAAGGACAATGCCTAGAAGAGTGGTTCTTCGAGTT
TGGCTTTGTGATCCCTAACTCCACAAACACCTGGCAGTCCTTGATAGAAGCAGCGCCTGAG
TCCCAGATGATGCCCGCCAGCGTCCTAACGGGCAATGTCATCATAGAGACAAAGTTCTTTG
ACGATGATCTTCTTGTCAGCACATCCAAAGTGANGCTTTTCTATGTTTGAGAGAATTTGTG
TGCACTTTTTAAAATTTGGNCCGGGGCA

Clone E62, nil amino acid sequence identity.

>CGAATTCGCGGCCGCGTCGACGAGCTGCAGCCGCCTCAGAGGCTTGTGTAATCCAGAAGT
TCAAAAGTCAAGGCTGCCTCATCCCGGCCTGGTGGGTATTGCATGGCTGAACATCTGGGAA
TGCTGTTCTATGCTGTCTCGTGAGTCAGAGGATGAGAAGATGGGCCCCAGGTGTCTTGCAG
TCCACTGCACCTTAAAGATTGCTGACACTAATCCACCTAGCACCGTGCTGTCAAGTCGGAT
GACTCCAGGGAAGCTGCTGATGTAAAGCATAAACAGCCCAAGGGAGATAACTGTGCTTAGT
GATAAAAATGCACAGTTTTCCTAGAATTATAATTTATTCCAGAATTTCCTTCCAGAGCATG
TCCTGGTAAATGACTTAGATTTGAGTGAAAAATAAACTGAAAGGGAAAAAAAAAAAAAAAA
AAAA

Clone F7, nil amino acid sequence identity.

>CGAATTCGCGGCCGCGTCGACCTGGCCGCCACCGCTGTTCACCTGAGGTGTCCGGCCGGG
CTGCGGCTGTCCTCGGTTCCTGGGTACCGTCTGCGAGGCCCCGNTTNAGCCAGCGTGGGAG
GGCCGCGGGCGGCAGGCGCCGCATCATGTCAGCCAAGGACGAGCGGGCCAGGGATATCCTG
AGAGGCTTCAAACTAAATTGGATGAACCTTCGGGATGCCGAAACAGGGAAGATACTCTGGC
AAGGAACAGAAGACCTGTCTGTCCCTGGTGTGGAACATGAAGCCCGTGTGCCCAAGAAAAT
CCTCAAGTGCAAGGCAGTGTCTCGAGAATTGAACTTTTCTTCAGCAGAACAAATGGAAAAA
TTCCGCCTGGAACAAAAAGTTTACTTCAAAGGACAATGCCTAGAAGAGTGGTTCTTCGAGT
TTGGCTTTGTGATCCCTAACTCCACAAACACCTGGCAGTCCTTGATAGAAGCAGCGCCTGA
GTCCCAGATGATGCCCGCCAGCGTCCTAACGGGCAATGTCATCATAGAGACAAAGTTCTTT
TGACGATGATCTTCTTGTCAGCACATCCAAAGTGAGGCTTTTCTATGTTTGAGAGAATTTG
TGTGCACTTTTAAGAATTTGGCCGGGGGCAGGA

Clone G23, nil amino acid sequence identity.

>CGAATTCGCGGCCGCGTCGACAGAAAAAGACCACGGTGTGTTAAACATTCCATATTTAGA
ATATGTCTGTTTTACACTAGGTTCTAGAAGTAATTGGATCCCAAAGTTCTTTGGCTATTAT
AAAGTATAGACACTATATAGCTACCTATAAACAGTATAGCAGTGAAATGTGTGCATTTTAA
GAGATCTGCACTCTTGGGTCTTGTCAGTGGCATAATCTCTGACTTGTTTGAAGACACTCCA
AGCCTTTCCAATGTCATCCTGCTCTCATTACAAACCTTCACTTTTGGAATGGATATTTTTT
GTGCATCATACACATGCTTTTTGCCTGTAGTTGCCTTAGTCATCTTTCTGCAGTAGCATCT
GGACATCACAAGTCCTGCTATTCATTAATCAAGGAAATCTGAAGAGATGACAGTGCCCTAA
AGTAGTTTACATCCTTTCAGAAAGTATGTGTAAGTGCATGCTTCTATGCACCTTCTTATGT
AGTACTTTTTTTACAGATATGCTTATTTTTATTTAATGACCCGGGTTAATGCCCTTCATAA
GTATATTGGCAATTATGAGCCTTTATACCCTAGCTACAAATTTAAAT

Clone G56, encodes 158 amino acids that showed 100% identity to Ubc9 (Gottlicher *et al.*, 1996). Accession number: MMU76416.

>CGAATTCGCGGCCGCGTCGACCTCTGCCGCCGCGACGGTCCGGGCCGCGGTCGCCCAGGG
ACTTTGAATATGTCGGGGATCGCCCTCAGCCGCCTTGCGCAGGAAAGGAAAGCCTGGAGGA
AGGACCACCCTTTTGGCTTTGTAGCTGTCCCAACAAAGAACCCTGATGGCACAATGAACCT
GATGAACTGGGAGTGCGCTATCCCTGGAAAGAAGGGGACTCCATGGGAAGGAGGCTTGTTC
AAGCTACGGATGCTTTTCAAAGATGACTATCCGTCCTCACCACCAAAATGTAAATTTGAGC
CCCCACTGTTTCATCCAAACGTGTATCCTTCTGGCACAGTGTGCCTGTCCATCCTGGAGGA
AGACAAGGACTGGAGGCCAGCTATCACCATCAAACAGATCTTATTAGGAATACAAGAACTT
CTAAATGAACCAAATATTCAAGACCCAGCTCAAGCAGAGGCCTACACAATTTACTGCCAAA
ACAGAGTGGAATATGAGAAAAGGGTCCGAGCACAAGCGAAGAAGTTTGCCCCCTCATAAGC
AGCGGCCCTGGGCTCCATGACGAGGAAGGGATTGGCTTGGCAAGAACTTGTTTACAACCTT
TTGCAGATCTAAGTCGCTCCGTACAGTTACTAGTCGCCTGGGA

Clone G71, nil amino acid sequence identity.

>CGAATTCGCGGCCGCGTCGACAGAAAAAGACCACGGTGTGTTAAACATTCCATATTTAGA
ATATGTCTGTTTTACACTAGGTTCTAGAAGTAATTGGATCCCAAAGTTCTTTGGCTATTAT
AAAGTATAGACACTATATAGCTACCTATAAACAGTATAGCAGTGAAATGTGTGCATTTTAA
GAGATCTGCACTCTTGGGTCTTGTCAGTGGCATAATCTCTGACTTGTTTGAAGACACTCCA
AGCCTTTCCAATGTCATCCTGCTCTCATTACAAACCTTCACTTTTGGAATGGATATTTTTT
GTGCATCATACACATGCTTTTTGCCTGTAGTTGCCTTAGTCATCTTTCTGCAGTAGCATCT
GGACATCACAAGTCCTGCTATTCATTAATCAAGGAAATCTGAAGAGATGACAGTGCCCTAA
AGTAGTTTACATCCTTTCAGAAAGTATGTGTAAGTGCATGCTTCTATGCACCTTCTTATGT
AGTACTTTTTTACAGATATGCTTATTTTTATTTAATGACCGGGTTAATGCCCTTCATAAGT
ATATTGGCAATTATGAGCTTTATACCTAGCTACAAATTAAATAAAATCCTTTTGAAAAACA
TTTTCTGATTATTGGTAAATTTTGTAAATCATCCTTATNTATGTCTAAAGAGATATTAAGT
ATGTACTAAATTACTGGAACATTTAAATTGCAATAACTNGCATAATTAAGTTGCAAATAAA
ATATTGTTTCTATNTTACACAAATAAGTTTACATAAAAAAAAAAAAAAAAAAA

Clone G113, nil amino acid sequence identity

>CGAATTCGCGGCCGCGTCGACTGTGGGAGAGCAAATGACAGGAAGACTGGACCTGATACA
GCTGCCATCACCTGGGAACAACAGACTCCAGTACCAGGAAGCCTCCAGACCTTCAGCCTCA
GACTAGGGCTGCATCATTGGTCCAGCCTATAGGAAGTTGGTGAGGCTGGTGTGAGGAATTT
CATCAGTGTGTGATGCTTCCAGCTACTGGGACCTACAGCATGCAGATGGTCACTGTGGGAC
TCTTCTGTCTGCAGTTTTGTAAACCAATCCAATGAATGTTTCCATAATCATTTTATAATTC
CATATATAACAATATGTGGTGTGTATTACCACAC

Clone G117, encodes 433 amino acids that showed 100% identity to CRTR-1 (Rodda *et al.*, 2001). Accession number: AF311309.

>CGAATTCCTCGACTACAGTATGTGTTGTGTGCCGCCACTTCTCCAGCGGTGAGGCTACAT
GAAGAGACCTTAACATACCTCAATCAAGGTCAGTCGTATGAAATCCGGCTGCTGGNTAATC
GGANGGTNGGGGACTTCCAAGATCTGAACACGAAATATGTGAAGAGCATCATCCGTGTCGT
TTTCCATGACCGCCGGCTGCAGTACACAGAGTACCAACAGTTGGAGGGTTGGCGGTGGAGT
CGGCCTGGGGACCGCATCCTGGACATTGATATTCCACTGTCTGTTGGTATCTTGGACCCCA
GGGCCAGCCCAACCCAGCTGAATGCTGTGGAGTTTTTGTGGGACCCATCGAAGAGAGCATC
TGCATTCATTCAGGTGCACTGTATCAGCACGGAATTCACCCCCAGGAAGCATGGGGGTGAG
AAGGGAGTGCTTTTTCGGGTGCAGATTGACGCGTTTAAGCAGAATGAGAGTGGGGACTACT
CGGAGCATCTACACTCTGCCAGCTGCCAGATCAAGGTGTTCAAGCCCAAGGGAGCTGATCG
GAAACAGAAGACTGACCGGGAAAAGATGGAAAAAAGAACCGGCTCAAGAGAAGGAGAAATA
CCAGCCATTCTATGAAACCACCATCCTTACCGAGTGTTCTCCATGG

A1.2 Yeast-2-hybrid screen 2.

Clone A1, nil amino acid sequence identity.

>CGAATTCGCGGCCGCGTCGACCGCGTTGGCTGCGGGAAGGTAAAGCAGTGCTGGAAGCGG
ATTGCAGGGGTCATGCCGTGGGGTCGTCTCTGCGCCTGCGCCCTACCGGGATGCTGAACCT
GGCGGCGCTGCTGTGGCGCCGGCTCCTGCGCAAGCGCTGGGTGCTCGCCCTGGTTTTCGGC
CTCTCGCTAGTTTACTTCCTCAGCAGCACCTTCAAGCAGGAAGAGAGGGCTGTGAGAGACC
GGAACCTCCTCCAGGTTCAAGACCGGGAACAGCCCATCCCGTGGAAGGTGCAGTTTAACCT
AGGCAACAGCAGCCGACCCAGCAACCAGTGCCGGAACTCCGTCCAAGGAAAACACCTCCTC
ACTGATGAGCTGGGCTATGTCTGCGAGAGGAAGGACCTGCTGGCGAATGGCTGTTGCGACG
TCAGCGTCCCCAGCACAAAGCAGTACTGCTGTGATGGGTGCCTGGCCAATGGCTGCTGTGA
AGCCTACGAGTACTGCGTCTCCTGCTGCCTGCAGCCCAGCAAGCAACTTCTCCTGGAGCGC
TTCCTCAACCGGGCAGCTGTGGCCTTCCAGAACCTCTTCATGGCAGTTGAAGACCACTTCG
AACTGTGCTTGGCTAAATGCAGGACCT

Clone A2, nil amino acid sequence identity.

CGAATTCGCGGCCGCGTCGACCGGGCGCGGGGAGCCCGGGTCTCTCCTAAACCCCGCAAAG
GTCAGACGTCCTCTCCGGGACCCAAGCGATGTATCTACGGGCAGGCTCCCGGACCTCTGCG
TGTTAAAGAGACGAGCACGCACATCACTGTAAGCGGCGGCGGCGGCGGCGCCCTGGTC
GAATTAGAATTTAAATACTCTGAGCACCATGACACTGAGACTTCTAGAAGACTGGTGCAGA
GGGATGGATATGAATCCTCGGAAAGCACTATTGGTTGCCGGCATCCCTCCGACCTGCGGAG
TGGCAGACATAGAGGAGGCCCTGCAGGCTGGCCTTGCTCCCTTAGGGGAACACAGACTGCT
TGGGAGGATGTTCAGGAGGGATGAGAACAAGAATGTAGCCCTGATTGGGCTTACAGTAGAG
ACTGGCAGTGCCCTGGTCCCCAAGGAAATACCTGCAAAAGGAGGTGTCTGGAGAGTGATCT
TTAAGCCTCCTGATACTGATAGTGACTTTTTGTGCAGATTAAATGAGTTTTTAAAGGGGGA
GGGCATGACGATGGGTGAATTAACCAGAGTTCTNGGGAATCGGAATGACCCTCTCGGNCTA
GACCCGGGCATAATGATCCCTGAAATTCGAGCACCCATGTTAGCACAGGCATTAAATGA

Clone A9, nil amino acid sequence identity.

>CGAATTCGCGGCCGCGTCGACCACTCTCTCTCCTTCCCTCCCCCCTCTCTCCTTGCATTC
ATCTTCCCCCTCCTGCCCCCCTCCCCCATTCTTTTCCTGCCTACACTCTGACTCTAGTCCC
TTCTCAACCTTGCTTCCCAAGCCCCAGATAAACTCTATTCTATACTAGCAGGCCTGGCAGG
GCTGTCAGCCACTCAAAGCACCAGTAAAAGGAAGTGACTTTNGTTGCTGTGTGGAGATAGT
AGTAGGCAGGACAGGTGTTCCGCAGGCAAGCTTCTGCCAGTCTCCAGTGTCCAACTGCCAA
GGACTCGGTGTTAGGCATTAAATGTAGGACATTGAACATGCCGGACATGCTTTGATACAGA
GCTCCATCTCCCACCACTCCAGTCTTCATCGAGGCATT

Clone A11, encodes 158 amino acids that showed 100% identity to Ubc9 (Gottlicher *et al.*, 1996). Accession number: MMU76416.

>CGAATTCGCGGCCGCGTCGACCTTGCGCAGGAAAGGAAAGCCTGGAGGAAGGACCACCCT
TTTGGCTTTGTAGCTGTCCCAACAAAGAACCCTGATGGCACAATGNTTCTGATGAACTGGG
AGTGCGCTATCCCTGGAAAGAAGGGGACTCCATGGGAAGGAGGCTTGTTCAAGCTACGGAT
GCTTTTCAAAGATGACTATCCGTCCTCACCACCAAAATGTAAATTTGAGCCCCCACTGTTT
CATCCAAACGTGTATCCTTCTGGCACAGTGTGCCTGTCCATCCTGGAGGAAGACAAGGACT
GGAGGCCAGCTATCACCATCAAACAGATCTTATTAGGAATACAAGAACTTCTAAATGAACC
AAATATTCAAGACCCAGCTCAAGCAGAGGCCTACACAATTTACTGCCAAAACAGAGTGGAN
TATGAGAAAAGGGTCCGAGCACAAGCGAAGAAGTTTGCCCCCTCATAAGCAGCGGCCCTGG
GCTCCATGACGAGGAAGGGATTGGCTTGGCAAGAACTTGTTTACAACCTTTTGCAGATCTA
AGTCGCTCCGTACAGTTACTAGTCGCCTGGGANGGGTGAGCGGGCGCCATTTTNCATTTNC
GNCACTGGCATATTCANGCCTTTGNATTTTTGATTATTAAGNAAACTTGCTTTATTTTNAA
AAAAAAAAAAAAAAAAAAAAAAAAAA

Clone A12, nil amino acid sequence identity.

>CGAATTCGCGGCCGCGTCGACGGGCTGGGTGCCAGCGCCTCGCGTCTCCCCAGCCCTCAA
AACCCGCCTAGGGTGCCCAGGACCCTAGTGCCCTCTGATGCGCTGGGGACCCTGCCTCCGA
CATCCCCTGGGCTGCCTGGGTCCGCGGCCACCTGTGGACCTGATTACCTTCCACACCTAGA
AAACCTCACCATCCTCCAGATTTCCCTCAGACTCAGCACACAGACCTCGGCGGCTTCAGGG
CCCGAGACCGTTATATCTTCTCCAGAAGTCCAACAGATCCCCTCTGGTCCAACTCCAAAGC
TTCGGGTCTCCAGGCCCATAGACTCCCATCCTTTCTTGGGTGGAGAGGCCTTCAGACCCAC
CTATCCCTAGAGACCTTCCTTCCTAGGATCTCCTGCCTCCTGCTCTCTATCTTGAGGTCCA
CAGAGCTTTTCAGCTTCTACTAGAGTGTTCTTGAGGTGGTACTCACCTTAGCGCCTCACCA
GGGTGTCTGCTATCCAAGTATAAGACACACTGCTTCCGGTAGGGGGAAGAGGGACTGAAGT
CAGCGGGGGAGTCCTGAAGGAAAGGGGAAGGTACTGAAGGAGACGAGTTCGGATGTGTACC
TGGAGGGGTTGAAGCTGCAAGCAGGAGGTGTCGATGTCCAGACAGGATTCACAGATGGAGT
CAGTGGAAAAGACCACGAACAGAAGTGAGCAGAATGCAGAAAGGTTTTAAAGAGCCTCATT
AGAAAGCAACCCCAGGACCTGCTCTTGGTAATTGGGACTGGCGTGAGCGCA

Clone A13, encodes 265 amino acids that showed 100% identity to Hsp40 DNAJ-like 2. (Royaux *et al.*, 1997). Accession number: NM_008298.

>CGAATTCGCGGCCGCGTCGACAAAAAGGCATATAGAAAATTGGCCTTGAAGTACCACCCT
GATAAGAATCCAAATGAAGGGGAAAAGTTTAAACAGATTTCTCAAGCTTATGAAGTTCTTG
CTGATTCCAAAAAAAGGGAACTATATGATAAAGGAGGGGAGCAGGCGATTAAAGAGGGCGG
AGCAGGTGGTGGTTTTGGCTCACCCATGGATATCTTTGATATGTTCTTTGGAGGAGGAGGA
AGAATGCAAAGAGAAAGGAGAGGTAAAAATGTTGTTCATCAGCTCTCAGTGACCTTAGAAG
ACTTATATAATGGTGCAACAAGAAAACTGGCTCTGCAAAAGAATGTGATTTGTGACAAATG
TGAAGGCCGAGGTGGTAAGAAAGGAGCAGTAGAGTGCTGTCCCAACTGCCGGGGGACAGGT
ATGCAGATAAGGATTCATCAGATTGGACCAGGAATGGTTCAGCAAATTCAGTCAGTGTGCA
TGGAGTGCCAGGGTCATGGAGAACGCATCAGTCCAAAAGACAGATGTAAAAGCTGCAATGG
AAGAAAAATAGTTCGAGAGAAGAAAATTTTAGAAGTTCATATTGATAAAGGCATGAAAGAT
GGTCAGAAGATAACATTCCACNGNGAANGAGACCAAGAACCA

Clone A14, encodes a 265 amino acids that showed 100% identity with mDj4, mammalian related DNAJ heat shock J2 protein, direct submission. Accession number: AB028854.

>CGAATTCGCGGCCGCGTCGACCAGAGACATGCCTCACCTGAGGACATTAAAAAGGCGTAT
CGAAAACAGGCACTTAAATGGCACCCGGACAAAAATCCTGAAAATAAAGAAGAAGCAGAGC
GGAAGTTCAAACAAGTAGCTGAGGCATATGAAGTGTTATCGGATGCTAAAAAGCGGGACAT
CTACGACAAATATGGCAAAGAAGGATTAAATGGTGGTGGAGGAGGAGGTGGAATTCATTTT
GACAGTCCATTTGAGTTTGGCTTCACATTCCGGAACCCAGATGATGTCTTCAGGGAATTTT
TTGGTGGAAGGGACCCATTTTCATTTGACTTCTTTGAAGACCCATTTGATGACTTTTTTGG
AAACCGAAGGGGTCCCCGAGGAAATAGAAGCCGAGGTGCCGGCTCATTTTTCTCTACCTTC
AGTGGATTTCCTTCTTTTGGAAGTGGATTTCCTGCTTTTGATACAGGCTTCACTCCATTTG
GGTCACTAGGTCATGGGGGTCTCACTTCATTTTCTTCAACGTCATTTGGCGGCAGTGGAAT
GGGCAACTTCAAATCAATATCAACTTCAACTAAGATAGTTAATGGCAAAAAAATCACGACA
AAGAGGATTGTGGAGAACGGTCAAGAAAGAGTAGAAGTTGAAGAAGATGGGCAGTTAAAGT
CCTTGACAATAAATGGTGTGGCCGACGAGAACGCCCTTGCAGAANAATGCCAGCGGAGAGG
CCAACCCACACCAGCTCTGGCCCTGGCCCGGCCCAGCACCAGT

Clone A16, nil amino acid sequence identity

>CGAATTCGCGGCCGCGTCGACAGCGTTTTGGGAGTCACGGGCTGGCCACTGGGCCGGGGG
TCCGGCTCCAGGGCAGTTTTACCGCATCCCGGCCACCCCCAGCGGCCTCATGGACCCGGCG
TCGGCGCCCTGCGAGGGTCCCATCACTCGGACCCAGAACCCCATGGTGACCGGGACATCGG
TACTCGGGGTGAAGTTCGACGGCGGAGTGGTGATTGCTGCAGACATGCTGGGCTCCTACGG
CTCCCTGGCTCGTTTCCGCAATATCTCTCGTATTATGCGAGTCAACGACAGCACTATGCTG
GGTGCCTCGGGAGACTACGCTGATTTCCAGTATTTGAAACAAGTTCTCGGCCAGATGGTGA
TTGATGAAGAGCTGCTGGGAGATGGACACAGCTATAGCCCTAGAGCTATTCATTCATGGTT
GACAAGAGCCATGTACAGCCGCCGCTCCAAGATGAATCCCCTGTGGAACACCATGGTCATT
GGAGGCTATGCTGACGGAGAAAGCTTCCTCGGTTATGTGGACATGCTTGGTGTAGCTTATG
AAGCCCCTTCACTGGCCACTGGTTATGGTGCATACTTGGCTCAGCCTCTGCTTCGAGAAGT

TCTAGAGAAGCAGCCAGTGCTGAGTCAGACTGAGGCTCGGGAGCTTGTGGAGCGCTGCATG
AGAGTGCTGTACTACAGAGATGCCCCGTTCGTATAACCGGTTTCAATTG

Clone A33, nil amino acid sequence identity.
>CGAATTCGCGGCCGCGTCGACTTGGAGACCGCCCCGGGTCGAGGAGCTGCTGGCCGAGGC
CCGCCGGGCCTTCATGGAGGAGTTTGGAGCCGAGCCGGAGCTGNTTNTGTCGGCGCCGGGC
CGCGTCAACCTCATCGGGGAGCACACGGACTACAACCAGGGCCTGGTGCTGCCCATGGCAC
TGGAGCTCGTGACCGTGATGGTTGGCAGCCCCCGGACAGATGGGCTTGTTTCTCTTCTCAC
CACTTCCAAAGATGCAGACGAGCCCCAAAGACTGCAGTTCCCACTGCCCTCAGCCCAGTGG
TCCTTGGAGCCTGGAATCCCACAGTGGGCCAATTATGTCAAGGGAGTGATTCAACATTACC
CAGCTTCCCCGCTCGTTGGCTTCAGTGCAGTGGTGGTCAGCTCAGTGCCCCTGGGGGGTGG
GCTTTCCAGCTCAGCGTCTCTGGAAGTGGCCACGTACACCTTCATCCAGCAGCTCTGCCCA
GACTCGGGGGCAATAGCTGCCCGGGCCCAGGTGTGTCAACGGGCTGAGCACAGCTTCGCAG
GGGTGCCCTGNGGCATCATGGACCAACTCATCGCGCTGCTGGGGCAGAAAGGCTATGCACT
ACTCATTGACTGCAGGNCCCTGGAAACAAGCCTGGGGCCACTGNCAGACCCCAAGCTGGNC
GGGCTCATCACAACTCAATGTCCGCCATTCCCTGGGCTCAGCGAGTACCCGGTTCGCGGCG
ACAGNGNGAAAANNGGCCCAGGCCCTGGGCANGAGAGC

Clone A43, encodes a 190 amino acids that showed 100% identity to BCL2-associated athanogene5 (Bag5), direct submission. Accession number: XM_127149.
>CGAATTCGCGGCCGCGTCGACCGGCTGCCCGGGTCAGACCTAGCCGGGAAGGGCGAGGGG
GGATGCCGCTGGGGGCACCGCTGCGGTGCCGCTCCCCGTGAANGTTNNATCTCCCACCTCG
CAGCCCCCCGCGGCAGCCAAGACCGCCGCCGCCCAGACCTCCCGGGTGCTTGTGAAACTGA
ACACAGAAGTATGGATATGGGAAACCAACACCCCTCCATTAGTAGGCTTCAGGAGATCCAG
CGGGAAGTAAAGGCTATAGAGCCGCAAGTGGTCGGCTTCAGCGGCCTGTCGGATGACAAGA
ACTACAAGAGGCTGGAGAGGATTCTGACCAAACAACTTTTTGAAATTGACTCTGTGGATAC
NGAAGGAAAAGGAGATATCCAGCAAGCTAGGAAGCGGGCGGCACAGGAGACAGAACGTCTC
CTCAAGGAGTTGGAGCAGAATGCCAACCACCCACACCGCATCGAAATCCAGAATATCTTTA
AGGAGGCTCAGGCCCTTGTGAAAGACAAGATTGTGCCCTTTTACAGCGGAGGCAACTGTGT
GACTGACGAGTTTGAGGAAGGCATCCAGGACAT

Clone A44, nil amino acid sequence identity.
>CGAATTCGCGGCCGCGTCGACAAATTTCCATGATCACACACTAATAATTGTTTTCCTAAT
TAGCTCCTTANTCCTCTATATCATCTCGCTAATATTAACAACAAAACTAACACATACAAGC
ACAATAGATGCACAAGAAGTTGAAACCATTTGAACTATTCTACCAGCTGTAATCCTTATCA
TAATTGCTCTCCCCTCTCTACGCATTCTATATATAATAGACGAAATCAACAACCCCGTATT
AACCGTTAAAACCATNGGGCACCAATGATACTGAAGCTACGAATATACTGACTATGAAGAC
CTATGCTTTGATTCATATATAATCCCAACAAACGACCTAAAACCTGGTGAACTACGACNGC
TAGAAGTTGATAACCGAGTCGTTCTGCCAATAGAACCTCCAATCCGTATATTAATTTCATC
TGAAGACGTCCTCCACTCATGAGCAGTCCCCTCCCTAGGACTTAAAACTGATGCCATCCCA
GGCCGACTAAATCAAGCAACAGTAACATCAAACCGACCAGGGTTATTCTATGGCCAATGCT
CTGAAATTTGTGGATCTAACCATAGCTTTATGCCCATTGTCCTAGAAATGGTTCCACTAAA
ATATTTCGAAAACTGATCTGCTTCAATAATTTAATTTAAAAAAAAAAAAAAAA

Clone A46, encoded 336 amino acids that showed 100% identity to NF2d9 (Sueyoshi *et al.*, 1995). Accession number: AAC52244.
>CGAATTCGCGGCCGCGTCGACTGGAAGTGGAATCGTCCAGGAGACAGGCTTCTCGATTTA
GATATTCCAATGTCTGTGGGAATAATTGACACAAGGACAAATCCAAGCCAGTTAAATGCAG
TTGAATTTCTGTGGGACCCTGCAAAGCGCACATCTGCCTTCATTCAGGTACATTGTATCAG
CACAGAATTCACCCCGCGTAAGCATGGAGGTGAAAAAGGAGTGCCTTTCAGGATCCAGGTT
GACACCTTTAAGCAGAATGAAAACGGGGAATACACAGATCATCTTCACTCAGCCAGCTGCC
AAATCAAAGTCTTTAAGCCTAAAGGTGCAGACAGAAAACAAAAAAATGACCGAGAAAAGAT
GGAGAAGAGAACAGCTCATGAAAAAGAAAAGTACCAGCCATCTTACGACACCACCATTCTC
ACAGAGTGTTCTCCATGGCCCGACACCCCTACAGCCTATGTGAACAACAGCCCTTCCCCAG

CGCCCACTTTCACCTCCTCACAACCGAGCACGTGCAGTGTCCCAGACAGCAATTCTTNATC
CCCAAATCACCAGGGAGATGGAGCTGCACAAGCGTCTGGGGAACAAATTCAGCCTTCAGCT
ACAACCCAAGGAAACACAGCAGTGGCTGCTCAAAAACANGTTCTCTTTCTACACAAGACTG
GTCTCTAATTTTTCA

Clone A49, encodes 193 amino acids that showed 100% identity to DnaJ (Hsp40) homologue, subfamily B, member 3 (Berruti *et al.*, 1998). Accession number: NM_008299.
>CGAATTCGCGGCCGCGTGACATCGGACGTCCGCAAGCGCGAGGTGTACGACCGCTGCGGC
GAAGTGGGCGAGGTGGGCGGAGGCGGCGCGGCGGGCAGCCCGTTCNNCGATGCCTTCCAGT
ACGTCTTCTCCTTCCGAGACCCCGCGGAGGTCTTCAGGGAGTTCTTCGGGGGCCACGACCC
TTTCTCCTTCGACTTCTTCGGGGGAGACCCCCTTGAGAACTTTTTTGGGGATCGGAGGAGC
ACCCGTGGAAGCAGAAGCCGAGGGGCTGTACCCTTCTCTACCTCCTTTACCGAATTCCCAG
GATTTGGGGGTGGCTTCGCTTCGTTAGATACTGGATTCACATCCTTCGGTTCTCCAGGAAA
CTCGGGCCTTTCTTCTTTCTCCATGTCTTGCGGCGGTGGGGCGGCAGGCAACTACAAGTCG
GTGTCAACCTCCACCGAAATAATTAATGGCAAAAAAATCACCACCAAGAGAATCGTTGAGA
ATGGTCAAGAAAGGGTGGAAGTGGAAGAAGATGGAGAGTTAAAGTCCCTGATAATTAATGG
CAAAGAGCAGCTGCTTCGCATCAATACTCAGTAAAGACTAAAGTGTCTTTTAAGGACTATC
ANGACTTTTTTGTTTAAACGCGTTTTCAANGGAACTCTACTTTCAGAACAACTGTATTCCA
GACATTATATGCAGCTCATGTCGGTGCCTCTTG

Clone A50, encodes a 167 amino acids that showed 100% identity to DnaJ (Hsp40) homolog, subfamily B, member 10 (Dnajb10), direct submission. Accession number: NM_020266.
>CGAATTCGCGGCCGCGTCGACACGGGCCCTTTCTTCACTTTCTCTTCTTCCTTTCCTGCC
AACTCCGATTTCTCCTCCTCATCTTTCTCCTTCAGCCCGGGGGCTGGTGCTTTCCGNTCCG
TTTCTACGTCCACCACCTTTGTCCAAGGCCGCCGCATCACCACACGCAGAATCATGGAGAA
CGGGCAGGAGCGGGTAGAAGTGGAAGAGGATGGACAACTGAAGTCAGTGTCAATCAATGGT
GTCCCAGATGACCTGGCACTAGGCTTGGAGCTGAGCCGTCGTGAGCAGCAACCTTCAGTTG
CCCCTGGGCTGGGGGTCATGCAGGTCCGGCCGACCTCTCTCTCTCGTCCCCCTGACCATGA
TCTTTCTGAGGATGAGGACCTGCAGCTCGCCATGGCTTACAGCCTGTCAGAGATGGAGGCG
GCTGGGCAGAAGCCAGCAGCCCAGGAAGATGCTGGGGCCAGTGGGTCCATAAGATCTTCTG
GGGAGGCTAGCTAGGTGGGGTGGAAGCCTCTCAGCCCTCCAGAGTCTCAACCAGAGTCAAC
TCCTCTTCCCTTCTTGCTGCCTCAGCCTGCCCTGGCCACAGGACTAGGCAGAGATGTGTTC
TGAGCTGGATATCCTGGATACAGAGTTGCTGCAGTTCCAACAGGACAGCGCCCTCCCCAGT
GTGCTAGGANGGAACACTACATTCCTCTCCCTCATTTATGCTGAGTGTAAAGCTGGGGTTG
GGGTGGGGGTGAGACTGA

Clone A54, encodes 192 amino acids that showed 67% identity to hypothetical protein, PR01741.
>CGAATTCGCGGCCGCGTCGACGGAAACAGCAGAGACTCAGTGATGGCTGTCACGACCGGA
GAGGGGACTCTCAGCCTTTCCAGGCACTTAAGTATTCTTCAAAGAGTCACCCCAGTAGTGG
TGATCACAGACATGAAAAGATGCGAGACGCCGCAGATCCTTCACCACCAAACAAAATGTTG
CGGAGATCTAATAGTCCTGAAAACAAATACAGTGACAGCACAGGTCACAACAAGGCCAAAA
ACGTGCACACTCAGAGAGTTAGAGAAAGGGACGGTGGGACCAGTTACTCTCCACAAGAAAA
CTCACACAACCACAGTGCACTTCATAGTTCAAATTCACATTCTTCTAATCCAAGCAATAAT
CCAAGCAAAACTTCAGATGCACCTTATGATTCTGCAGATGATTGGTCTGAGCACATTAGCT
CATCTGGAAAAAAGTACTACTACAATTGTCGCACAGAAGTTTCACAGTGGGAGAAACCAAA
AGAGTGGCTTGAAAGAGAACAGAGACAAAAAGAAGCAAATAAGCTGGCAGTTAATAGTTTC
CCAAAAGACAGGGATTACAGAAGAGAGGTGATGCAAGCAACAGCCACTAGTGGGTTCACCA
GTGGAATGGAAGACAAGCATTCCAGCGATGCCAGTAGTTTGCTCCCACAGAATATTTTGTC
TCAAACAAGCAGACACAATGACAAAGACTACAGACTGGCAAGAGCAGAGACTCACAGTAGC
T

Clone A56, nil amino acid sequence identity.

>CGAATTCGCGGCCGCGTCGACCGCAGGTAAACGAGCAGCGAAACCTGGAGCAGTCGGGTG
CTGGTCTCCTCGCAGCCGGGATCCGCTTCAGCCGGCCACTTCATCCTCTCTCTCCTTTCTT
GTTCAGTCCCCACTACGGAGTTCTGTCCCCTACCTGTTAGCAATGACACCCCACACCCCCA
CCCCACCCCCGCTCCCCGAGTATTTTATGTTACTAAGGAATTATCGTCATCCTTATCGAAC
GTGCTTACGGTAATGTGGCTTTAAATTTAAAACACACACACACACACACACACACACTAAA
GTGTTGATAGGTAAAATGATCCAATACGTTTATTTTGTTTTAGACTAAGCCTCTGGAAACT
GGGAAAGAAGGGAATAAATTAACTGAGGATGTCCTTCGATGTTGGAGCTGTGGGATATGTA
CACATATGCTATCATACCAGTATTTATGTCTCAAATTGTTCATAATAAAGTTTTGGTTTGG
GGGGAAAAAAAAAAAAAAAAA

Clone A57, encodes a 153 amino acids that showed 99% identity to the hypothetical protein AL136561 and 67% identity to Antennal specific membrane protein, direct submission. Accession number: NP_493948.

>CGAATTCGCGGCCGCGTCGACATTCAGTCCACACCTCTGAACTTGGCGGTGAACTGGCGC
TGTGAGCCTGCGAGCACTGACCTGCGCATAGACTATAAGTACAACACGGATGCCATGTCCA
CTGCAGTGGCCCTCAACAACGTGCAGTTCCTGGTCCCCATAGACGGAGGAGTGACCAAGCT
TCAGGCTGTGCTTCCTCCAGCAGTCTGGAATGCTGAACAACAAAGAATATTATGGAAGATT
CCCGATATCTCCCAGAAGTCAGAAAATGGAGGCGTAGGTTCTTTACTGGCGAGATTTCAGT
TATCCGAAGGCCCGAGCAAACCTTCCCCGCTGGTTGTGCAGTTCACAAGTGAAGGAAGTAC
TCTCTCTGGCTGTGACATTGAACTTGTTGGAGCAGGCTACCGGTTTTCACTCATTAAGAAG
AGGTTTGCTGCAGGAAAGTACTTGGCAGATAACTAATAAAAGGTCATGCAAGGATTTGGAA
GATCAAAAAAAAAAAAAAAAA

Clone A58, encoded 122 amino acids that showed 100% identity to the hypothetical protein A930031F18, direct submission.

>CGAATTCGCGGCCGCGTCGACCGGCAACCGGCGAGGAAACGGCTGGCGTCTGTGCAGTGG
GCGCCTGGGGACCCTCGGAGCGAGCGGGCGCGTGATCGAGCTAAGGCCGGCGACCCTTCTC
CGCGAACTTCGGACACGGAGTGCATGAGTCAGAGATGGCTCTCCCCACGTTGCCCTCTTAT
TGGTCCAGCCGGAAACACATGGATCTTCGCCAGCGCCAGCATCAGCAGGAGGACCAGTTTC
GCCAGCAGTGGGAACAGAACAGTCGCTATTTCAGGACCTGGGACATCCACAACTCGAAACA
GATCGAATGGAGCTCCAAGATCTCCTACCAGCGGAGCATGCACGCCTACCATTGTGAGAAG
ATGAAGGAGGAGAAGAGGAAGATTCTGGAGCTCCGGCGGGAGAGGCTCAGAGAGCTTCTCC
TGGAGGAACAGGACTTGCTGGCTGCGGAGCTGGATGAGCTCAGGCTGAGCATGGGCCTGCG
GGAGCAAAGGCTCCGCGAGCAGCACCAGGACCTGAAGTCAGCCCGAGAAGAACAGAGGAAG
CTGATTGCTGAACGACTTTTACATGAACACTGGAAGAAGAACAACCCCAAACTTCGAGAGC
TTGAGTTGGACCTTCACAAGAAGCACGTGATAAATTCTTGGGCAACACAGAAAGAAGAAAA
AAAA

Clone A59, encoded 651 amino acids that showed 100% identity to PIAS1 (Liu *et al.*, 1998). Accession number: AAC36701.

>CGAATTCGCGGCCGCGTCGACGCGGACAGTGCGGAACTAAAGCAAATGGTTATGAGCCTT
AGAGTTTCTGAACTCCAAGTACTGTTGGGCTACGCTGGGAGGAACAAGCACGGACGCAAAC
ACGAACTTCTTACAAAAGCCCTGCATTTGTTAAAGGCTGGCTGTAGTCCTGCTGTACAAAT
GAAAATTAAAGAACTCTACAGGAGGCGGTTCCCTCAGAAAATTATGACGCCTGCGGACTTG
TCTATCCCCAACGTACATTCAAGTCCTATGCCTNCGACTCTTTCTCCATCCACCATTCCAC
AGCTCACTTATGATGGCCACCCTGCATCATCCCCACTACTCCCTGTTTCTCTTCTGGGACC
CAAACATGAACTGGAACTCCCACATCTCACGTCAGCGCTGCACCCAGTCCACCCGGACATA
AAGCTGCAGAAGCTACCATTCTATGACCTGTTGGATGAACTGATCAAGCCCACCAGTCTAG
CTTCAGACAACAG

Clone A62, nil amino acid sequence identity.

>CGAATTCGCGGCCGCGTCGACCAGGGCAGCAGTGTCCCCTCAGAAGAGAAAAGCAGACGG
ACCCTGACCCTGCATTTACTCTCGCTCGGAGGGTCCTGGGAGCAGCTGGTACCTCACGCAG
GATTAACATTTCTCTGCAAAGGCAGGGGGTTGCCTAGTAGCTCTTCAGTGTTGCTGCTGGC
CCCTGACCCTCTGCCACCACTGGCCTGGACTCCTCTGCTCTCCTTGGGATCTGCACAACTG
TGGCCCTGAAGCCGACCTGGCCAAGCAAGACTGTGGTCTCCATCCCTGACCAGCCCACTCC
CTCCCAAAGAATAACTTCTTCAGCGTCCCCCCACAGAGAGACCAGGCCTCATCCAGAGTCA
CAGCAGCAGTCCTCCTGACCTTGAACCCTGGCCCTCAGCCACTGAGCCAGGCCCAACTGTA
GGAGGAACTCTCAAGCAGACATGTTGGGCCTTGTCCACCCTGACTGCCGCTGGAGAGGACG
GCTTCCCAGGCTTTCTCAGGGCTATTCCTGGNGTCTGCCTCTCAGATTCCAAAGACTGGAA
TAAAATTTAACCTGGGAAA

Clone A66, tetratricopeptide repeat domain 1, clone MGC:7871, direct submission. Accession number: BC010236.

>CGAATTCGCGGCCGCGTCGACGAAAAGTCAGAAGACTGTAAGGTCCCAGAAGATCTGTTC
AATGGTCTGAAAGTCGCAGATCCTCAAGAAGGAGAGTCTGCCAGCCCCATGGTTTCTGATC
CCAAGGGTCAGCACTGCCAGAGCAAGCTGCCGAAGGCTGCTGAAGCCCATCCACAGGACGA
CCATGTTGAAGAGGAATGTTTTCATGACTGTAGTGCCTCGTTTGAGGAGGAACAGCCAGGG
GCGCACGTGGCAGGGAGCAAAGCCAGCGATGATTCTAGTTCTGAACTGGATGAAGAATACC
TAATTGAACTGGAAAAAAACATGCCAGAAGAAGAGAAACAGAAAAGAAGAGAGGAGAGCGC
TAAACTAAAGGAGGAGGGGAATGAGCGGTTTAAGAGAGGAGATTACATGGAAGCTGAGAGT
TCTTACAGTCAAGCCCTTCAGATGTGCCCAGCCTGTTTCCAGAAAGATCGATCTGTTCTGT
TTTCAAATAGAGCTGCTGCGAGGATGAAACAGGACAAGAAGGAGACGGCCATCACTGACTG
CAGCAAAGCAATTCAGTTAAACCCCACCTATATCCGTGCAATACTGAGGAGAGCTGAATTA
TATGAGAAAACAGACAAACTAGATGAAGCACTGGAAGATTATNAATCAGTATTAGAAAAGA
TCCATCAGTACATCAAGCAAGAGAGCTTGTATGAGATTACCTAAGC

Clone A68, encoded 651 amino acids that showed 100% identity to PIAS1 (Liu *et al.*, 1998). Accession number: AAC36701.

>CGAATTCGCGGCCGCGTCGACGCGGACAGTGCGGAACTAAAGCAAATGGTTATGAGCCTT
AGAGTTTCTGAACTCCAAGTACTGTTGGGCTACGCTGGGAGGAACAAGCACGGACGCAAAC
ACGAACTTCTTACAAAAGCCCTGCATTTGTTAAAGGCTGGCTGTAGTCCTGCTGTACAAAT
GAAAATTAAAGAACTCTACAGGAGGCGGTTCCCTCAGAAAATTATGACGCCTGCGGACTTG
TCTATCCCCAACGTACATTCAAGTCCTATGCCTCCGACTCTTTCTCCATCCACCATTCCAC
AGCTCACTTATGATGGCCACCCTGCATCATCCCCACTACTCCCTGTTTCTCTTCTGGGACC
CAAACATGAACTGGAACTCCCACATCTCACGTCAGCGCTGCACCCAGTCCACCCGGACATA
AAGCTGCAGAAGCTACCATTCTATGACCTGTTGGATGAACTGATCAAGCCCACCAGTCTAG
CTTCAGACAACAGCCAGCGCTTTCGGGAAACCTGTTTTGCATTTGCCTTGACACCACAACA
GGTGCAGCAGATCAGCAGCTCCATGGATATTTTCTGGGACCAAATGTGACTTCACAGTGCA
GGTCCAATTAANGGTTTGTTTATCAGAAACCAGTTGTCCACAAGAAGATCACTT

Clone A73, nil amino acid sequence identity.

>CGAATTCGCGGCCGCGTCGACGGGAAGCCTTCCTTCCTGAGGCAAAGGCCATCTCCTGAG
GGACCAAGGACACCAGAGCCTACCTTCGTGACGCTGCCTTTTTATACACTGTCCTTCTTCC
TCGATGGACCCTAACTGATGCCCCTGCCTTGGCTGGACTTAGCCCTTCCACTCTGCCCCAC
AGACAAACTGTTCCCAGTTACTCTAACCAACATTTCATTTAGCTTCCACGTATATTTTCTT
ACCTAAGAGAATAGTTTCCTGCTTTAAGCAAAAGACCTACAATAGGTGGTGGAATTATAGG
TTGGGGTGGAGTGTTGATATAAATATATAAATACAAATGTATATTTTTCAGGATGTGGTTT
AGGAACTGGGAATAACGTTTTCTGTTACTCCTGATGGTGCCATGAAAGATTATGTAATAAA
ATATTTGAAAATCA

Clone B4, encodes 204 amino acids that showed 100% identity with T complex testis-specific protein 2 (TCTEX-2) (Huw *et al.*, 1995). Accession number: P11985.

>CGAATTCGCGGCCGCGTCGACCCCGGTGAGCCGATCAAGATGGAGCGGCGAGGCCGAATG
GCGAAGACGCCCACCGGCCAAACGCATCAATCCCCGGTGTCTAAGAGNGAAAGGAAGCCTA
GCATGTTCGAGAAGGAGTCATATGCACAGATCTTAAGAGAAAGACTGAGAGAGTCTTTTCA
TGATGTTCAGTACGTGGAACCTCCGTTTGATGACTCAATTGCTGATGTAGGCAAAGAATGG
AAAAGTGCCCTGGCAAAATTAAAGTTTGCTAATTCATACAGAATGGAGCCACTGAAGAAAT
TTCAAGCACATTTGGTAGAAACTAAAATCCAGCAGATATTAAAGGACAGTCTTAAAGATGT
CAAATATGATGACAAAGCCCCTCATTTGTCACTTGAATTGGCAGATCGAATATTGGCAGCA
GTCAAAGAATTTGCATACCATCGTTATAAATTCATTATACAAGTATTATTTATTCAAAAGA
CTGGTCAAGCAATAAATATTGCCAGCAGATGGATCTGGGATGTGGCATGGGACAACTGGGT
AGAAGCTAAACATGAAACAGAGTCTTACGTGGTATTGGCCTTGGTGTTTGCTCTCTATTGT
GAATAGCTCAGGACCAGCATTTTCACCCCCCATCCTTCAAAATAAATGATATATACAAAAA
AAAAAAAAAAAA

Clone B5, nil amino acid sequence identity.

>CGAATTCGCGGCCGCGTCGACGTGAGGGTGTTTCGACGCGCTGGGCGGTTTGTGCTTTCA
TCACATTTGTTAACAGGTCAAAATGCAGATCTTCGTGAAGACCCTGACCGGCAAGACCATC
ACCCTAGAGGTGGAGCCCAGTGACACCATCGAGAACGTGAAGGCCAAGATCCAGGATAAAG
AGGGCATCCCCCCTGACCAGCAGAGGCTGATCTTTGCCGGCAAGCAGCTGGAAGATGGCCG
CACCCTCTCTGATTACAACATCCAGAAGGAGTCAACCCTGCACCTGGTCCTTCGCCTGAGA
GGTGGCATGCAGATCTTCGTGAAGACCCTGACCGGCAAGACCATCACCCTGGAGGTGGAGC
CCAGTGACACCATCGAGAACGTGAAGGCCAAGATCCAGGATAAAGAGGGCATCCCCCCTGA
CCAGCAGAGGCTGATCTTTGCCGGCAAGCAGCTGGAAGATGGCCGCACCCTCTCTGATTAC
AACATCCAGAAAGAGTCAACCCTGCACCTGGTCCTCCGTCTGAGGGGTGGCTATTAATTAT
TCGGTCTGCATTCCCAGTGGGCAGTGATGGCATTACTCTGCACTCTAGCCACTTGCCCCAA
TTTAAGTTTAGAAATTACAAGTTTCAATAATAGCTGAACCTCTGNTAAAAATGTTAATAAA
GGTTTTGTTGCATGGTAAGCNAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Clone B8, encodes 208 amino acids that showed 100% identity with T complex testis-specific protein 2 (TCTEX-2) (Huw *et al.*, 1995). Accession number: P11985.

>CGAATTCGCGGCCGCGTCGACCGGAGGCGTCCCGGTGAGCCGATCAAGATGGAGCGGCGA
GGCCGAATGGCGAAGACGCCCACCGGCCAAACGCATCAATCCCCGGTGTCTAAGAGAGAAA
GGAAGCCTAGCATGTTCGAGAAGGAGTCATATGCACAGATCTTAAGAGAAAGACTGAGAGA
GTCTTTTCATGATGTTCAGTACGTGGAACCTCCGTTTGATGACTCAATTGCTGATGTAGGC
AAAGAATGGAAAAGTGCCCTGGCAAAATTAAAGTTTGCTAATTCATACAGAATGGAGCCAC
TGAAGAAATTTCAAGCACATTTGGTAGAAACTAAAATCCAGCAGATATTAAAGGACAGTCT
TAAAGATGTCAAATATGATGACAAAGCCCCTCATTTGTCACTTGAATTGGCAGATCGAATA
TTGGCAGCAGTCAAAGAATCTGCATACCATCGTTATAAATTCATTATACAAGTATTATTTA
TTCAAAAGACTGGTCAAGCAATAAATATTGCCAGCAGATGGATCTGGGATGTGGCATGGGA
CAACTGGGTAGAAGCTAAACATGAAACAGAGTCTTACGTGGTATTGGCCTTGGTGTTTGCT
CTCTATTGNGAATAGCTCAGGACCAGCATTTTCACCCCCCATCCTTCAAAATAAATGATAT
ATACAAAAAAAAAAAAAAAAA

Clone B17, nil amino acid sequence identity.

>CGAATTCGCGGCCGCGTCGACACACGGCGGAGGGCGGAGGGCGGAGGGCTGGAGGCGTCC
CGGTGAGCCGATCAAGATGGAGCGGCGAGGCCGAATGGCNAANACTNTTTCCGGCCNGNCN
CATCAATCCCCGGNGTCTAAGAGAGAAAGGAAGCCTANCATGTTCGAGAAGGAGTCATATG
CACAGATCTTAAGAGAAAGACTGAGAGAGTCTTTTCATGATGTTCAGTACGTGGAACCTCC
GTTTGATGACTCAATTGCTGATGTAGGCAAAGAATGGAAAAGTGCCCTGGCAAAATTAAAG
TTTGCTAATTCATACAGAATGGAGCCACTGAAGAAATTTCAAGCACATTTGGTAGAAACTA
AAATCCAGCAGATATTAAAGGACAGTCTTAAAGATGTCAAATATGATGACAAAGCCCCTCA
TTTGTCACTTGAATTGGCAGATCGAATATTGGCAGCAGTCAAAGAATTTGCATACCATCGT
TATAAATTCATTATACAAGTATTATTTATTCAAAAGACTGGTCAAGCAATAAATATTGCCA
GCAGATGGATCTGGGATGTGGCATGGGACAACTGGGTAGAAGCTAAACATGAAACAGAGTC

TTACGTGGTATTGGCCTTGGTGTTTGCTCTCTATTGTGAATAGCTCANGACCAGCATTTTC
ACCCCCATNCTTCAAATAATGATTTTACAGAAAAAAAAAA

Clone B27, nil amino acid sequence identity.
>CGAATTCGCGGCCGCGTCGACGCAAGCTGCTCTATAGCGTTTGTTTGGAGGAGCTTGGAG
CAGAGAGGTCCTGCTGTGCCTTCTCCCAAAGATGGAGATGTAAATGAAGAAATGCAGATGG
AGCATGGACAGAAACACTGACAGAGATCCGCAGAAGCAAGAGAACAAGAAGACCATGAATG
ACCTCGCCCAAACCTCACAGGATGGATTGCTGGTTACACATGTTAACCAGACCCAGGACTT
ACTGATTCATTCCGTGTACAAATGACCCTCTACCTAAGATCATGGACAACTCTTGAATTCT
CTCTACCATGGAAGCTATCATCCCTACAAGTTGTAAGTGGAAGCAGCTGCAGGGACGTGAG
ACCCAGACGTCGGCTCTGGAAGACTCTGAAGACTGGCTTGCAATGCACAGTTTAAAGTTCG
AGAAGCTCACCTTGGCTGACTTGATAAGCCAGGGCACAGTTGAGCTGGAGGATTGTAACAA
TGCTGTGCCCAAAGTGCATTTCACCACCCAGACCATCCACCACTTCAAGTCAAGGCTTTCT
GACACTATTGAACTCTATCAGCAGAGGATGCGGTGGCTCACAGAGAACAGCAAGAAGGCAT
TTGGCCTCATCAAGGGATCAAGAGTGGGCCTCCTCATAGATTCCTCGCAGGTCAGCAGCGG
CTCTCAGACAAAGGAATTCCAAATGACCTCACAGGTCTCATTGATGAGCAGTTGAGCCTCA
AGAAAA

Clone B28, encodes 216 amino acids that showed 100% identity with T complex testis-specific protein 2 (TCTEX-2) (Huw *et al.*, 1995). Accession number: P11985.
>CGAATTCGCGGCCGCGTCGACACACGGCGGAGGGCGGAGGGCGGAGGGCGGAGGCGTCCC
GGTGAGCCGATCAAGATGGAGCGGCGAGGCCGAATGGCGAAGACGCCCACCGGCCAAACGC
ATCAATCCCCGGTGTCTAAGAGAGAAAGGAAGCCTAGCATGTTCGAGAAGGAGTCATATGC
ACAGATCTTAAGAGAAAGACTGAGAGAGTCTTTTCATGATGTTCAGTACGTGGAACCTCCG
TTTGATGACTCAATTGCTGATGTAGGCAAAGAATGGAAAAGTGCCCTGGCAAAATTAAAGT
TTGCTAATTCATACAGAATGGAGCCACTGAAGAAATTTCAAGCACATTTGGTAGAAACTAA
AATCCAGCAGATATTAAAGGACAGTCTTAAAGATGTCAAATATGATGACAAAGCCCCTCAT
TTGTCACTTGAATTGGCAGATCGAATATTGGCAGCAGTCAAAGAATTTGCATACCATCGTT
ATAAATTCATTATACAAGTATTATTTATTCAAAAGACTGGTCAAGCAATAAATATTGCCAG
CAGATGGATCTGGGATGTGGCATGGGACAACTGGGTAGAAGCTAAACATGAAACAGAGTCT
TACGTGGTATTGGCCTTGGTGTTTGCTCTCTATTGTGAATAGCTCANGACCAGCATTTTCA
CCCCCCATCCTTCAAAATAAATGATATATACAGAAAAAAAAAAAAAAA

Clone B40, encodes 240 amino acids that showed 100% identity with T complex testis-specific protein 2 (TCTEX-2) (Huw *et al.*, 1995). Accession number: P11985.
>CGAATTCGCGGCCGCGTCGACGGCGGAGGGCGGAGGCGTCCCGGTGAGCCGATCAAGATG
GAGCGGCGAGGCCGAATGGCGAAGACGCCCACCGGCCAAACGCATCAATCCCCGGTGTCTA
AGAGAGAAAGGAAGCCTAGCATGTTCGAGAAGGAGTCATATGCACAGATCTTAAGAGAAAG
ACTGAGAGAGTCTTTTCATGATGTTCAGTACGTGGAACCTCCGTTTGATGACTCAATTGCT
GATGTAGGCAAAGAATGGAAAAGTGCCCTGGCAAAATTAAAGTTTGCTAATTCATACAGAA
TGGAGCCACTGAAGAAATTTCAAGCACATTTGGTAGAAACTAAAATCCAGCAGATATTAAA
GGACAGTCTTAAAGATGTCAAATATGATGACAAAGCCCCTCATTTGTCACTTGAATTGGCA
GATCGAATATTGGCAGCAGTCAAAGAATTTGCATACCATCGTTATAAATTCATTATACAAG
TATTATTTATTCAAAAGACTGGTCAAGCAATAAATATTGCCAGCAGATGGATCTGGGATGT
GGCATGGGACAACTGGGTAGAAGCTAAACATGAAACAGAGTCTTACGTGGTATTGGCCTTG
GTGTTTGCTCTCTATTGTGAATAGCTCANGACCAGCATTTTCACCCCCCATCCTTCAAAAT
AAATGATNTATACAAAAAAAAAAAAAAAA

Clone B54, nil amino acid sequence identity
>CGAATTCGCGGCCGCGTCGACGAAGATGTGGAATTTGCGGTGACTTTTCAGAATTGAATA
ATCTGGACTGTGGGCAGGGCAGTGCTTTCCTGCTGATTCTGCCCACCCTTGAGTACGCAAG
AAATTGGTTCCCGAACCTCTCGGTTCTCAAAATCTGAAGATACTTTATAAAGTGATGACAC
AGATTTTGCCTGTCAACCTACATTCATCTTTGTATTTTTAGTCATCCCCAGATTACCTCCA
AGAATCTACTACAACAAAAGCATCATCTGAGAGTCCTCAGACTGCGCTGCTTAGGGGATAA

TGGCAATTAAACCAGGCTTAATACCCTTACTGTGCCAGTTTCCTTCAGAAAACTGTTCATC
CAGGCTAGCTAAGTCACACAAGTGGGGCTCGCGCATGCAGTCAGGAGAATTATAAAGTGAT
TAAACTATGTGTGCAGCATAAATATTGTATATTGTTGCATGAATTTGGGTGTGGATCTTTG
TNAGAACACAATGAATTCTTTGTTGGGTTTTGATCTTTGAGACATTTTTTTTTTCTCAGTA
TAGCCCTGGAACTCACTTTGNAGATCNATCCTGGCCTCTGTCTCAGAGATCTGCCTCTGCC
TCTGNCTCTGCCTCTGCCTTTGCCTCTGCCTCTGCCTCTGCCTCTGCCTCTGCCTCTGCCT
CTGCCTCTGCCTTGCCTCTGCCTCTGCCTTTGCCTCTGCCT

Clone B73, nil amino acid sequence identity
>CGAATTCGCGGCCGCGTCGACACACNGGCGGAGGGCGGAGGGCGGAGGGCGGAGGCGTCC
CGGTGAGCCGATCAAGATGGAGCGGCGAGGCCGAATGGCGAAGACGCNCACCGGCCAAACG
CATCAATCCCCGGTGTCTAAGAGAGAAAGGAAGCCTAGCATGTTCGAGAAGGAGTCATATG
CACAGATCTTAAGAGAAAGACTGAGAGAGTCTTTTCATGATGTTCAGTACGTGGAACCTCC
GTTTGATGACTCAATTGCTGATGTAGGCAAAGAATGGAAAAGTGCCCTGGCAAAATTAAAG
TTTGCTAATTCATACAGAATGGAGCCACTGAAGAAATTTCAAGCACATTTGGTAGAAACTA
AAATCCAGCAGATATTAAAGGACAGTCTTAAAGATGTCAAATATGATGACAAAGCCCCTCA
TTTGTCACTTGAATTGGCAGATCGAATATTGGCAGCAGTCAAAGAATTTGCATACCATCGN
TATAAATTCATTATACAAGTATTATTTATTCAAAAGACTGGTCAAGCAATAAATATTGCCA
GCAGATGGATCTGGGATGNGGCATGGGACAACTGGGTAGAAGCTAAACATGAAACAGAGTC
TTACGTGGGATTGGCCTNGGGGNTNGCTCTCTATTGGGAATAGCTCANGACCAGCATTTTC
ACCCCCATCCTTCAAAATAAATGATATATACAGAAAAAAAAAAAAAAAAA

Clone B77, encodes a 231 amino acids that showed 100% identity to succinate dehydrogenase complex, subunit B (Carninci *et al.*, 2000). Accession number: AK003052.
>CGAATTCGCGGCCGCGTCGACCGAATGCAGACGTACGAGGTGGATCTGAATAAGTGCGGA
CCTATGGTGTTGGATGCTTTAATCAAGATCAAGAATGAAGTGGATTTTACTTTAACCTTCC
GAAGATCTTGTAGAGAAGGCATCTGTGGCTCTTGCGCCATGAACATCAACGGAGGCAATAC
TCTGGCGTGCACACGCAGGATCGACACGGACCTCAGCAAAGTCTCCAAAATCTACCCTCTT
CCACATATGTATGTGATCAAGGATCTAGTCCCTGATTTGAGTAACTTCTACGCACAATACA
AATCCATTGAGCCTTATCTGAAGAAGAAGGATGAGTCCCAGGAGGGCAAGCAACAGTATCT
GCAGTCCATCGAGGACCGGGAGAAGCTGGACGGGCTGTACGAGTGCATCCTGTGTGCCTGC
TGCAGCACCAGCTGCCCCAGCTACTGGTGGAACGGAGACAAGTACCTGGGGCCTGCAGTTC
TCATGCAGGCCTATCGCTGGATGATCGACTCCAGAGACGACTTCACAGAGGAACGCCTGGC
CAAGCTGCAGGACCCCTTCTCTGTCTACCGCTGCCACACCATCATGAACTGCACACAGACC
TGCCCCAAGGGTCTGAATCCNGGGAAAGCGATTGCGGAAATCAAGAAGATGATGGCGACCT
ACAAGGAGAAGCGAGCATTGGCTTAACCCAGTCCGTGCTGAGTGNGCCTGNCAACTCTGGG
GCNAACAGCTGGAGTCTCCCTAAGTNCAGCATATGTAATAACATTTAAGATAAAAAAAAAA
AAAAAAA

Clone B87, encodes 334 amino acids that showed 100% identity to FKBP4 (Schmitt *et al.*, 1993). Accession number: P30416.
>CGAATTCGCGGCCGCGTCGACAACGCCACACTTGTATTTGAGGTGGAGCTGTTTGAGTTC
AAAGGAGAAGATCTTACAGAAGAAGAAGATGGCNGGGATCATCCGCAGAATACGGACTCGG
GGTGAAGGCTATGCCAGGCCCAATGATGGTGCTATGGTGGAAGTGGCCCTGGAAGGCTACC
ACAAGGACCGCCTCTTTGACCAGCGGGAGCTCTGCTTTGAAGTCGGGGAAGGGGAAAGTCT
AGATCTGCCCTGTGGGCTGGAGGAGGCCATTCAGCGCATGGAGAAAGGAGAGCATTCCATC
GTGTACCTCAAACCTAGCTATGCTTTTGGCAGTGTGGGGAAGGAGAGGTTCCAGATCCCAC
CGCACGCTGAGCTGAGGTATGAAGTGCGGCTGAAGAGCTTTGAGAAGGCCAAGGAGTCTTG
GGAGATGAGCTCCGCGGAGAAGCTGGAGCAGAGCAACATAGTGAAAGAGAGGGGCACCGCG
TACTTCAAGGAAGGCAAGTACAAGCAGGCGTTACTGCAGTACAAGAAGATCGTGTCTTGGC
TAGAATACGAGTCTAGCTTCTCCGGTGAGGAAATGCAAAAGGTCCATGCACTCCGACTGGC
CTCACACCTCAATCTGGCCATGTGTCACCTGAAACTGCANGCCTTCTCAGCTGCCATCGAA
AGCTGCAACAAGGCCTTGGAGCTGGACAGCAACAACGAGAAGGCCTGTTTCGCCCGGGAGA
GCCCACCTGGCCGTGAATGACTTTGACCTGGCAAGAGCTGACTTCAAAAAGTCCTGCAGCT
NTTTCCANCACAAAGCCGC

Clone B101, encodes 229 amino acids that showed 100% identity with T complex testis-specific protein 2 (TCTEX-2) (Huw *et al.*, 1995). Accession number: P11985.

>CGAATTCGCGGCCGCGTCGACTGCTCCGCGGCATCTGAGCAGGGCCGTTGGGCAGTTACA
CGGCGGAGGGCGGAGGGCGGAGGGCGGAGGCGTCCCGGTGAGCCGNTCAAGATGGAGCGGC
GAGGCCGAATGGCGAAGACGCCCACCGGCCAAACGCATCAATCCCCGGTGTCTAAGAGAGA
AAGGAAGCCTAGCATGTTCGAGAAGGAGTCATATGCACAGATCTTAAGAGAAAGATTGAGA
GAGTCTTTTCATGATGTTCAGTACGTGGAACCTCCGTTTGATGACTCAATTGCTGATGTAG
GCAAAGAATGGAAAAGTGCCCTGGCAAAATTAAAGTTTGCTAATTCATACAGAATGGAGCC
ACTGAAGAAATTTCAAGCACATTTGGTAGAAACTAAAATCCAGCAGATATTAAAGGACAGT
CTTAAAGATGTCAAATATGATGACAAAGCCCCTCATTTGTCACTTGAATTGGCAGATGGAA
TATTGGCAGCAGTCAAAGAATTTGCATACCATCGTTATAAATTCATTATACAAGTATTATT
TATTCAAAAGACTGGTCAAGCAATAAATATTGCCAGCAGATGGATCTGGGATGTGGCATGG
GACAACTGGGTAGAAGCTAAACATGAAACAGAGTCTTACGTGGTATTGGCCTTGGTGTTTG
CTCTCTATTGTGAATAGCTCA

Clone B102, encoded 264 amino acids that showed 100% identity to the putative protein AK016993, direct submission.

>CGAATTCGCGGCCGCGTCGACCGCGGCCGCGTCGACCCGCGTCCTGGGGCCCAGAAGTCA
TCCGGAGCAGTAACCAAGAAGGGGGATCGGGCTGCTAAAGATAAGACAGCAAGCACCCTGC
CCCCGGTGGGAGAGGACGAGCCAAAAAATCCGGAGGAGTACCAGTGCACGGGGGTCCTGGA
GACAGACTTCGCTGAGCTCTGCACACGGTCAGGCTACGTGGACTTTCCCAAAGTTGTCACC
CGGCCACGGGTCCAACAGAGCTCTGTCCCTTCCGCCTCCACGTCAGAAAAACCTGTCCTAG
ATGACCAGAGACCGTCGGCATCCTGCAGCCAGAGCAGCCTGGAGAGCAAGTATGTGTTTTT
TCGGCCCACTATTCAAGTGGAGCTGGAGCAGGAGGACAGCAAGGCCGTGAAGGAGATCTAC
ATTCGAGGCTGGAAAGTTGAAGATCGGATTCTGGGTATTTTCTCCAAATGTCTGCCCTCCC
TCAGCCAGCTGCAGGCCATCAACTTGTGGAAGGTGGGGCTGACGGATAAGACCCTGACCAC
TTTCATCGCCCTTTTGCCTCTTTGTTCCTCCACACTCAGGAAGGTATCTCTGGAGGGGAAC
CCGATACCAGAGCAGTCCTTTAGTAAGCTGATGGGACTGGACAGCACGATTGTTCACTTGT
CTCTGAGGAACAACAACATCAACGACCACGGGGCGCAGCTCCTGGGCCA

Clone C1, encodes 167 amino acids that showed 100% identity to a protein similar to eukaryotic translation elongation factor 1alpha, direct submission. Accession number: BC003969.

>CGAATTCGCGGCCGCGTCGACCTTCCTGGGGACAATGTGGGCTTCAATGTAAAGAACGTG
TCGGTCAAAGATGTTAGACGAGGCAATGTTGCTGGTGACAGCAAAAACGACCCACCAATGG
AAGCAGCTGGCTTCACTGCTCAGGTGATTATCCTGAACCATCCAGGCCAAATCAGTGCTGG
CTACGCTCCTGTTCTGGATTGTCACACAGCCCACATAGCATGCAAGTTTGCTGAGCTTAAA
GAAAAGATCGATCGTCGTTCTGGTAAGAAGCTGGAAGATGGCCCCAAGTTCCTGAAGTCTG
GCGATGCTGCCATTGTTGATATGGTCCCTGGCAAGCCCATGTGTGTTGAGAGCTTCTCTGA
CTACCCTCCACTTGGTCGCTTTGCTGTTCGTGACATGAGGCAGACAGTTGCTGTGGGTGTC
ATCAAAGCTGTGGACAAGAAGGCTGCTGGAGCTGGCAAAGTCACCAAGTCTGCCCAGAAAG
CTCAGAAGGCTAAATGAATATTACCCCTAACACCTGCCACCCCAGTCTTAATCAGTGGTGG
AAGAACGGTCTCAGAACTGTTTGTCTCAATTGGCCATTTAAGTTTAATAGTAAAAGACTGG
TTAATGATAACAATGCATCGTAAAACCTTCAGAAGGAAAGAATGTTGTGGACCATTTTTTT
TGTGTGTGGCAGTTTTAAGTTATTAGTTTCAAAATCAGTACTTTTTAATGGAAACAACTTG
ACCAAAATCTGTCACAGAATTTTGAGACCATTNAAACAAGTTAATGAGAAAAAAAAAAAAA
A

Clone C2, encodes 167 amino acids that showed 100% identity to a protein similar to eukaryotic translation elongation factor 1alpha, direct submission. Accession number: BC003969.

>CGAATTCGCGGCCGCGTCGACCTTCCTGGGGACAATGTGGGCTTCAATGTAAAGAACGTG
TCGGTCAAAGATGTTAGACGAGGCAATGTTGCTGGTGACAGCAAAAACGACCCACCAATGG
AAGCAGCTGGCTTCACTGCTCAGGTGATTATCCTGAACCATCCAGGCCAAATCAGTGCTGG

CTACGCTCCTGTTCTGGATTGTCACACAGCCCACATAGCATGCAAGTTTGCTGAGCTTAAA
GAAAAGATCGATCGTCGTTCTGGTAAGAAGCTGGAAGATGGCCCCAAGTTCCTGAAGTCTG
GCGATGCTGCCATTGTTGATATGGTCCCTGGCAAGCCCATGTGTGTTGAGAGCTTCTCTGA
CTACCCTCCACTTGGTCGCTTTGCTGTTCGTGACATGAGGCAGACAGTTGCTGTGGGTGTC
ATCAAAGCTGTGGACAAGAAGGCTGCTGGAGCTGGCAAAGTCACCAAGTCTGCCCAGAAAG
CTCAGAAGGCTAAATGAATATTACCCCTAACACCTGCCACCCCAGTCTTAATCAGTGGTGG
AAGAACGGTCTCAGAACTGTTTGTCTCAATTGGCCATTTAAGTTTAATAGTAAAAGACTGG
TTAATGATAACAATGCATCGTAAAACCTTCAGAAGGAAAGAATGTTGTGGACCATTTTTTT
TGTGTGTGGCAGTTTTAAGTTATTAGTTTTCAAAATCAG

Clone C3, encodes a 267 amino acids that showed 92% identity to the human hypothetical protein KIAA1840, direct submission.

>CGAATTCGCGGCCGCGTCGACCACACTCTCATAAGCAAGTGCCTCCATGGGAAGAACTAC
TGCCGGCAGGTCCTCTGTCTGTATGAACTTGCCAAGGATTTGGGCTGTTCCTATGGAGATG
TTGCTGCTAGGGACAGTGAAGCCATGCTCCGAGCAATCTTGGCTTCTCAGCGGCCTGACCG
ATGTAGACAAGCCCAGGTCTTCATCAACACCCAGGGCCTTGAAGCAGACACTGTGGCTGAA
CTAGTGGCTGAAGAGGTGACACGGGAGCTACTGACTCCATCAGAGGGAACAGGAGAAAAGC
AACCTTTCAACCCAGCAGAGGAAAGCCAGACATTTCTTCAGCTAACTGCTTTGTGTCAAGA
CCGTACATTGGTAGGCATGAAGTTGTTGGACAAGATTCCCTCTGTCCCCCACGGGGAGCTG
TCTTGCACCACAGAGCTCCTGATCCTGGCCCACCACTGCTTTACCTTCACGTGCCACATGG
AAGGCATCACCAGAGTCCTGCAAGCCGCCCGCATGCTCACAGATAACCACTTGGCTCCCAA
TGAGGAGTACGGATTAGTGGTCCGCCTCCTCACTGGCATCGGGCGCTACAATGAGATGACG
TACATATTTGACTTGCTGCATCAAAAGCATTATTTCGAAGTTCTGATGAGAAAGAAGCTAG
ACCCAACCGGCACCCTGAAGACAGCCCTACTAGACTACATCAAACGTTGCCGCCCTGGAGA
CAGCGAAAAGCACAATATGATTGCCCTGTGCTTCAGTATGTGCCCGGAGATAGGAGAGATC
ACGAGGCAGCG

Clone C4, encodes a 267 amino acids that showed 92% identity to the human hypothetical protein KIAA1840, direct submission.

>CGAATTCGCGGCCGCGTCGACCACACTCTCATAAGCAAGTGCCTCCATGGGAAGAACTAC
TGCCGGCAGGTCCTCTGTCTGTATGAACTTGCCAAGGATTTGGGCTGTTCCTATGGAGATG
TTGCTGCTAGGGACAGTGAAGCCATGCTCCGAGCAATCTTGGCTTCTCAGCGGCCTGACCG
ATGTAGACAAGCCCAGGTCTTCATCAACACCCAGGGCCTTGAAGCAGACACTGTGGCTGAA
CTAGTGGCTGAAGAGGTGACACGGGAGCTACTGACTCCATCAGAGGGAACAGGAGAAAAGC
AACCTTTCAACCCAGCAGAGGAAAGCCAGACATTTCTTCAGCTAACTGCTTTGTGTCAAGA
CCGTACATTGGTAGGCATGAAGTTGTTGGACAAGATTCCCTCTGTCCCCCACGGGGAGCTG
TCTTGCACCACAGAGCTCCTGATCCTGGCCCACCACTGCTTTACCTTCACGTGCCACATGG
AAGGCATCACCAGAGTCCTGCAAGCCGCCCGCATGCTCACAGATAACCACTTGGCTCCCAA
TGAGGAGTACGGATTAGTGGTCCGCCTCCTCACTGGCATCGGGCGCTACAATGAGATGACG
TACATATTTGACTTGCTGCATCAAAAGCATTATTTCGAAGTTCTGATGAGAAAGAAGCTAG
ACCCAACCGGCACCCTGAAGACAGCCCTACTAGACTACATCAAACGTTGCCGCCCTGGAGA
CAGCGAAAAGCACAATATGATTGCCCTGTGCTTCAGTATGTGCCCGGAGATAGGAGAGATC
ACGAGGCAGCG

Clone C5, encodes a 216 amino acids that showed 100% identity to hypothetical proteins KIAA1840 and FLJ21439, direct submissions.

>CGAATTCGCGGCCGCGTCGACCACACTCTCATAAGCAAGTGCCTCCATGGGAAGAACTAC
TGCCGGCAGGTCCTCTGTCTGTATGAACTTGCCAAGGATTTGGGCTGTTCCTATGGAGATG
TTGCTGCTAGGGACAGTGAAGCCATGCTCCGAGCAATCTTGGCTTCTCAGCGGCCTGACCG
ATGTAGACAAGCCCAGGTCTTCATCAACACCCAGGGCCTTGAAGCAGACACTGTGGCTGAA
CTAGTGGCTGAAGAGGTGACACGGGAGCTACTGACTCCATCAGAGGGAACAGGAGAAAAGC
AACCTTTCAACCCAGCAGAGGAAAGCCAGACATTTCTTCAGCTAACTGCTTTGTGTCAAGA
CCGTACATTGGTAGGCATGAAGTTGTTGGACAAGATTCCCTCTGTCCCCCACGGGGAGCTG
TCTTGCACCACAGAGCTCCTGATCCTGGCCCACCACTGCTTTACCTTCACGTGCCACATGG
AAGGCATCACCAGAGTCCTGCAAGCCGCCCGCATGCTCACAGATAACCACTTGGCTCCCAA
TGAGGAGTACNGATTAGTGGTCCGCCTCCTCACTGGCATCGGGCGCTACAATGAGATGACG

TACATATTTGACTTGCTGCATCAAAAGCATTATTTNAAGTTCTGATGAGAAAGAACTAGAC
CCAACCGGCACCCTGAAGACAGCCCTACTAGACTACATTAAACGTTGCCGCCCTGGAGACA
GCGAAAAGCACAATATGATTGCCCTGTGCTT

Clone C7, encodes 167 amino acids that showed 100% identity to a protein similar to eukaryotic translation elongation factor 1alpha, direct submission. Accession number: BC003969.

>CGAATTCGCGGCCGCGTCGACCTTCCTGGGGACAATGTGGGCTTCAATGTAAAGAACGTG
TCGGTCAAAGATGTTAGACGAGGCAATGTTGCTGGTGACAGCAAATTTNACCCACCAANGG
AAGCAGCTGGCTTCACTGCTCAGGTGATTATCCTGAACCATCCAGGCCAAATCAGTGCTGG
CTACGCTCCTGTTCTGGATTGTCACACAGCCCACATAGCATGCAAGTTTGCTGAGCTTAAA
GAAAAGATCGATCGTCGTTCTGGTAAGAAGCTGGAAGATGGCCCCAAGTTCCTGAAGTCTG
GCGATGCTGCCATTGTTGATATGGTCCCTGGCAAGCCCATGTGTGTTGAGAGCTTCTCTGA
CTACCCTCCACTTGGTCGCTTTGCTGTTCGTGACATGAGGCAGACAGTTGCTGTGGGTGTC
ATCAAAGCTGTGGACAAGAAGGCTGCTGGAGCTGGCAAAGTCACCAAGTCTGCCCAGAAAG
CTCAGAAGGCTAAATGAATATTACCCCTAACACCTGCCACCCCAGTCTTAATCAGTGGNGG
AAGAACGGTCTCAGAACTGTTTGTCTCAATTGGCCATTTAAGTTAATAGTAAAAGACTGGG
TAATGATAACAATGCATCGTAAAACCTTCAGAAGGNAAGAATGTTG

Clone C8, encodes 167 amino acids that showed 100% identity to a protein similar to eukaryotic translation elongation factor 1alpha, direct submission. Accession number: BC003969.

>CGAATTCGCGGCCGCGTCGACCTTCCTGGGGACAATGTGGGCTTCAATGTAAAGAACGTG
TCGGTCAAAGATGTTAGACGAGGCAATGTTGCTGGTGACAGCAAAAACGACCCACCAATGG
AAGCAGCTGGCTTCACTGCTCAGGTGATTATCCTGAACCATCCAGGCCAAATCAGTGCTGG
CTACGCTCCTGTTCTGGATTGTCACACAGCCCACATAGCATGCAAGTTTGCTGAGCTTAAA
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TGTGTGTGGCAGTTTTAAGTATTAGTTTCAAATCAGTACTTTTTATGGNAACAACTTGACC
AAAAATCTGTACAGAATTTTGAGACATTAAACAAGTTTAATGAGAAAAAAAAAAAAAAAAA
AA

APPENDIX 2

APPENDIX 2: GENERATION OF CRTR-1 SPECIFIC POLYCLONAL SERA.

A2.1 Design and Construction of a GST-CRTR-1 Fusion Protein for Rabbit Immunisation

Two criteria were considered in the selection of a CRTR-1 peptide for polyclonal antiserum production:

• Antigenicity, which is expected to be higher for hydrophilic regions of the protein as these are more likely to be exposed on the protein surface, and

• Specificity. The antibody should be generated against a region that is not highly conserved between same species family members.

The hydrophobicity of CRTR-1 was determined a by Kyte and Doolittle plot carried out using the program DNAsis (2.3.1.16) (Figure A2.1). This analysis revealed the protein to be hydrophilic overall having an average of –0.48. The central portion of the protein was the most hydrophilic, however this portion is highly conserved between CP2 family members (figure 3.11 and 3.12) and was not considered for antibody generation. The N terminal region (amino acids 8-177) of CRTR-1 was selected for antibody generation. This region displays low hydrophobicity (average of –0.43; Figure 3.12b) and is located N terminal to the regions implicated in DNA and Protein binding in other CP2 family members.

A2.1.2 GST-CRTR-1(8-177) expression plasmid construction.

The 510 bp *Eco*RI fragment of clone 8.2.1 (3.2.1 and Figure 3.1) (encoding sequence at the 5′ end of *CRTR-1* excluding the most N-terminal 7 amino acids) was excised by *Eco*RI digestion (2.3.1.1), end-filled (2.3.1.4), gel purified (2.3.1.3) and ligated (2.3.1.6) into *Eco*RI digested and end-filled pGEX-2T (2.2.6.1) such that *CRTR-1* was cloned in frame with GST generating the plasmid pGEX-2T-CRTR-1(8-177) (Figure A2.2a). The correct orientation and frame was confirmed by *Bam*HI/*Pst*I digestion and BDT automated sequencing (2.3.1.14) using the RACE1 primer (2.2.7.2).

FIGURE A2.1

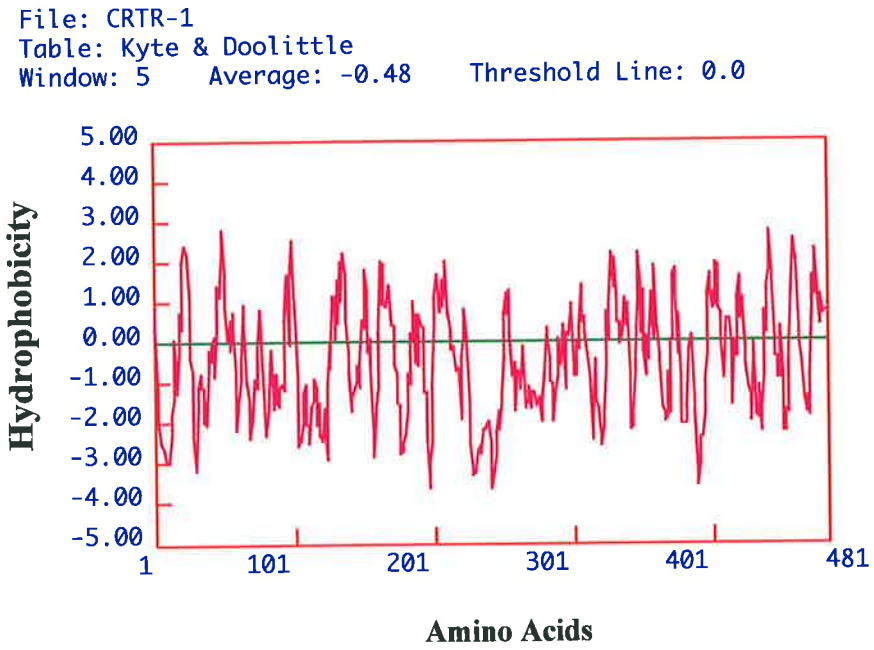
CRTR-1 Kyte and Doolittle plot

Predicted hydrophobic regions of the protein sequence are graphed below the zero line while hydrophilic regions are graphed above the line. Analysis was carried out using the computer program DNasis.

A) Full length CRTR-1.

B) CRTR-1 amino acids 8-177.

A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4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00
1
101
201
301
401
481
Amino Acids

B

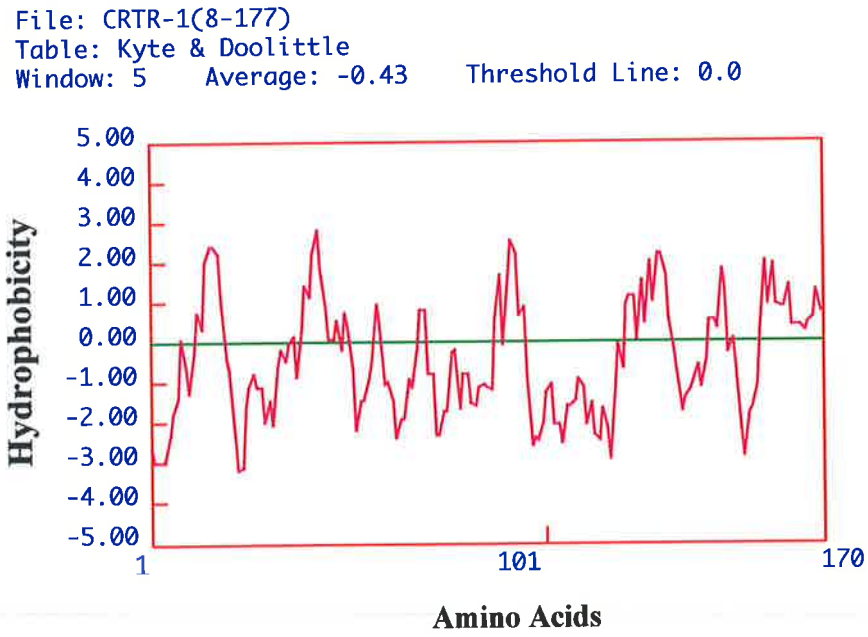
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Table: Kyte & Doolittle
Window: 5 Average: -0.43 Threshold Line: 0.0
Hydrophobicity
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00
1
101
170
Amino Acids

FIGURE A2.2

Cloning strategy to generate GST-CRTR-1(8-177) and expression of fusion protein.

A) Clone 8.2.1 was digested (2.3.1.1) with *Eco*RI to obtain the 510bp cDNA fragment which encodes amino acids 8-177 of CRTR-1. This fragment was subsequently blunt ended (2.3.1.4) and ligated (2.3.1.6) into the pGEX-2T vector digested with *Eco*RI and blunt.

B) BL21 bacteria transformed with pGEX-2T-CRTR-1(8-177) were cultured over night at 37°C. A sample of the culture (non-induced) was taken prior to induction of fusion protein expression by the addition of 0.2 mM IPTG and culturing for 2 hours at 30°C (2.3.6.1.4). Cellular extracts from induced bacteria were produced by sonication as described (2.2.3.6.1.4). Soluble and insoluble lysates were separated by centrifugation, resuspended in TBS and 20 µL samples were separated by 10% SDS-PAGE (2.3.6.4). Total cell lysates of non-iduced bacteria were also analysed. Proteins were visualised by coomassie staining (2.3.6.5).

Lanes were loaded as follows:

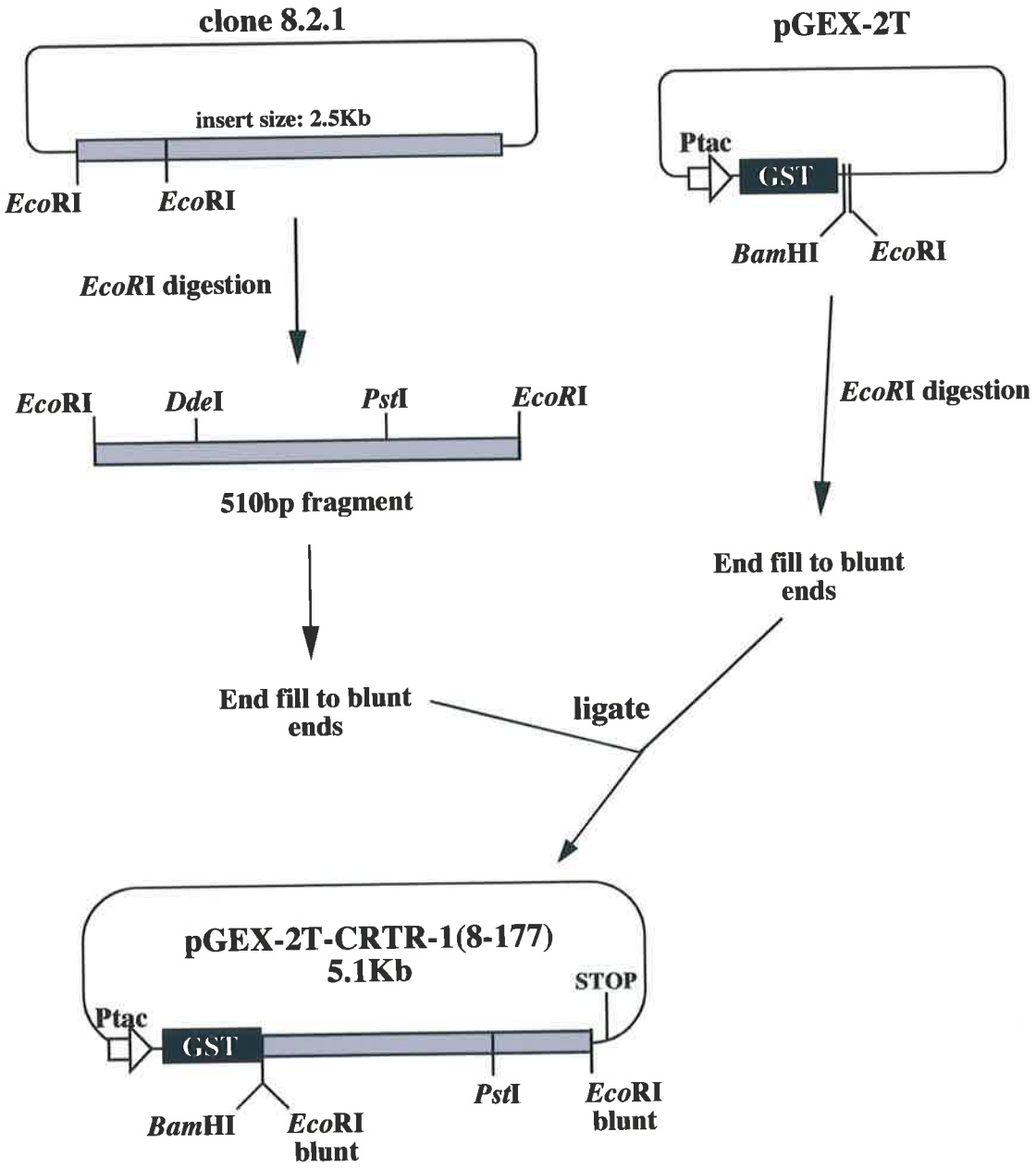
1 + 5: Benchmark pre-stained molecular weight markers (2.2 .14)

2: Induced insoluble

3: Induced soluble

4: Pre-induced total lysate

A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EcoRI
EcoRI digestion
EcoRI
DdeI
PstI
EcoRI
510bp fragment
End fill to blunt ends
pGEX-2T
Ptac
GST
BamHI
EcoRI
EcoRI digestion
End fill to blunt ends
ligate
pGEX-2T-CRTR-1(8-177)
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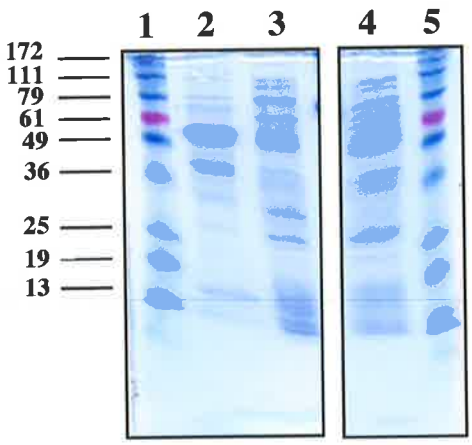
Ptac
GST
PstI
EcoRI
blunt
BamHI
EcoRI
blunt

B


1 2 3 4 5
172
111
79
61
49
36
25
19
13

A2.1.3 Expression of the GST-CRTR-1(8-177) fusion protein.

BL21 bacteria (2.2.9) were transformed with clone pGEX-2T-CRTR-1(8-177). Single colonies were picked and tested for their ability to produce GST-CRTR-1(8-177) fusion protein upon induction with IPTG (2.3.6.1.4). Briefly, 50ml cultures were grown at 30°C to log phase (OD_{600} = 0.6). A pre-induced sample of culture was collected prior to induction of fusion protein expression by addition of 0.2 mM IPTG. Cultures were grown for 2 hours at 30°C prior to the production of lysates from induced and non-induced cultures and separation of lysates into soluble and insoluble fractions by centrifugation (2.3.6.1.4). 20 µl samples of each fraction was added to 20 µl of 2x SDS-PAGE load buffer (2.2.5) and analysed by 10% SDS-PAGE (2.3.6.4) prior to visualisation of proteins by Coomassie staining (2.3.6.5).

IPTG induced protein of the correct molecular weight (50 kDa) was detected in the insoluble fraction (Figure A2.2b), however the expressed protein was undetectable in the soluble fraction. Variation of induced BL21 transformants, IPTG induction concentrations, temperature/time cultured and alternative *E. coli* strains produced comparable induction results to those shown (data not shown).

A2.1.4 Purification of insoluble GST-CRTR-1(8-177) fusion protein.

Protein concentration of the insoluble fraction of the induced GST-CRTR-1(8-177) band was quantitated against BSA standards (2.3.6.1.6) and determined to be approximately 0.5 mg/ml. Approximately 150 µg of protein was loaded into a large well and was separated by 10% SDS-PAGE (2.3.6.4). The region of the gel expected to contain GST-CRTR-1(8-177) based on pre-stained size markers (2.2.14) was excised from the gel which was then stained with Coomassie (2.3.6.5) to confirm that the majority of GST-CRTR-1(8-177) had been excised. The excised acrylamide band was puréed manually by pushing through a series of 5 syringe needles of increasing gauge syringes increasing from 18 to 25 gauge with the addition of 800 µl of TBS to maintain a paste-like consistency. Purée was stored at -20°C. This process

was repeated prior to each round of rabbit immunisations to ensure the integrity of injected GST-CRTR-1(8-177).

A2.2 Rabbit Immunisation

Adjuvants were used to maximise phagocytosis and prolonged persistence of the injected fusion protein. In this regime the polyacrylamide present with the proteins, heat killed *mycobacteria*, and muramyl dipeptide (a component of the bacterial cell wall known to activate macrophages, added at 100 μg per rabbit) were all used as adjuvants in the primary immunisation (2.3.6.1.7). Adjuvants were not used in subsequent immunisations.

Approximately 50 μg of GST-CRTR-1(8-177) was injected intravenously into each of two rabbits 3 times at 3 week intervals (2.3.6.1.7). A pre-immunisation serum sample was collected from both rabbits prior to the initial immunisation. 10 days after the final immunisation a post-immune sera samples were collected from each rabbit and are named, CRTR-1 #94 and CRTR-1 #95. These sera were then characterised and compared with the preimmune serum. Both rabbits were bled and the final sera collected (2.3.6.1.7).

A2.3 Antibody characterisation.

A2.3.1 Detection of CRTR-1 by Western blot.

The two polyclonal anti-CRTR-1 sera were characterised in association with Michaela Scherer (The Department of Molecular Biosciences, The University of Adelaide). Western blot analysis (2.3.6.6) 25 μg of protein fractions from IPTG-induced BL21 GST-CRTR-1(8-177) expressing bacteria separated by 10% SDS-PAGE (2.3.6.4). Polyclonal serum CRTR-1 #94 did not detect the GST-CRTR-1(8-177) fusion protein and was discarded (Scherer, 1999). Polyclonal serum CRTR-1 #95 detected the GST-CRTR-1(8-177) fusion. Cleanest results were achieved using a 1/500 dilution in PBT with 2% milk probed at room temperature for 1 hour.

The specificity of CRTR-1 #95 antiserum was tested against of 25 µg of whole cell extracts (2.3.6.2) made from P19 EC cells 24 hours after transfection (2.4.5) with 1.5 µg of mammalian expression vectors for CRTR-1 and CP2, pTRE-CRTR-1 and pTRE-CP2, respectively (Scherer, 1999) and whole cell extracts of untransfected P19 EC, 293T, COS-1 and ES D3 cells separated by 12.5% SDS-PAGE and transferred for Western analysis (2.3.6.6). Duplicate filters were produced for analysis using the preimmune #95 serum.

CRTR-1 #95 antiserum detected a 64 kDa protein within pTRE-CRTR-1 transfected P19 EC cells (Figure A2.3), consistent with the expected size of CRTR-1 (3.3.2 and Figure 3.3c). No protein was detected in untransfected P19 EC or pTRE-CP2 transfected P19 EC cells using CRTR-1 #95 antiserum or pre-immune serum confirming specific detection of protein.

A 64 kDa band was detected in untransfected 293T whole cell extracts. This protein could be the human homologue of CRTR-1, LBP-9, and is consistent with conservation of amino acid sequence across amino acids 8-177 of CRTR-1 with LBP-9. Furthermore, detection of LBP-9 in the kidney cell line is consistent with the observed expression of CRTR-1 demonstrated previously (1.12.2). Together, these results demonstrate that the CRTR-1 #95 antisera can be used to detect CRTR-1 expressed from transfected vectors and endogenous protein specifically.

A2.3.2 Detection of CRTR-1 protein by immunocytochemistry.

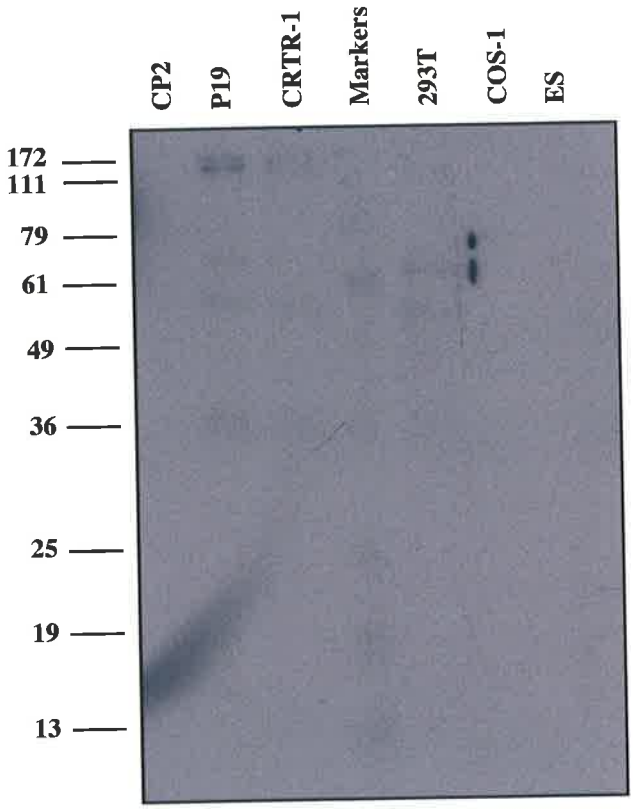
Immunohistochemistry (2.3.6.9) was carried out on 293T cells transfected (2.4.5) with pTRE-CRTR-1. Initial characterisation suggested that for immunohistochemistry CRTR-1 #95 antiserum was best used at a 1/200 dilution PBT with 3% BSA (Sherer 1999; data not shown). 293T cells seeded at 10^5 cells per 35 mm well in a 6 well tray on glass cover slips were transfected (2.4.5) with 1.5µg of pTRE-CRTR-1 and cultured for 24hrs. Cells were methanol fixed (2.3.6.9), blocked in 3% BSA in PBT prior to incubation with the CRTR #95

FIGURE A2.3

Characterisation of CRTR-1 #95 antisera by Western analysis.

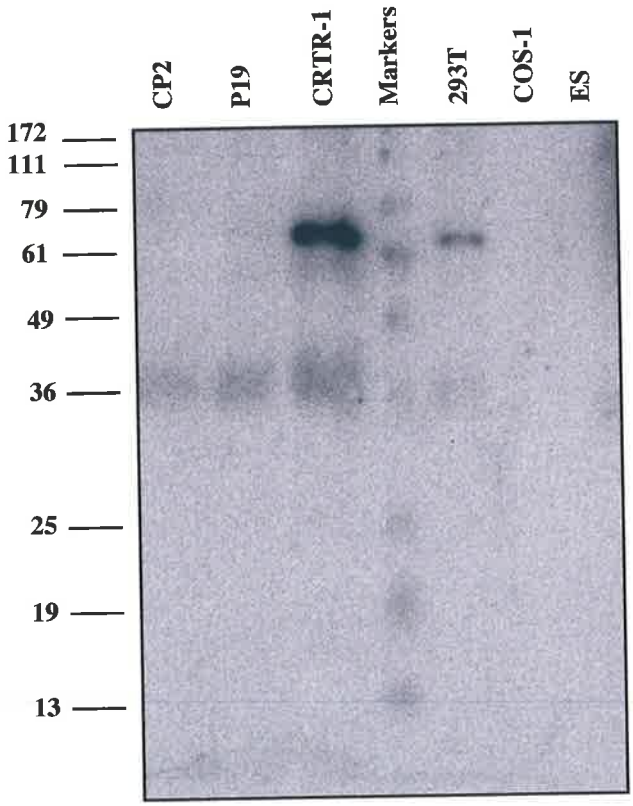
$3x10^5$ P19 EC, 293T, COS-1 and ES cells were grown in 60 mm diameter tissue culture dishes. P19 EC cells were transfected with 1.5 μg of pTRE-CRTR-1 or pTRE-CP2. Whole cell extracts were produced 24 hrs after culture (2.3.6.2). Protein concentrations were determined by Bradford assays (2.3.6.1.3) and 25 μg of cell extracts were separated by 12.5 % SDS-PAGE (2.3.6.4) prior to Western analysis (2.3.6.6). Filters were probed with pre-immune serum **(A)** or CRTR-1 #95 antiserum **(B)** prior to incubation with horseradish peroxidase (HRP) conjugated goat anti-rabbit secondary antibody (2.2.8). Filters were visualised using chemiluminescence (2.3.6.6). CP2, P19 EC cells transfected with pTRE-CP2; P19, untransfected P19 EC cells; CRTR-1, P19 EC cells transfected with pTRE-CRTR-1; 293T, COS-1 and ES, untransfected cells; Markers; 10 μL of Benchmark pre-stained protein ladder (2.2.14).

A



Pre-immune

B



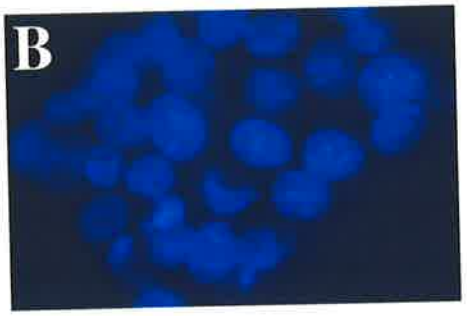
anti-CRTR-1 #95

FIGURE A2.4

Characterisation of CRTR-1 #95 antisera by immunohistochemistry.

10^5 293T cells, grown in 35 mm wells of a 6 well tray on cover slips and transfected (2.4.5) with pTRE-CRTR-1, were stained 24 hrs post transfection with pre-immune serum **(A)** or CRTR-1 #95 antiserum **(C)** and a goat anti rabbit FITC conjugated antibody (2.2.8) prior to staining with Hoechst **(B and D)**, following methanol fixation (2.3.6.9). Specific antibody staining was visualised and photographed on a Zeiss fluorescent microscope at 100X magnification with oil emersion under the FITC excitation wavelength, 465-495 nm, **(A and C)** and under the UV excitation wavelength, 330-380 nm, **(B and D)**. Staining observed in **(C)** and **(D)** were overlayed using Photoshop (Adobe) producing **(E)**.


A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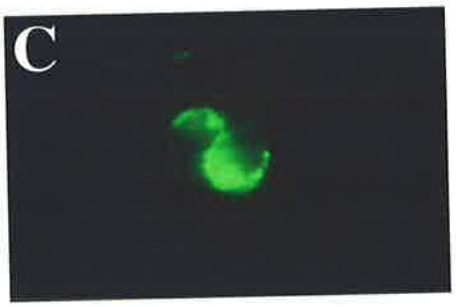
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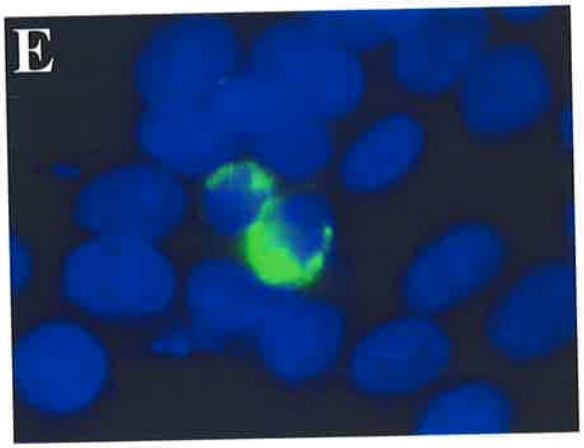
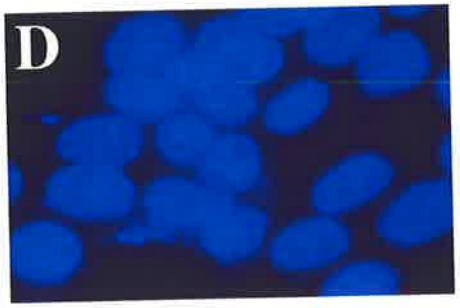

E


D

antiserum or pre-immune serum at a 1/200 dilution in 3% BSA in PBT for 1hr (2.3.6.9). Cells were then probed with a Goat anti-rabbit FITC-conjugated antibody (2.2.8 and 2.3.6.9).

pTRE-CRTR-1 transfected cells probed with CRTR-1 #95 antiserum showed intense staining that appeared perinuclear and polarised to only one outer side of the nucleus (Figure A2.4c and e). 293T cells transfected with pTRE-CRTR-1 probed with the pre-immune serum (Figure A2.4 a and b) did not show staining confirming the specificity of CRTR-1 #95 antiserum. CRTR-1 #95 antiserum can therefore be used to visualise the localisation of CRTR-1 in cells using immunohistochemistry. However, under these conditions, levels of expression of endogenous protein were too low for detection.

CHAPTER 11

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Stephen James Rodda
Corrections for thesis entitled:

"Isolation and Characterisation of CRTR-1 and altCP2: Negative Regulators of CP2 Transcription Factor Family Activity"

For the award of Doctor of Philosophy.

Examiner 1:
CRTR-1: CP2 Related Transcriptional Repressor 1.

Page 14, paragraph 3, line 3:
The word 'Uropophyyrinogen' is misspelt and should be spelt 'Uroporphyrinogen'.

Page 17, paragraph 3, line 2:
The word EST's is incorrect and should be written as ESTs. The word ESTs should replace all occurrences of the word EST's throughout the thesis.

Page 21, section 1.13:
The last sentence reads 'The purpose of this work was to understand the specific function of CRTR-1, achieved via investigation of the following aspects of transcription factor action;'
This sentence should read:
'The purpose of this work was to understand the specific function of CRTR-1, achieved via investigation of the following aspects of transcription factor action:'
Where the semi-colon at the end of the sentence is replaced by a colon.

Page 26, line 1:
The word 'Alkalynie' is misspelt and should be spelt 'Alkalyne'

Page 75, paragraph 3, line 4:
It is stated that 'The ATG is preceded by a consensus Kozak sequence'. This is technically incorrect and should be re-worded to state that 'The ATG is surrounded by a nucleotide sequence that resembles a consensus Kozak sequence'.

Page 88, paragraph 2, line 2:
The Gal4 DNA binding domain is said to encompass amino acids 1-174. This should read as amino acids 1-147.

Page 96, paragraph 2, line 15:
The word 'β-galoctosidase' is misspelt and should be spelt 'β-galactosidase'.

Page 121, paragraph 2, line 6 and 9:
The word '*Hind*III' should be presented as '*Hin*dIII'

Page 143, paragraph 1, line 14:
The name 'Ed Setto' should be spelt 'Ed Seto'.

Page 162, paragraph 2, line 1:
The word 'heterologous' should read 'heterozygous'.

Page 163, paragraph 1, line 6:
The word 'update' should be omitted.

Acknowledgements:
The line 'Mikey B is treat to work with' should be replaced with 'Mikey B is a treat to work with'

The line 'you guys have mad the lab a great place to work' should be replaced with 'you guys have made the lab a great place to work'

Throughout the Acknowledgements section the word 'patients' should be replaced with the word 'patience'.

Examiner 2:
Page 6, paragraph 2, line 10:
The word 'nuroblast' is misspelt and should be spelt 'neuroblast'

Page 14, paragraph 2, line 4:
'An A for a G nucleotide polymorphism' should read 'An A for a G nucleotide transition'

Page 14, paragraph 2, line 5:
'appears to be protective of the AD phenotype' should read ' appears to be protective against the AD phenotype'

Page 14, paragraph 3, line 4:
'a C to an A nucleotide exchange' should read 'a C to an A nucleotide transition'

Page 61, section 2.3.6.1.3
'bradford' should read 'Bradford'.

Page 86, paragraph 2, line 1:
The words 'In sight' should be written as one word 'Insight'.

Page 86, paragraph 2, line 4:
The word 'charatcerisation' is misspelt and should be written as 'characterisation'.

Page 102, paragraph 3, line 7:
'Significance of the less abundantly, 74kDa' should be written as 'The significance of the less abundant 74kDa'.

Page 102, paragraph 3, line 8:
'is unclear but may' should be written as 'is unclear, but this may'.

Page 104, paragraph 2, line 6:
'formation of heteromeric complexes not' should read 'formation of heteromeric complexes does not'.

Page 115, paragraph 1, line 11:
'Identification of a CRTR-1 DNA binding sequences' should read 'Identification of a CRTR-1 DNA binding sequence'.

Page 115, paragraph 1, line 20:
The statement 'The ability of CRTR-1 to regulate transcription from this promoter would be consistent with the expression of CRTR-1 during the later stages of kidney development and would provide information about the biological function of CRTR-1' should be omitted and replaced with 'Demonstration of the ability of CRTR-1 to bind a CP2 consensus response element may open a number of possibilities of gene targets of CRTR-1 based upon CP2 consensus sites within their promoters. The observation that some of these genes have already been characterised to be expressed in ES cells or during kidney development may provide insight into CRTR-1 biological function, however, further reporter assay and EMSA experiments would be required to demonstrate the DNA binding ability of CRTR-1 to the promoters of these genes'.

Page 123, paragraph 1, line 3:
'of the CRTR-1 repression domain to can repress Gal4' should read 'of the CRTR-1 repression domain to repress Gal4'.

Page 164, paragraph 4, line 1:
The expression pattern described is suggested for 15.5 d.p.c. kidneys. This should be corrected to more accurately describe the data presented where the expression of CRTR-1 was investigated using kidney explants taken from between 11.5-12 d.p.c. and cultured for several days in vitro.

Page 164, paragraph 4, line 3:
The line 'these are cell types derived from the mesonephros and the metanephros' incorrectly describes the origin of these cell types. The ureteric bud arises from the mesonephric duct (also referred to as the Wolffian duct) in response to signals from the metanephric mesenchyme. The ureteric bud and metanephric mesenchyme are of independent origin. The first inductive event in the development of the kidney is the initial outgrowth of the ureteric bud into the metanephric mesenchyme.

Page 165, paragraph 3, line 4:
The line 'These techniques would also be employed if kidneys are formed incorrectly resulting in embryonic lethality at a later stage of development' should be omitted and replaced with the line 'These techniques would also be employed if kidneys are formed incorrectly resulting in postnatal lethality'.

Page 165, paragraph 3, line 6:
The line 'Correct function of the kidney would be investigated by analysis of blood pH and ion levels' should be omitted and replaced with 'Correct development of the kidney would simply be investigated by anatomical assessment and correct kidney function would be initially investigated by testing urine for the presence of proteinurea or hematuria, indicative of protein or blood in the urine'.

Page 175, paragraph 2, line 15:
The line 'If mice survive to birth then the effect of CRTR-$1^{-/-}$ mutation on kidney physiology will be analysed by morphological and histological criteria as well as testing the composition of the urine and blood' should be omitted. As with the above point, correct development of the kidney would be investigated by anatomical assessment and correct kidney function would be initially investigated by testing urine for the presence of proteinurea or hematuria, indicative of protein or blood in the urine.

Figure 6.4 and 6.9 B:
Both figures have two heteromeric arrows indicating the existence of two distinct protein complexes formed under the described experimental conditions that are unique and intermediary from the complexes indicated as homomeric.

Figure 9.1 B and C:
The structure being highlighted as the ureteric bud tip (ubt) is incorrect and should be corrected to indicate that the structure being highlighted is the developing distal convoluted tubule.

Acknowledgements:
The line 'and manuscripts in preparation (there coming soon)…' should be replaced with the line 'and manuscripts in preparation (they're coming soon)…'