CLONING AND

CHARACTERISATION OF THE

HUMAN UROPLAKIN 1B GENE

A thesis submitted to the University of Adelaide as the
requirement for the Degree of Doctor of Philosophy

by

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TABLE OF CONTENTS

CHAPTER 3   85

CLONING AND CHARACTERISATION OF THE HUMAN UROPLAKIN 1B GENE

CHAPTER 4   103

CHROMOSOMAL LOCALISATION OF THE HUMAN AND MOUSE UROPLAKIN 1B GENES

CHAPTER 5   126

ANALYSIS OF EXPRESSION OF HUMAN UROPLAKIN 1B MRNA

CHAPTER 6   145

MOLECULAR MECHANISMS OF THE FREQUENT LOSS OF UROPLAKIN 1B EXPRESSION IN BLADDER CANCER

ANTI-PROLIFERATIVE ACTIVITY OF UROPLAKIN 1B

Summary

The uroplakin 1B gene has been cloned and characterised in only two species; mink (designated TI1) and cow. The cDNA sequences of mink TI1 and bovine uroplakin Ib have been isolated and putative protein sequences predicted. The mink TI1 gene is preferentially expressed during growth arrest mediated by transforming growth factor beta. The bovine uroplakin Ib gene is expressed as a terminal differentiation product of the asymmetric unit membrane of the bovine bladder and has urothelial-specific expression. The uroplakin 1B protein belongs to the tetraspan family of proteins, all of which have a similar protein structure with four highly conserved transmembrane domains interspersed with two more diverse extracellular domains. The tetraspan proteins are involved in a variety of cell functions including motility, activation and development. Recent studies have found that some of the tetraspan genes have altered patterns of expression in cancer and may act as metastasis suppressors.

This thesis describes the first cloning and characterisation of the human uroplakin 1B (UPK1B) gene, thereby confirming the existence of a human homologue of the mink TI1 and bovine uroplakin Ib genes. The cloning, by PCR techniques of the cDNA coding for the open reading frame of the human uroplakin 1B gene revealed homologies to both mink TI1 and bovine uroplakin Ib cDNA of greater than 90%. The cloning of 2.5 kb of contiguous human uroplakin 1B genomic sequence is described, along with the discovery of a *Taq*I restriction fragment length polymorphism. Chromosome mapping using two independent human uroplakin 1B genomic probes located the gene to human chromosome 3q13.3-21, a region with synteny to the location of the bovine UPKIb

gene to bovine chromosome 1. Mapping to mouse chromosomes revealed the location of mouse Upk1b to chromosome 16B5-C2, a region syntenic with human chromosome 3q.

Expression of the human uroplakin 1B gene in normal human urothelium was determined by Northern and RT-PCR analysis. A loss or marked reduction of expression of UPK1B mRNA was observed in approximately 70% of bladder carcinomas. All five bladder cancer cell lines analysed have no expression of uroplakin 1B mRNA detectable by Northern analysis. A search for a possible molecular mechanism for the observed frequent down-regulation of human uroplakin 1B mRNA expression involved both allelic loss studies and detection of UPK1B gene rearrangements. Using polymorphic markers located either side of the uroplakin 1B gene on chromosome 3q13.3-21, allelic loss was not detected in this chromosome region. Southern analysis using human UPK1B genomic probes did not detect gross rearrangements of the UPK1B gene, suggesting UPK1B gene rearrangements are not responsible for the down-regulation of UPK1B expression in bladder cancer.

To examine the biological function of UPK1B, the highly homologous mink TI1 cDNA, under the control of a constitutive cytomegalovirus (CMV) promoter, was transfected into the mink CCL64 cell line and two bladder cancer cell lines, T24 and 5637. The failure to propagate any stable clones expressing exogenous TI1 in any of the three cell lines suggested expression of TI1 was antiproliferative. There was an eight-fold reduction in the number of colonies propagated from

T24 cells transfected with the TI1/CMV plasmid when compared to vector-transfected cells, supporting this hypothesis.

In summary, this thesis reports the partial cloning and characterisation of the human uroplakin 1B gene. Cloning of partial human uroplakin 1B genomic sequences has allowed analysis and characterisation of the gene with regard to its structure, chromosomal localisation and integrity. Sequence comparisons of human UPK1B to mink TI1, bovine UPKIb and other tetraspan proteins were made possible by the cloning of the open reading frame of the human uroplakin 1B cDNA. The cloning of human uroplakin 1B cDNA has also enabled expression studies of UPK1B mRNA in normal urothelial tissue, bladder carcinomas and bladder cancer cell lines. Absent or greatly-reduced expression of UPK1B mRNA in a high proportion of bladder cancers and functional studies suggesting that the UPK1B gene is antiproliferative, all point to a potential role for UPK1B in the pathogenesis of bladder cancer.

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 previously published or written by another person, except where due reference has been made in the text.

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

Jennie Louise Finch

10 December 1998

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Publications arising from this thesis

Finch JL, Miller J, Aspinall JO and Cowled PA. Cloning of the human Uroplakin 1B cDNA and analysis of its expression in urothelial tumor cell lines and bladder carcinoma tissue. *International Journal of Cancer* (In press)

Webb GC, **Finch JL** and Cowled PA. Assignment of the uroplakin 1b (Upk1b) gene to mouse chromosome 16 band(s) B5-C2 by in situ hybridization. *Cytogenetics and Cell Genetics* (In press)

Finch JL, Webb GC, Evdokiou A and Cowled PA. Chromosomal localization of the human urothelial "tetraspan" gene, UPK1B, to 3q13.3-q21 and detection of a *Taq*I polymorphism. *Genomics* (1997), 40:501-503

Presentations arising from this thesis

Finch JL, Miller J, Aspinall JO and Cowled PA. Molecular mechanisms of the frequent loss of uroplakin 1B expression in bladder cancer. Molecular Genetics of Cancer, Second Joint Conference of the American Association of Cancer Research and the European Association of Cancer Research, Oxford, England (1997)

Cowled PA, **Finch JL,** Miller J and Aspinall J. Loss of expression of uroplakin 1B in bladder cancer is not due to allelic loss or gene rearrangement. National meeting of the Australian Society of Medical Research, Adelaide (1997)

Finch JL, Miller J, Aspinall J and Cowled PA. Loss of expression of the human urothelial "tetraspan" gene, UPK1B, in bladder cancer is not due to allelic loss or gene rearrangement, Australian Society of Medical Research Annual Society Meeting, Adelaide (1997)

Finch JL, Webb GC, Miller J, Aspinall J and Cowled PA. Loss of expression of the human "tetraspan" gene UPKIB in bladder cancer. 9th Lorne Cancer Conference, Joint Special conference with the American Association of Cancer Research, Lorne, Victoria (1997)

Finch JL and Cowled PA. *Taq*I polymorphism of the human TI1 gene. 8th Lorne Cancer Conference, Lorne, Victoria (1996)

Abbreviations

ATCC	American Type Culture Collection
AUM	asymmetric unit membrane
bp	base pair
BSA	bovine serum albumin
CIP	calf intestinal alkaline phosphatase
cDNA	complementary DNA
CPM	counts per minute
CDK(I)	cyclin-dependent kinase (inhibitor)
CMV	cytomegalovirus
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DMEM	Dulbecco's modified Eagle's medium
FCS	fetal calf serum
hr	hour
kb	kilobase
LOH	loss of heterozygosity
μg	microgram
μl	microlitre

mg	milligram
ml	millilitre
min	minute
mRNA	messenger ribonucleic acid
MOPS	3-morpholinopropanesulfonic acid
ng	nanogram
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RISH	radioactive *in situ* hybridisation
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
TRE	tetracycline responsive element
TCC	transitional cell carcinoma
TGFβ	transforming growth factor β
TM4SF	transmembrane 4 superfamily
UPKIb	uroplakin Ib (bovine)
UPK1B	uroplakin 1B (human)
Upk1b	uroplakin 1b (mouse)
UPKII	uroplakin II (bovine)
UPKIII	uroplakin III (bovine)

CHAPTER 1

LITERATURE REVIEW

1.1 THE CELL CYCLE

The cell cycle of the mammalian cell can be divided into distinct stages. The gap 1 (G1) phase is followed by DNA synthesis (S), gap 2 (G2) and mitosis (M). Entry into and progression through each phase of the cell cycle is controlled by a number of regulatory control mechanisms or "checkpoints" responsible for the correct order of the stages of the cell cycle. After the completion of mitosis, the cells enter the growth arrest (G0) phase. From the G0 stage, a cell may re-enter the cycle and either replicate, differentiate or become quiescent and eventually undergo apoptosis. The control of cellular proliferation is important in the homeostasis of normal tissue and is maintained by the balance between positive and negative regulators. Cell cycle regulation involves the interactions of oncogenes, tumour suppressor genes, growth factors and other genes which act to determine the fate of a cell. Disruption of the genes involved in cell cycle regulation may result in uncontrolled cellular proliferation, leading to cancerous cell growth. This neoplasia may result from a disorder in the checkpoint of a specific cell cycle phase transition, for example, G1-to-S.

1.1.1 Growth Arrest

From the growth arrest stage of the cell cycle, a cell may either proliferate, enter a quiescent state, differentiate to a particular cell type, or apoptose, depending on the external stimuli. The micro-injection of messenger RNA species of growth-arrested cells can cause the recipient growing cells to undergo growth arrest (Pepperkok et al., 1988). Therefore, there must be genes, preferentially expressed during G0 that are essential for the induction and maintenance of growth arrest. Genes induced during growth arrest have varying properties and functions and a

2

role in the regulation of cellular proliferation has been demonstrated for some of these genes. Examples of genes preferentially expressed during growth arrest include p53, GAS (growth arrest-specific) genes 1-6, transforming growth factor beta (TGFβ) and MyoD. The nuclear phosphoprotein p53, includes induction of growth arrest as just one of its many functions as a tumour suppressor (reviewed in Levine, 1997). The GAS genes are related only by their preferential expression in growth arrest and have varying functions. GAS1, for example, inhibits G0/G1 phase transition in fibroblasts (Del Sal et al., 1992). TGFβ induces growth arrest and differentiation in many cell types, including epithelial and endothelial cells (Massague, 1990). The MyoD gene induces growth arrest and terminal differentiation of myoblasts (Crescenzi et al., 1990).

Other genes up-regulated during growth arrest may have roles in the control of cell cycle progression and differentiation. For example, the cyclin-dependent kinase inhibitors (CDKIs) are a family of growth-inhibitory proteins which physically associate with and inhibit the activity of the cyclin/CDK (cyclin-dependent kinases) protein complexes. Cyclin/CDK complexes trigger cell cycle transitions by phosphorylation of downstream targets, for example, the retinoblastoma protein (reviewed in Grana et al., 1995). The tumour suppressor retinoblastoma protein is found in a hypophosphorylated state as cells exit mitosis and is subsequently hyperphosphorylated during late G1 phase thereby, acting as a cell cycle oscillator, regulating progression through the cell cycle (reviewed in Sherr, 1994).

Many of these growth-inhibitory gene products interact with each other in common signalling pathways to control cellular proliferation and prevent neoplasia. For example, p53 modulates the activity of TGFβ (Blaydes et al., 1995), including the p53-dependent repression of CDK4 by TGFβ-induced growth arrest (Ewen et al., 1995). Many CDKIs, including p15 (Hannon et al., 1994) and p27 (Polyak et al., 1994), interact with TGFβ to induce growth arrest.

1.1.2 Differentiation

Differentiation and cellular proliferation are believed to be mutually exclusive events. Withdrawal from the cell cycle appears to be a prerequisite for differentiation and may be an early event in terminal differentiation (Philipson et al., 1991, Olson, 1992). Differentiating cells display extended G1 phases and inhibition of G1/S transition (Liebermann et al., 1995). The induction of differentiation can result in the hypophosphorylation of the retinoblastoma protein and the onset of growth arrest (Pardee, 1989). Differentiation may affect the phosphorylation state of pRb through expression of the CDKI, p21. Up-regulation of expression of p21, has been observed upon addition of differentiation-inducing agents, including dimethylsulfoxide (DMSO) and butyrate, to K562 haematopoietic and Hep3B hepatoma cell lines (Jiang et al., 1994, Steinman et al., 1994). Up-regulation of p21 by differentiation-promoting agents occurs in both p53-positive and negative cells, suggesting that p21 induction can be p53-independent. Differentiation can occur without the involvement of p53, as observed in mice deficient for p53 which are developmentally normal

4

(Donehower et al., 1992). Therefore, the growth arrest stage preceding terminal differentiation may also be p53-independent.

1.1.3 MyoD

In muscle cells, proliferation and differentiation are mutually exclusive events. The MyoD family of muscle-specific, transcription factors activate muscle differentiation and inhibit cell proliferation. Transcription of muscle-specific genes is initiated only when myoblasts are growth arrested in the G0/G1 phase. Therefore, the myogenic differentiation regulatory program may be dependent on the functions of genes expressed in G0/G1 or it may be that inhibitory factors expressed at other phases of the cell cycle are incompatible with the myogenic differentiation program (Olson, 1992). Expression of the MyoD gene can inhibit cell proliferation in both normal and transformed cells, therefore overriding the mitogenic signals of these cells. The transformed cells in which MyoD induced growth arrest, included NIH3T3 cells transformed with H-ras, src or fos oncogenes as well as KLN 205, a squamous cell carcinoma cell line and LL/2, a lung carcinoma cell line (Crescenzi et al., 1990). The growth arrest and differentiation properties of the MyoD gene appear to act via independent pathways, as, in several cell types, inhibition of DNA synthesis occurs, but not muscle differentiation. Basic domain mutants of MyoD retain their capacity to inhibit DNA synthesis, although are unable to activate myogenesis (Sorrentino et al., 1990). Transfection of the MyoD gene into both murine myocytes and non-myogenic cells induced the expression of cyclin-dependent kinase inhibitor, p21 mRNA. The MyoD-mediated induction of expression of p21, which inhibits CDK activity, is correlated with growth arrest of the cells (Halevy et al., 1995). These

observations imply that MyoD may naturally induce myoblasts to exit the cell cycle via growth arrest before terminal differentiation occurs.

1.1.4 DCC

The DCC (deleted in colorectal cancer) gene is an example of a candidate tumour suppressor gene involved with differentiation. The DCC gene was originally isolated because of its chromosomal location, 18q, a region often deleted in colon cancer (Vogelstein et al., 1988). The mRNA expression of DCC is absent or decreased in more than half of primary colorectal tumours analysed. Although loss of heterozygosity of 18q, including the DCC locus is observed in 70% of colorectal cancers, the retained allele of DCC is somatically altered in only approximately 10-15% of these cancers (Fearon et al., 1990). Abnormalities of DCC are thought to be part of a multi-step model of colorectal carcinogenesis with DCC mutations involved in the later stage of tumour progression. The dedifferentiation of the goblet cells in the normal colonic epithelium coincides with the loss of DCC expression, which may be a consequence of, or have a causative role in the loss of differentiation (Hedrick et al., 1992). Loss of DCC expression in neuroblastoma is correlated with disease dissemination. Expression of DCC could not be detected in 60% of metastatic tumours compared with only 15-20% of non-metastatic tumours (Reale et al., 1996). DCC is frequently inactivated in several other types of cancers through either altered expression or 18q loss of heterozygosity (reviewed in Cho et al., 1995). Transfection of a DCC cDNA into transformed keratinocytes lacking endogenous expression of DCC, suppressed tumorigenicity of these cells in nude mice (Klingelhutz et al., 1995). Experiments were set up to evaluate the activity of DCC in the neuroblastoma cell

line, IMR-32. These DCC-expressing IMR-32 cells differentiate in response to nerve growth factor (NGF). Expressing antisense DCC in the IMR-32 cells failed to elaborate neuritic processes and the cells continued to divide even in the presence of NGF (Hedrick et al., 1992).

The discovery of DCC-homologues and DCC-related proteins has aided the search for the functions of DCC. The DCC protein has significant amino acid sequence similarity with the neural cell adhesion molecule (N-CAM) cell-surface glycoproteins, including four immunoglobulin-like domains and a fibronectin-type III-related domain (Fearon et al., 1990). The DCC gene may play a role in cell-cell or cell-matrix interactions and disturbance of this function by mutation may lead to tumour invasion or metastasis. The DCC-related neogenin protein, originally isolated in the developing nervous system of chicks, is induced in neural cells immediately before cell cycle withdrawal and the initiation of neurite outgrowth (Vielmetter et al., 1994). UNC-40, the *C.elegans* homologue of DCC, is involved in guidance of circumferential migrations of axons and mesodermal cells through interactions with UNC-6. The vertebrate homologues of UNC-6 are netrin-1 and netrin-2. Netrins have been implicated in the guidance of commissural axons in the spinal cord. These proteins can function as attractants or repellents for developing axons to their targets in establishing connections (Chan et al., 1996). DCC is expressed on rat spinal axons and possesses netrin-1 binding activity. DCC, therefore, may act as a receptor for netrin-1 or may be a component of a receptor that mediates the effect of netrin-1 on axons (Keino-Masu et al., 1996). DCC may have a role in differentiation and migration in the nervous system. It has also been proposed that netrins may provide growth

inhibitory or differentiation signals to epithelial cells (Meyerhardt et al., 1997). DCC inactivation may lead to a loss of growth or differentiation control through the inability of cells to respond to interactions at the cell surface. Abnormalities of DCC may allow uncontrolled migration of cancer cells leading to metastasis as seen in tumours without DCC expression (Reale et al., 1996).

1.1.5 Transforming Growth Factor β (TGFβ)

Transforming growth factor beta is a family of multifunctional cytokines which are believed to play regulatory roles in the cell cycle. In particular, TGFβ1 promotes cell proliferation in mesenchymally-derived cells but induces growth arrest in other cells including epithelium, endothelium and lymphocytes (Massague, 1990). There are three mammalian isoforms, TGFβ1, 2 and 3, one isoform, TGFβ4, only found in chickens and TGFβ5 is only found in Xenopus. All five isoforms have a high degree of structural and functional homology and bind to the same cell-surface receptors. TGFβ1 and 2 are expressed in almost all mammalian cell types but TGFβ3 is only expressed in cells of mesenchymal origin (reviewed in Massague, 1990).

Transforming growth factor beta 1 is the best characterised member of the family of transforming growth factor betas and has 98% conservation of amino acid sequence between human, mouse, pigs and cow (Sporn et al., 1988). The mRNA coding for human transforming growth factor beta is 2439 base pairs in length which produces a 391 amino acid polypeptide (Derynck et al., 1985). TGFβ1 is

8

synthesised as the C-terminal segment of a precursor polypeptide which is made up of the first 279 amino acids of the full length 391 amino acid polypeptide. Therefore, the mature TGFβ1 is only 112 amino acids long. The TGFβ1 monomer is cleaved from the precursor at the arginine-arginine dipeptide immediately preceding the TGFβ amino-terminus. It is this cleavage which activates the TGFβ1 molecule and allows it to bind to its receptor (Derynck et al., 1985).

The TGFβs exert their biological effect through binding to various cell surface receptors and binding proteins. Nine TGFβ-specific receptors have been isolated. The most important receptors for signal transduction of TGFβ-mediated effects are TGFβ Type I and Type II which have ubiquitous tissue distribution (Laiho et al., 1991). Type II receptor can bind ligands in the absence of Type I but requires Type I receptor for signalling. Type I receptor can not bind ligands without the presence of the Type II receptor (Okadome et al., 1994). This suggests that the ligand binding induces formation of a heterotetramer receptor complex consisting of the TGFβ dimer ligand and two each of Type I and Type II receptors (Ventura et al., 1994). Both Type I and Type II receptors have serine/threonine kinase activity which is essential for signalling activity for growth inhibition. The kinase activity is constitutively active for Type II receptors, but not for Type I. After ligand binding and formation of a heteromeric receptor complex, Type II transphosphorylates the GS domain of the Type I receptor which may then activate the Type I kinase (Carcamo et al., 1995). The GS domain is a glycine and serine rich sequence in the region preceding the kinase domain of the Type I

receptor. This domain is conserved in other Type I receptors for proteins in the TGFβ family but is not present in the Type II receptor (Franzen et al., 1995). The mechanism by which the Type I receptor signals the specific response to the cell has not been elucidated.

Cell Cycle Regulation by TGFβ

Transforming growth factor beta 1 is known to regulate the expression of a number of genes and growth factors, many of which are involved in extracellular matrix formation. TGFβ1 up-regulates the expression of, for example, collagen and other extracellular matrix proteins and inhibitors of proteases, but down-regulates the expression of a number of proteases including collagenase. TGFβ1 up-regulates a number of growth factors including TGF-α and PDGF, but can have a variety of effects on a number of immune factors. TGFβ induces the expression of a number of nuclear proteins, including fos and jun, but down-regulates the expression of the nuclear protein, myc and prevents the phosphorylation of the retinoblastoma protein, keeping retinoblastoma in its growth suppressive form (reviewed in Newman, 1993).

TGFβ1 is believed to exert its control on the cell cycle by inhibiting certain biochemical targets, by down-regulating transcription of genes, decreasing phosphorylation of target proteins and inactivating cell cycle-regulated enzymes. For example, c-myc, a potential upstream positive regulator of cyclins A and E, is required for entry into S phase. TGFβ1 decreases c-myc expression in the mouse

keratinocyte cell line (BALB/MK) by inhibiting c-myc transcription (Alexandrow et al., 1995a). The addition of TGFβ1 to CCL64 cells in late G1, can cause growth arrest by inhibiting the CCL64 cells from entering S phase (Pietenpol et al., 1990). As cells are sensitive to TGFβ1 in both early and late G1, it is possible that TGFβ-induced growth arrest may occur by two separate pathways depending on the presence or absence of TGFβ1 expression at various stages of G1. When TGFβ1 is added in late G1, TGFβ1 may prevent entry into S phase by post-transcriptional or post-translational inhibition of genes as no *de novo* RNA synthesis is required during the last few hours of the G1/S phase transition once the cells have passed the R (restriction) point and are committed to enter the S phase (Alexandrow et al., 1995b).

TGFβ1 inhibition of cell cycle progression into S phase has been linked to the cyclin-dependent kinases (CDKs) which are important in promoting cell proliferation. TGFβ may inactivate CDKs which are normally responsible for the phosphorylation of the retinoblastoma protein, pRb. TGFβ1 down-regulates CDK4 expression by inhibiting its translation (Ewen et al., 1995) and CDK4 is involved with cyclin D proteins in phosphorylating pRb (reviewed in Cordon-Cardo, 1995). Expression of the CDKI, (CDK inhibitor), p15, is greatly up-regulated by TGFβ, suggesting that p15 may act as a mediator of TGFβ-induced growth arrest by inhibiting the activity of the CDK4/cyclin D2 complex (Hannon et al., 1994). Therefore, TGFβ may induce growth arrest by inhibiting the phosphorylation of pRb, through activation of the p15 cyclin-dependent kinase inhibitor. Both p21

and p27 (growth arresting-CDKIs) are also transcriptionally up-regulated upon TGFβ treatment, suggesting that TGFβ can act through multiple signalling pathways to induce growth arrest (Polyak et al., 1994, Datto et al., 1995).

1.2 THE MINK TI1 GENE

Another gene preferentially expressed during growth arrest is the TI1 gene. This gene was isolated by screening cDNA libraries made from TGFβ1-induced growth arrested cells (Kallin et al., 1991). The novel clone they isolated was named "TI1" and this gene will be the focus of this thesis. The aim of the study was to isolate genes that were preferentially expressed during growth arrest induced by TGFβ1 (Kallin et al., 1991). A library screening method termed "differential screening" was used to select only those clones which hybridised to one particular probe and not the other. A library was made from cDNA synthesised from RNA isolated from growth-arrested, TGFβ1-treated mink lung epithelial cells (CCL64). Total cDNA from serum-stimulated and actively proliferating CCL64 cells was used as the first probe. The second probe was derived from total cDNA from TGFβ1-treated, growth-arrested CCL64 cells (Figure 1.1). The clones which were positive only when hybridised with the second probe, were used as probes on Northern blots containing RNA from either serum-stimulated, serum-starved or TGFβ1-treated CCL64 cells. The clones which hybridised only to the TGFβ1-treated RNA were subcloned and sequenced. Four genes already known to be regulated by TGFβ1 were isolated by this differential screening method. These genes included mink homologues of the human collagen α type I and fibronectin genes,
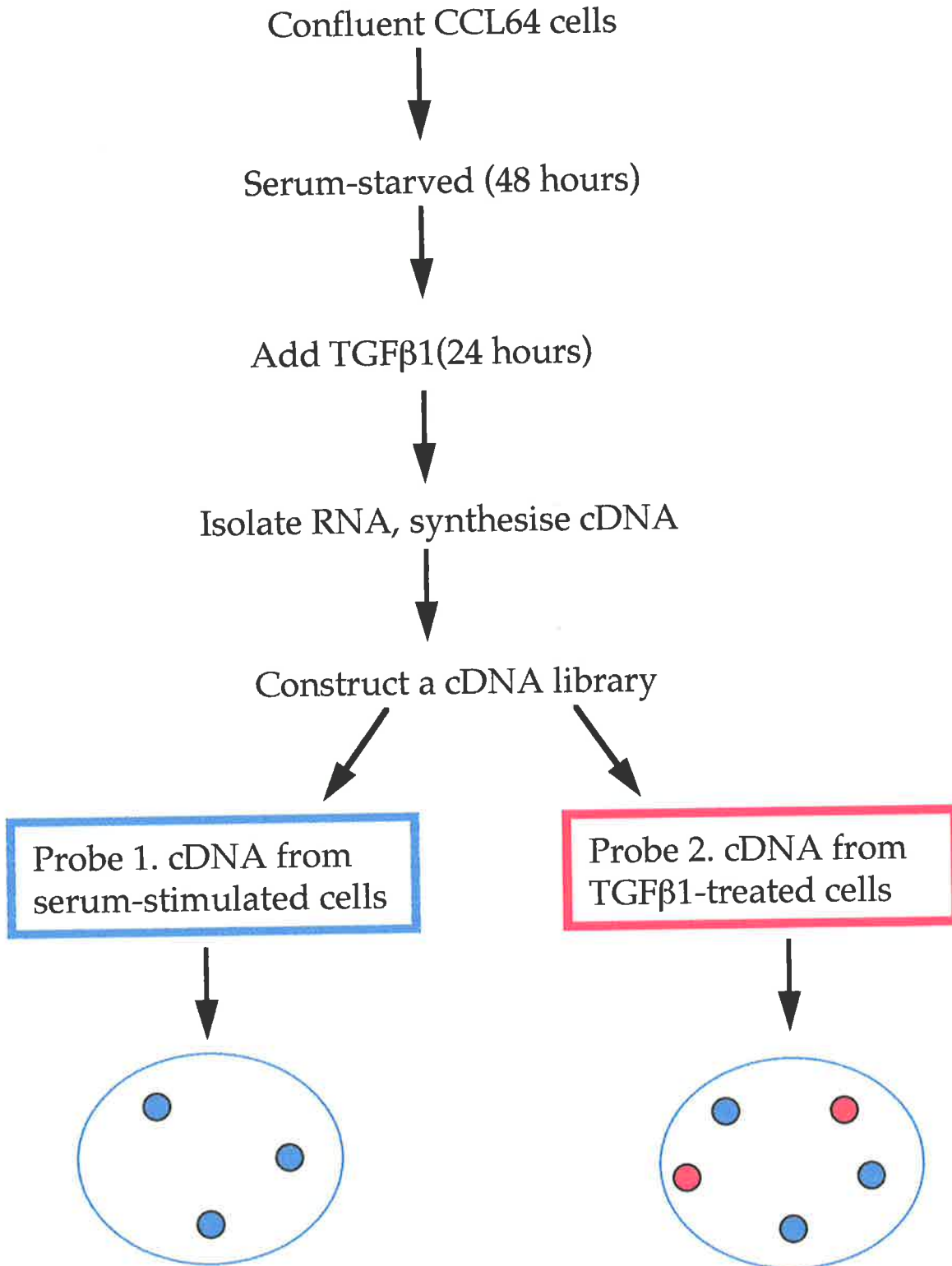
Figure 1.1
Selection of genes specifically expressed in growth-arrested and TGFβ-treated CCL64 cells using a differential screening approach. Only clones positive for Probe 2. (red) and not Probe 1. (blue) were selected.

plasminogen activator inhibitor I and the monocyte chemotactic cell-activating factor (JE gene). The only novel gene isolated by the differential screening and cDNA cloning experiments was named "TI1".

The cDNA of TI1 is 1807 base pairs in length and codes for a putative open reading frame of 260 amino acids, resulting in a protein with a molecular weight of 28,500 Daltons. The open reading frame begins at base 69 extending to base 848, which is followed by a 3' untranslated region consisting of 951 base pairs and then a poly (A) tail (Kallin et al., 1991).

1.2.1 Expression of TI1 mRNA

A series of experiments were conducted to determine the effects of TGFβ1 and various growth conditions on the expression of the TI1 gene in CCL64 cells (Kallin et al., 1991). The effect of serum starvation on the level of expression of TI1 mRNA was analysed by growing CCL64 cells for 3 days , then starving the cells for 2 subsequent days followed by serum-stimulation of the cells. RNA was isolated from these cells daily and then at 1, 2, 4 and 6 hours after the cells were serum-stimulated. An increase in TI1 mRNA expression was observed from day 1 to day 3, as the cells became more confluent. The levels of expression of TI1 mRNA remained high when the cells were placed in serum-starvation conditions. However, after serum-stimulation, the level of TI1 mRNA expression decreased dramatically to a basal level within one hour. When TGFβ1 was added to growing CCL64 cells, the expression of TI1 was transiently increased. However, when TGFβ1 was added to growth-arrested CCL64 cells, the expression

Figure 1.2   TI1 mRNA expression studies

A.   When CCL64 cells were serum-starved, the expression of TI1 was
      increased.

B.   When TGFβ was added to growing CCL64 cells, the expression of
      TI1 was transiently increased.

C.   When TGFβ was added to CCL64 cells in serum-starvation
      conditions, the expression of TI1 was transiently decreased.

- **Effect of serum starvation**
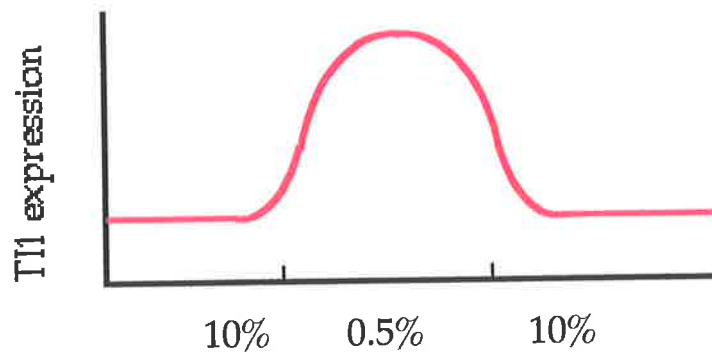
- **Effect of TGFβ on growing cells**
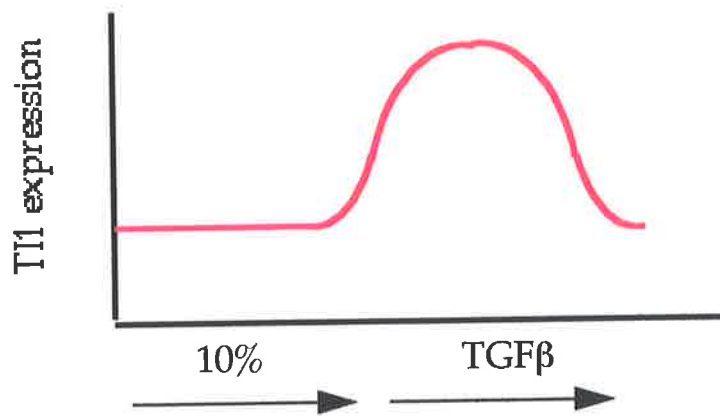
- **Effect of TGFβ on growth-arrestedcells**
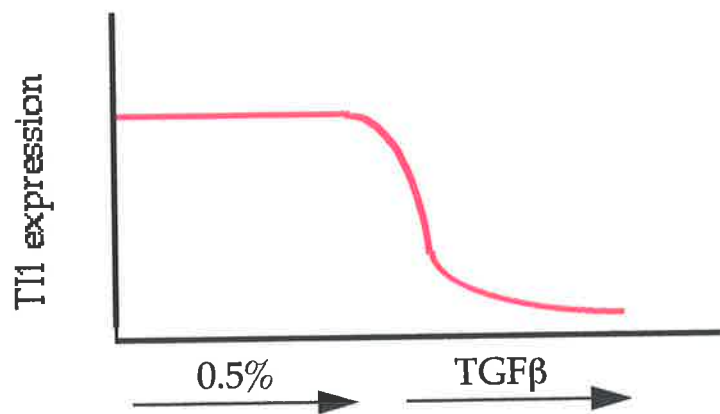
of TI1 was transiently reduced (Figure 1.2). These experiments indicate that the expression of the mink TI1 gene is regulated by both TGFβ1 and by growth arrest induced by confluence and serum deprivation but the exact mechanism of this regulation is unknown.

1.3 STRUCTURAL FEATURES OF THE MAMMALIAN BLADDER

1.3.1 Asymmetric unit membrane

It was discovered in 1994 that the bovine uroplakin Ib protein, expressed in bovine urothelium, had considerable sequence homology to the mink TI1 putative protein (Yu et al., 1994). The putative protein product of the mink TI1 gene has 93% amino acid sequence homology with the bovine uroplakin Ib (UPKIb) protein and this high degree of sequence identity suggested that UPKIb was the bovine homologue of the mink TI1 gene (Yu et al., 1994). The uroplakin Ib gene was discovered in a search for the protein constituents of the asymmetric unit membrane (AUM) of the bovine bladder. The urothelium is composed of three urothelial cell layers, the basal cells, the intermediate cells and the umbrella cells, where expression of the uroplakins is confined to the umbrella cells (Figure 1.3). The structural characteristics of the bladder urothelium are associated with its particular role as an accommodating tissue structure and a permeability barrier. Urothelium is derived from the endoderm and is normally considered a slow turn-over epithelium (Marceau, 1990), however, rapid proliferation is a feature of the bladder epithelium during fetal development. The mammalian urothelium elaborates, as a terminal differentiation product, a plasma membrane
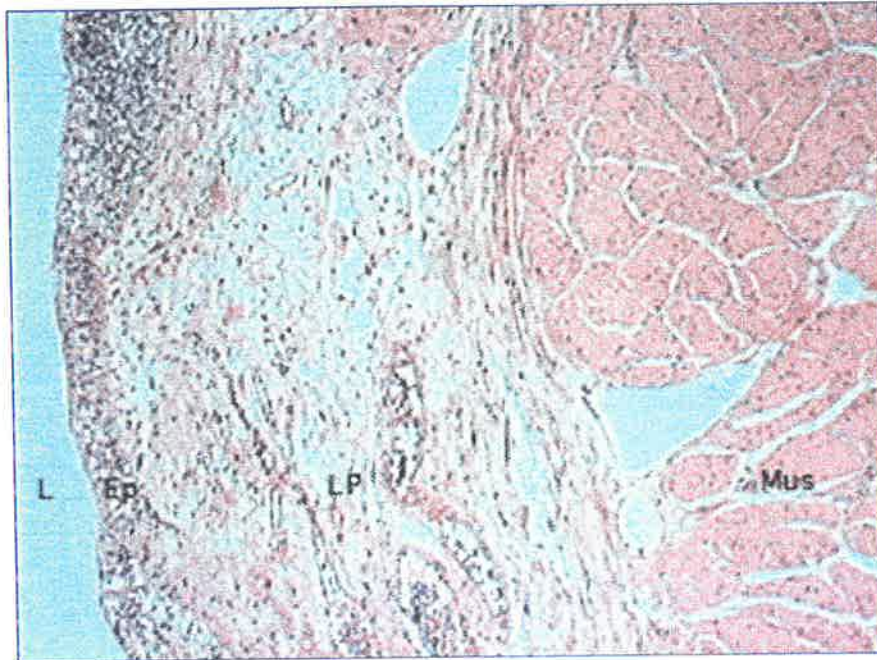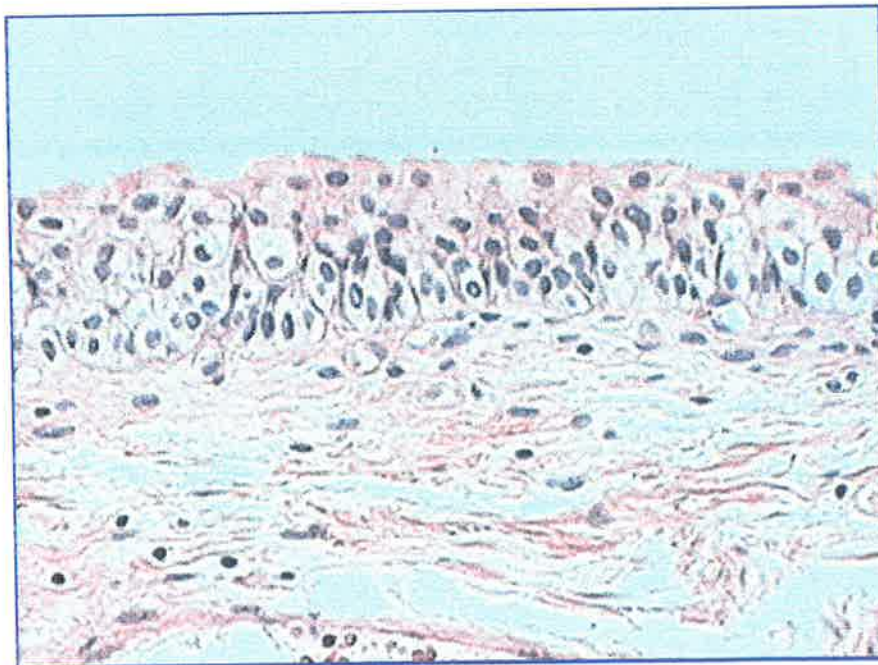
Figure 1.3
A. Histology of the normal bladder. The lumen of the bladder (L) is on the left and lining the bladder wall is the epithelium (Ep), also known as urothelium. Beneath the epithelium is the connective tissue called the lamina propria (LP). The bladder musculature is labelled (Mus).

B. Histology of the normal bladder. The urothelium is made up of three layers. The most superficial cells of the urothelium are the umbrella cells which cover over several underlying urothelial cells, including the intermediate and basal cells.

(This figure was taken from Burkitt, 1996)

called the asymmetric unit membrane. The AUM is so-called because its outer, luminal leaflet (8nm) is almost twice as thick as the inner, cytoplasmic membrane leaflet (4nm) (Hicks, 1965, Koss, 1969). The thickened outer leaflet of the membrane is composed of semi-crystalline, hexagonal arrays of 12nm particles. These tightly-packed particles protrude above the luminal membrane and account for the thickness of the outer membrane leaflet (Hicks et al., 1970, Knutton et al., 1976). The plaque-associated particles are believed to play a role in establishing a permeability barrier between the hypertonic urine stored in the bladder and the adjacent tissues (Hicks, 1966a). These plaques may also strengthen the urothelial apical surface through its interactions with an underlying cytoskeleton, to prevent rupturing of the membrane during bladder distention (Hicks, 1965; Hicks, 1966b).

1.3.2 Uroplakins

The protein composition of the AUM has been studied by a number of groups, initially with conflicting results. Some studies have attempted to detach the AUMs from the underlying cytoskeleton with reducing agents and then purify the proteins by sucrose gradient centrifugation (Ketterer et al., 1973, Stubbs et al., 1979, Vergara et al., 1974, Caruthers et al., 1977). However, the protein patterns have varied greatly between these different AUM fractions and there is no evidence that these proteins were AUM-associated as no antibodies were available at that time. Other studies have used an immunological approach to identify the individual protein components of the AUM. Yu et al., (1990) generated a monoclonal antibody to the apical surface of bovine urothelium. This antibody, AE31, identified a urothelial-specific 27 kD protein, which was

named uroplakin I. Uroplakin I co-purified with a 15 kD and a 47 kD protein, named uroplakins II and III respectively. These three proteins were individually identified by the analysis of highly purified bovine AUM plaques by sucrose gradient centrifugation and detergent treatment and subsequent SDS-PAGE analysis of these membrane preparations. Immunoelectron microscopy determined that all three proteins were associated *in situ* with the AUM. These proteins are called uroplakins because they are associated with the *uro*thelial *pla*ques, which make up the AUM (Wu et al., 1990).

1.3.3 Uroplakin Ia and Ib

Initially, the bovine AUM was thought to consist of only 3 major proteins, uroplakin I, II, and III. However, a further biochemical and molecular cloning study by Yu et al., (1994) found that uroplakin I was composed of two separate protein components of sizes 27 kD and 28 kD. The 27 kD glycoprotein was named uroplakin Ia and the 28 kD protein, uroplakin Ib. These two proteins are 39% identical in their amino acid sequences and are both members of the transmembrane 4 superfamily (TM4SF), also called the tetraspan family (Chapter 1.4). The uroplakin I genes were cloned from a bovine urothelium cDNA library which was probed with PCR products amplified using degenerate primers designed from known amino acids of the uroplakin I proteins.

The uroplakin Ib gene (the bovine homologue of mink TI1) transcribes a 1,965 bp mRNA sequence which codes for a 260 amino acid protein. Hydropathy plots reveal the UPKIb protein consists of four regions of hydrophobic amino acids which are presumed to form transmembrane domains (Figure 1.4). The first and

ASYMMETRICAL MASS DISTRIBUTION OF UROPLAKINS I, II AND III
IN ASYMMETRICAL UNIT MEMBRANE
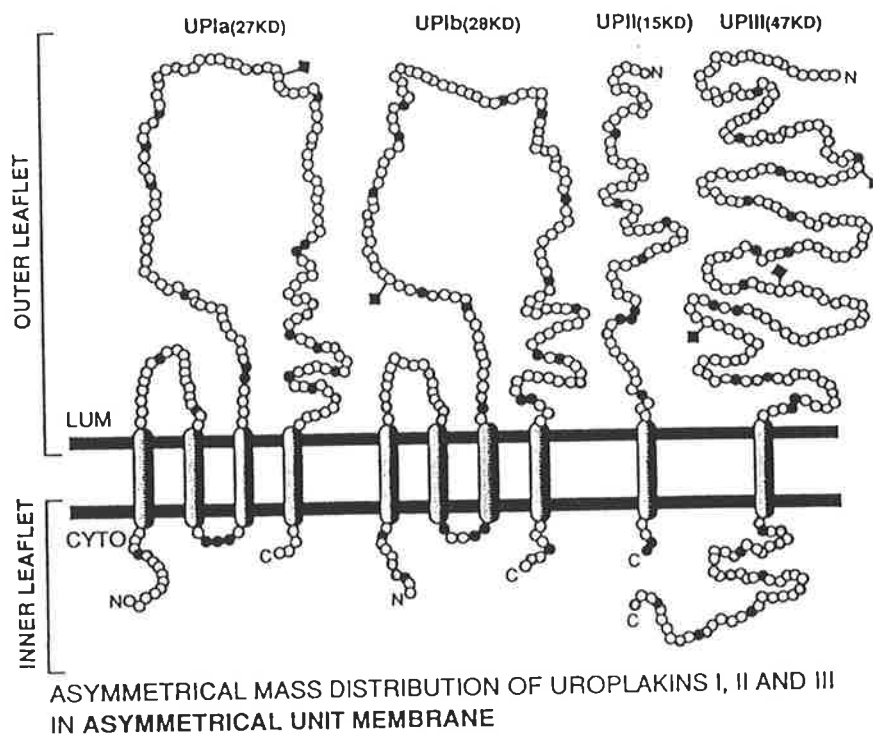
Figure 1.4
Schematic diagram of the four bovine uroplakin proteins, uroplakin Ia, Ib, II and
III. Uroplakin Ia and Ib have four transmembrane domains. Uroplakin II and III
are single transmembrane proteins. Uroplakins Ia, Ib and II have very short
cytoplasmic domains. The majority of the uroplakin III protein is also
extracellular. N and C denote the amino and carboxy termini respectively. Filled
boxes denote potential N-glycosylation sites and filled circles represent positively-
charged amino acids. The lipid bilayer is represented by the two horizontal bars,
with the luminal (*Lum*) and cytoplasm (*Cyto*) leaflets labelled.

(This figure was taken from Yu et al., 1994).

second transmembrane domains (TMD) are connected by 17 amino acids, while the second and third TMDs are separated by a short highly charged region of five amino acids. The third and fourth TMDs are connected by a long hydrophilic loop of 121 amino acids, which includes the putative N-linked glycosylation site (Yu et al., 1994). The UPKIb gene has been localised to bovine chromosome 1 by bovine x rodent somatic cell hybrids (Ryan et al., 1993).

1.3.4 Bladder-specific expression of uroplakins

Both UPKIa and UPKIb mRNAs have been readily detected in bovine bladder tissue by Northern analysis. Other bovine tissues, including esophageal epithelium, snout epithelium, intestinal epithelium, liver, kidney (excluding renal pelvis), lung and brain do not express UPKIa or UPKIb transcripts that are detectable by Northern analysis. This bovine bladder-specific expression contradicts the work by Kallin and colleagues who isolated the TI1 gene from lung epithelium (Kallin et al., 1991). However, the lung cells are derived from a cell line and not primary tissue, so may not reflect the normal expression pattern of TI1, but this does not change the fact that the TI1 gene has some level of expression in mink lung cells. The uroplakin III protein was not detected in human lung tissue by immunohistochemistry (Moll et al., 1995), however it is also possible, that different species have different tissue-specific patterns of expression of uroplakins or that there may be deregulated expression in cultured cells. For example, the rat mast cell antigen, AD1, the rat homologue of the human tetraspan gene, CD63, is expressed in other cell lineages in culture including fibroblasts and hepatocytes (Nishikata et al., 1992). The promoter region of the mouse uroplakin II gene is capable of driving expression of a foreign

gene in both the bladder and the hypothalamus. This study used a 3.6 kb 5′ flanking sequence of the mouse uroplakin II gene, in transgenic mouse experiments, to drive the expression of a bacterial *lacZ* reporter gene in urothelial tissue. The transgene product was also detected in the hypothalamus, but not any other tissue analysed (Lin et al., 1995).

1.3.5 Interactions of the uroplakin proteins

The uroplakin Ia protein forms a complex with uroplakin II and the uroplakin Ib protein has been shown to associate with the largest uroplakin molecule, uroplakin III (Wu et al., 1995). Both uroplakin II and III possess a single membrane spanning domain (Figure 1.4) and therefore do not belong to the tetraspan family of proteins. The 15 kDa uroplakin II protein is synthesised as a 19 kDa precursor protein of 185 amino acids. The precursor molecule consists of a cleavable signal peptide of 26 amino acids, a prosequence of 59 residues followed by 100 amino acids of the mature UPKII. Only the mature UPKII is present in the AUM and is capable of associating with uroplakin I proteins. Ultrastructural localisation studies suggest that the majority of the UPKII protein is exposed on the luminal side of the AUM, while the C-terminal is anchored to the membrane (Lin et al., 1994). The uroplakin III protein consists of a N-terminal, luminal domain of 189 amino acids and a C-terminal, cytoplasmic domain of 52 amino acids. Therefore the molecular mass of the luminal domain (20 kD, without glycosylation) greatly exceeds that of the cytoplasmic domain (5 kD) (Wu et al., 1993). The high extracellular/cytoplasmic mass ratio of both uroplakin II and III may contribute to the outer thickened luminal leaflet of the AUM.

A recent study revealed that, at least *in vitro*, type 1-fimbriated *E.coli.* can bind to uroplakins Ia and Ib. The uropathogenic *E.coli* which cause most urinary tract infections, expresses type 1 fimbriae containing adhesions that recognise cell receptors. Anchorage of *E.coli* to the urothelial surface via type 1 fimbriae-uroplakin I interactions may play a role in urinary bladder tract infections. The interactions between type 1 fimbriae and uroplakin I may aid bladder colonisation of *the E.coli* pathogen and allow its movement through the ureters to invade the kidneys (Wu et al., 1996b).

1.3.6 Evolutionary conservation of uroplakins

Asymmetric unit membranes have been isolated from cattle, human, monkey, sheep, pig, dog, rabbit, mouse and rat. All of these species have morphologically similar AUMs with crystalline patches of 12nm protein particles. Immunoblotting, using antibodies raised against synthetic oligopeptides or individual bovine uroplakins, established that the four uroplakins are present in the AUMs of all the abovementioned species (Wu et al., 1994). The urothelial specificity of at least one uroplakin, UPKIII, has been documented in human tissues (Moll et al., 1995).

1.3.7 Uroplakins and cancer

It has been suggested that uroplakin III may have potential as a histological marker of metastatic transitional cell carcinoma. Immunohistochemistry, using rabbit antibodies against uroplakin III, of paraffin sections of transitional cell carcinoma (TCC), detected positive reactions in all stages of bladder cancer. Of 16

papillary noninvasive TCC, 14 (88%) were positive, 53% of invasive TCCs and 66% of metastatic TCCs were positive for uroplakin III protein expression. An extensive screening of 177 primary tumour and metastatic carcinomas derived from other organs, ie. non-TCCs, revealed negative reactions for the uroplakin III antibody. The authors suggest that the uroplakin III marker could be used in cases of metastatic carcinomas of unknown origin to identify the metastasis as originating from a TCC (Moll et al., 1995).

Ogawa et al., (1996) studied the patterns of expression of uroplakins in various degrees of chemically-induced hyperplasia and carcinoma in the rat bladder. A rabbit anti-asymmetric unit membrane antibody was used which reacted strongly with uroplakin III, moderately with uroplakin Ia/Ib and weakly with uroplakin II. In control bladder sections, only the luminal surface membrane stained strongly positive. In chemically-induced simple hyperplasia, expression of uroplakins was observed in the intermediate cells as well as in the normal location on the luminal surface but the staining patterns remained orderly. In bladder carcinomas, expression patterns were disorderly and expression was absent from the luminal surface. Only a small amount of focal staining was observed in the intermediate cells. In a recent study, expression of uroplakin II was detected in only 40% of transitional cell carcinomas by immunohistochemical staining (Wu et al., 1998). These studies suggest that disruption of expression of uroplakins is involved in the progression of transitional cell carcinomas.

1.4   TRANSMEMBRANE 4 SUPERFAMILY

1.4.1 Introduction

The TI1/uroplakin Ib protein is a member of a protein family called the "transmembrane 4 superfamily", "tetraspans" or "tetraspanins" (reviewed in Wright et al., 1994 and Maecker et al., 1997). This family, as its name implies, is made up of a group of cell surface glycoproteins which span the cell membrane four times. These four hydrophobic transmembrane domains are interspersed with two hydrophilic extracellular domains and two short cytoplasmic domains (Figure 1.5). There are some defining characteristics of the tetraspan proteins which distinguish them from other four-membrane spanning proteins. The transmembrane domains, for example, include polar amino acids which are highly conserved. These charged residues include an asparagine (N) amino acid in transmembrane domain 1 and glutamate (E) or glutamine (Q) residues in transmembrane domains 3 and 4. There are three motifs in the large extracellular domain containing four cysteine residues which are highly conserved, CCG, PXSC and EGC. The cytoplasmic domains show little sequence homology between tetraspans, however, there is frequently a lysine (K) residue in the N-terminal region, a glutamate (E) residue between transmembrane 2 and 3 and an isoleucine (I) residue in the C-terminal region.

The four transmembrane domains contain most of the observed homology between the "tetraspan" family members. These domains are most likely to be involved in a signalling function common to all family members. The extracellular domains are the most diverse in length and sequence among the family members and are most likely to be involved in specific ligand binding and
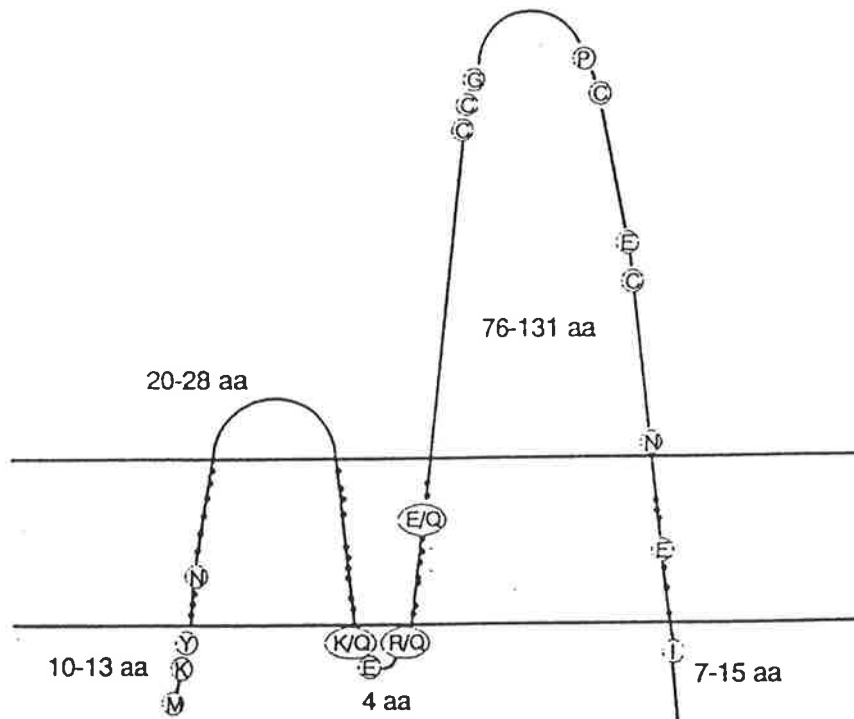
Schematic of TM4SF Protein Structures

Figure 1.5
Schematic diagram of the TM4SF proteins, showing four transmembrane domains, a small and large extracellular domain and short cytoplasmic domains. Conserved hydrophobic amino acid residues are represented by black dots and conserved hydrophilic residues are indicated in single letter code and circled.

(This figure was taken from Hemler et al., 1996).

it is the large extracellular domain which has the greatest amino acid variation. Antibody epitope mapping and glycosylation patterns have confirmed that the hydrophilic regions are extracellular, however this predicted membrane topology has yet to be proven by crystallography studies. While the tetraspans have no significant sequence homology with any other protein family, there are some structural similarities with the ligand-gated ion channels (Wright et al., 1994). The transmembrane domains are rich in polar residues which is a feature of ion channels. However, there is no evidence that the tetraspans can act as ion channels. In fact, the uroplakin Ia and Ib tetraspan proteins are unlikely to act as ion channels. The asymmetric unit membrane containing uroplakins may serve as a permeability barrier within the urothelium. Experiments show that vesicles from rabbit urothelium are unusually impermeable to urea, water and ions (Chang et al., 1994).

The transmembrane 4 superfamily members are listed in Table 1.1, and include a number of leukocyte markers as well as tumour-associated antigens. Also listed in Table 1.1 is the tissue distribution of each tetraspan and the known function of each tetraspan. While the majority of the tetraspans were identified in mammals, tetraspan proteins have been discovered in a range of species including *Schistosoma, Drosophila* and *Caenorhabditis elegans*.

Table 1.1   Members of the transmembrane 4 superfamily

Tetraspans (species)	Tissue distribution	Function	Reference
CD63 (human and rat)	platelets, monocytes and nonlymphoid cells	Induces adhesion, suppresses metastasis in melanoma	Hotta et al., (1988)
CD9 (human, monkey, rat, cat and cow)	platelets, pre-B and activated T cells, neural cells	Regulates motility, activation and adhesion	Ikeyama et al., (1993)
KAI1 (human)	lymphoid and myeloid cells, most tissues	Suppresses metastases in prostate cancer	Dong et al., (1995)
CD53 (human and rat)	lymphocytes, monocytes and granulocytes	Induces signal transduction	Gonzalez et al., (1993)
CD37 (human)	mature B cells	Antibodies induce homotypic aggregation of B cells	Barrett et al., (1991)
TAPA-1 (human, rat and mouse)	lymphocytes	B cell activation, induces T cell maturation	Takahashi et al., (1990)
L6 (human, hamster and mouse)	lung, breast, colon and ovarian carcinomas	Unknown	Hellstrom et al., (1986a)
SAS (human)	sarcomas	Amplified in sarcomas	Meltzer et al., (1991)
CO-029 (human)	colon carcinoma	Tumour-associated antigen	Szala et al., (1990)
TALLA-1 (human)	T cells	Associated with T-ALL	Emi et al., (1993)
UPKIa (cow)	urothelium	Bladder differentiation	Yu et al., (1994)
Sm23 (S.mansoni)	Schistosome membrane	Unknown	Reynolds et al., (1992)
PETA-3 (human and mouse)	most tissues, except brain	Platelet activation	Fitter et al., (1995)
IL-TMP (human)	intestinal epithelial cells	Associated with proliferation of cells in the crypt-villus	Wice et al., (1995)
late bloomer (Drosophila)	neurons	Promotes synapse formation	Kopczynski et al., (1996)
YKK8 (C.elegans)	C.elegans membrane	Unknown	Tomlinson et al.,(1996b)
Sj25/TM4 (S.japonicum)	Schistosome membrane	Unknown	Fan et al., (1997)
NAG-2 (human)	fibroblasts, endothelial cells	Unknown	Tachibana et al., (1997)
TM4SF5 (human)	pancreatic cancer	Unknown	Muller-Pillasch et al., (1998)
Tspans 1-6 (human)	various	Unknown	Todd et al., (1998)

The chromosomal location of those tetraspan genes which have been mapped are listed in Table 1.2. Only the human chromosomal location of each tetraspan gene is listed.

Table 1.2   Chromosomal location of the tetraspan genes

Tetraspan	Chromosomal location	Reference
CD63	12q12-14	Hotta et al., (1988)
KAI1	11p11.2	Dong et al., (1995)
CD53	1p13	Gonzalez et al., (1993)
CD9	12p13	Benoit et al., (1991)
CD37	19p13-q13.4	Virtaneva et al., (1993)
CD81	11p15.5	Virtaneva et al., (1994)
L6	3q21-25	Virtaneva et al., (1994)
SAS	12q13-14	Meltzer et al., (1991)
A15	Xq11	Virtaneva et al., (1994)
SFA-1	11p15.5	Hasegawa et al., (1997a)
TM4SF5	17p13.3	Muller-Pillasch et al., (1998)

1.4.2 CD63

CD63 was originally identified as a platelet antigen (Metzelaar et al., 1991) and independently as the melanoma antigen, Me491 (Hotta et al., 1988). CD63 is a major component of platelet lysosomal membranes and appears on the surface of activated platelets (Metzelaar et al., 1991). The 1.2 kb CD63 mRNA transcript codes for a protein of 237 amino acids with three potential N-glycosylation sites

(Hotta et al., 1988). The human CD63 gene consists of eight exons spanning 4 kb. (Hotta et al., 1992). Human CD63 and mouse CD63 share 79.4% homology at the amino acid level, whereas there is 95.4% amino acid homology between mouse and rat CD63 (Miyamoto et al., 1994).

Functional analysis of the CD63 protein has revealed its association with $\alpha3\beta1$ and $\alpha6\beta1$ integrins in the human fibrosarcoma HT1080 and the human erythroleukemia K562 cell lines (Berditchevski et al., 1995). On the surface of melanoma cells, the CD63 protein is associated with two other tetraspan molecules, CD9 and CD81 and with $\beta1$ integrins (Radford et al., 1996). These results suggest that CD63 is capable of forming multicomponent complexes with tetraspan proteins and $\beta1$ integrins. A study investigating the effect of CD63 monoclonal antibodies (mAbs) on neutrophil adhesion to human umbilical vein endothelial cells (HUVECs), revealed that CD63 mAb binding to the neutrophil surface triggers a transient activation signal that requires extracellular calcium and regulates the adhesive activity of CD11/CD18. These leukocyte markers, CD11 and CD18, which are major mediators of cell-cell adhesion in neutrophils, were found to be associated with the CD63 protein (Skubitz et al., 1996).

The potential role of CD63 in cancer has been investigated in melanoma. Although different levels of expression of CD63 were observed in a number of melanoma cell lines by Northern analysis, the human CD63 gene is not rearranged or amplified in melanoma (Hotta et al., 1988). The growth rate in nude mice of H-ras-transfected NIH3T3 cells, was decreased when the cells were co-

transfected with a plasmid expressing the human CD63 gene (Hotta et al., 1991). The CD63 gene, transfected into a CD63-negative human melanoma cell line, reduced the number of metastatic nodules formed in the lungs of nude mice, as well as suppressing the growth of the primary tumour. However, the expression of the CD63 gene had no impact on the growth of the melanoma cells *in vitro* (Radford et al., 1995). These results suggest that CD63 suppresses the growth of human melanoma and transformed mouse fibroblast cells *in vivo* and reduces metastasis. Recent studies have shown that expression of CD63 in melanoma cell lines suppressed random tumour cell motility, but increased migration and adhesion toward the β1 integrin substrates, fibronectin, laminin and collagen (Radford et al., 1997). CD63 may be therefore involved in the regulation of the motility of melanoma cells and their adhesion and migration on substrates associated with β1 integrins.

1.4.3 CD9

Expression of the CD9 protein has been detected on a variety of cells including platelets, murine mature T cells, rat neural cells and mouse myeloid cells (Tai et al., 1996, Kaprelian et al., 1995, Oritani et al., 1996). The human CD9 gene transcribes a 1.4 kb mRNA which codes for a protein of 227 amino acids which contains one potential glycosylation site (Bouchiex et al., 1991). The human CD9 gene contains 8 exons and spans more than 20 kb in length (Rubinstein et al., 1993a). The molecular cloning of the bovine and murine CD9 genes revealed amino acid homologies of 83.5% and 89% to the human CD9 protein for bovine and mouse respectively (Martin-Alonso et al., 1992, Rubinstein et al., 1993b).

CD9 may directly regulate cell motility as its overexpression in a CD9-positive human lung adenocarcinoma cell line (MAC10) and a CD9-negative human myeloma cell line (ARH77) results in suppressed cell motility as detected by penetration and phagokinetic track assays (Ikeyama et al., 1993). However, a more recent study has found conflicting results in a CD9-negative, human B cell line, Raji, where CD9 was found to enhance cell motility across laminin and fibronectin-coated filters (Shaw et al., 1995). These results suggest that CD9 does regulate cell motility, either enhancing or inhibiting motility depending on the cell type and other protein factors involved. The CD9 antigen may inhibit cell migration by associating with the β1 chain of integrins as shown with the pre-B cell line, NALM-6 and the megakaryocytic cell line, HEL (Rubinstein et al., 1994). CD9 may also regulate cell adhesion as ligation with anti-CD9 antibody promotes homotypic adhesion of the pre-B cell lines NALM-6 and HOON (Masellis-Smith et al., 1990). Fibronectin receptors on pre-B cells were induced after addition of an anti-CD9 monoclonal antibody which also stimulated platelet and pre-B cell aggregation (Massellis-Smith et al., 1994).

CD9 also associates with integrins α3β1 and α6β1 on the cell surface of the rat neural S16 Schwann cells (Hadjiargyrou et al., 1996). CD9 was found to be co-localised with the platelet receptor for fibronectin (αIIbβ3 integrin) on the inner face of α–granule membranes and on pseudopods of activated platelets (Brisson et al., 1997). The CD9-αIIbβ3 integrin complex is also found in resting, inactive platelets and may physically associate together via hydrophobic interactions (Indig

et al., 1997). Anti-CD9 provides a stimulatory signal, independent of CD28, to T cells in the absence of antigen-presenting cells (Tai et al., 1996). A further study suggests that CD9 and CD28 induce T cell co-stimulation using different signalling pathways, therefore creating a synergy in T cell activation (Toyo-oka et al., 1997). CD9 may therefore function as a key component of receptor signalling complexes in both immune and neural cells by regulating important cell functions such as cell motility, activation, adhesion and aggregation.

Studies in a variety of tumours support the hypothesis that CD9 is a negative regulator of a number of cellular functions including cell motility. The metastatic behaviour of a mouse melanoma cell line (BL6) transfected with CD9 was reduced in comparison to the parental cell line (Ikeyama et al., 1993). CD9 was also found to be expressed predominantly on primary melanoma rather than on metastatic tumours, suggesting a possible metastasis suppressor role for CD9 (Si et al., 1993). Immunohistochemical studies of the CD9 antigen in breast carcinoma, has revealed that almost 50% of patients have CD9 protein levels that are lower in metastatic lymph nodes than in corresponding matched primary tumours, suggesting that low CD9 expression may be associated with the metastatic potential of breast cancer (Miyake et al., 1995). Further studies in breast carcinoma indicate that a reduction in the expression of CD9 in tumours is strongly associated with an increased risk of recurrence among node-negative (N0) and N1-stage disease and with early-stage T1 and T2 tumours (Miyake et al., 1996). Low CD9 expression by non-small cell lung carcinomas may also be associated with poor prognosis. An RT-PCR-based study showed that the overall survival rate of lung adenocarcinoma patients with CD9-positive tumours was better than

those with reduced CD9 expression (Higashiyama et al., 1995). Expression levels of CD9, detected by immunohistochemistry, solely in the lung adenocarcinoma cells within the tumour tissue were inversely associated with nodal involvement and tumour stage (Higashiyama et al., 1997). A differential display study identified CD9 as highly expressed in primary tumours compared to metastatic human colon carcinoma cells. High expression levels of CD9 were correlated with a high migratory potential of primary colon cancer cells, assessed using Matrigel migration chambers (Cajot et al., 1997). This study is in direct contradiction to other CD9 studies described above whose findings have suggested that maintenance of CD9 expression is important to prevent cancer recurrence and reduce metastatic potential.

1.4.4 KAI1/CD82/R2/4F9/IA4/C33

The KAI1 (*kang ai*, Chinese for anticancer) gene has been cloned independently from four different aspects of its function. Originally, KAI1 was isolated by subtractive hybridisation of a cDNA library of phytohemagglutinin (PHA)/ phorbol myristate acetate (PMA)-stimulated Jurkat cells and a cDNA library of PHA-stimulated peripheral blood lymphocytes to isolate genes that are specifically inducible in normal lymphoid cells. The new clone was named R2 and its expression was found to be transiently induced in T and B cell lines after functional activation with PHA/PMA (Gaugitsch et al., 1991). In 1992, KAI1 was cloned as IA4, which was up-regulated upon activation of B lymphocyte cell lines (Gil et al., 1992). Independently, the protein 4F9 (KAI1) was found to be a co-stimulatory molecule for the proliferation of human T cells (Nojima et al., 1993). Later, it was discovered that this co-stimulatory molecule was CD3, which led to

strong IL-2 production and T-cell differentiation (Lebel-Binay et al., 1995). KAI1 was also isolated as the membrane antigen C33, which was capable of inhibiting syncytium formation in a human T-cell line (Fukudome et al., 1992). C33 forms a complex with another tetraspan protein, TAPA-1 and further associates with CD4 or CD8 in T cells (Imai et al., 1993). KAI1 is also associated with HLA class I molecules at the surface of human B cells (Lagaudriere-Gesbert et al., 1997).

The KAI1 protein is made up of 267 amino acids and has 76% amino acid identity with the mouse KAI1 protein (Nagira et al., 1994). The KAI1 gene spans about 80 kb of DNA consisting of 10 exons which transcribes a mRNA of 2.4 kb (Dong et al., 1997).

An independent study isolated the KAI1 gene as a metastasis suppressor gene on chromosome 11p11.2. Transfection of the KAI1 gene into rat AT6.1 prostate cancer cells reduced the number of lung metastases, compared to the parental line, when these cells were injected into nude mice (Dong et al., 1995). Overexpression of KAI1 did not affect the growth rate of the primary tumour. The KAI mRNA is expressed in many tissues, with the most abundant message levels in prostate, lung, liver, bone marrow and placenta. KAI1 protein levels are down-regulated during the progression of prostate cancer. The down-regulation of expression of KAI1 was not primarily caused by mutation or allelic loss of the KAI1 gene in a study of American patients (Dong et al., 1996). However, a study investigating prostate cancers in Japanese patients did find loss of heterozygosity at the D11S1344 locus, a polymorphic marker at 11p11.2 in seven out of ten

informative metastatic tumours, suggesting a race-related mutation (Kawana et al., 1997).

Expression levels of KAI1 mRNA have also been investigated in cancers of the pancreas, lung, breast and bladder. KAI1 mRNA expression is increased in 89% of pancreatic cancer samples in relation to levels of expression of KAI1 mRNA in normal pancreatic tissue. However, tumours in advanced stage had significantly lower expression levels of KAI1 than early stage tumours (Guo et al., 1996). A retrospective study of KAI1 mRNA expression levels by RT-PCR, suggested that high expression of KAI1 was correlated with low metastatic potential of non-small cell lung cancer. The overall survival rate over an average of 3 years of lung cancer patients with KAI1-positive tumours (77%) was significantly higher than those with KAI1-negative tumours (38%) (Adachi et al., 1996). Reduced levels of expression of KAI1 mRNA have been observed in metastatic human breast cancer cell lines (Yang et al., 1997) and also correlates with invasive bladder cancer (Yu et al., 1997). These findings suggest that loss of expression of the KAI1 gene is a poor prognostic factor in many cancers and is associated with high metastatic potential.

1.4.5 CD53

The CD53 gene consists of eight exons spanning at least 26 kb of genomic DNA (Korinek et al., 1993). The human CD53 protein has 82% amino acid homology with its mouse CD53 homologue (Wright et al., 1993). Epitope mapping of anti-rat CD53 monoclonal antibodies have confirmed the proposed membrane

orientation of the tetraspans with the hydrophilic domains of the protein located on the extracellular membrane (Tomlinson et al., 1993).

OX-44, the rat homologue of CD53, was isolated because of its ability to activate the phosphatidylinositol signalling pathway in RNK-16 (rat leukemia cell line with natural killer activity) cells (Bell et al., 1992). OX-44 co-precipitates with CD2 in RNK16 and rat splenic T cells, indicating that CD53 and CD2 form a physical association on the cell surface (Bell et al., 1992). Cross-linking of CD53, by an anti-CD53 antibody has been found to induce activation of human monocytes and B cells (Olweus et al., 1993). The CD53 protein has been implicated in signal transduction in rat macrophages, as antibody ligation induces the production of nitric oxide via a protein kinase C-dependent pathway (Bosca et al., 1994). Immune complexes of rat CD53 contains tyrosine phosphatase activity in rat lymph node cells and the rat W/Fu (C53NT)D thymoma cell line, although the phosphatase identity is unknown (Carmo et al., 1995).

1.4.6 CD37

CD37 is highly expressed on mature B cells but not on pre B-cells. Expression of CD37 is also found on the surface of some T cells and myeloid cells (Schwartz-Albiez et al., 1988). Antibodies to CD37 induce homotypic aggregation of human tonsillar B cells and B cell lines suggesting CD37 may be involved in cell-cell adhesion (Barrett et al., 1991). The mRNA transcript of CD37 is 1.2 kb in length and the human CD37 protein consists of 244 amino acids with 3 potential N-glycosylation sites (Classon et al., 1989). The mouse CD37 protein has 79% amino acid homology with human CD37 (Tomlinson et al., 1996a).

1.4.7 CD81/TAPA-1

The TAPA-1 antigen was first identified as a target for an antiproliferative antibody raised against a human B cell lymphoma and is also named CD81. The 26 kDalton (kDa) TAPA-1 protein is expressed on normal B and T lymphocytes, chronic and promyelocytic leukemia cells as well as on neuroblastoma and melanoma cells (Oren et al., 1990). TAPA-1 is non-covalently associated with the 16 kDa Leu-13 antigen on the cell surface of hematolymphoid cells (Takahashi et al., 1990). The TAPA-1 protein has 92% amino acid homology between mouse and human TAPA-1 (Andria et al., 1991).

Experimental results suggest that CD81 may have a role in early T cell development. The immature thymocytes isolated from fetal thymus organ cultures, which were treated with anti-CD81 antibodies, could not differentiate from CD4⁻CD8⁻ to CD4⁺CD8⁺ (Boismenu et al., 1996). CD81 is physically associated with CD4 and CD8 T cell receptors and crosslinking of CD81 with CD3 on T cells promotes DNA synthesis (Todd et al., 1996). CD81 may also be important for B cell activation as it has a co-stimulatory role as part of the CD21/CD19/Leu 13 complex (Fearon et al., 1995). A recent study investigating CD81-deficient (exons 2-8) mice revealed that the T cells are normal, disputing the importance of CD81 in T cell development. However, these mice have abnormal B cells with a decreased expression of CD19, suggesting CD81 is important for CD19 signalling and B cell function (Tsitsikov et al., 1997).

1.4.8 L6

The L6 cell surface antigen is highly expressed on lung, breast, colon and ovarian carcinomas. L6 expression was not found on peripheral blood lymphocytes or B or T cell lines (Hellstrom et al., 1986a). Mouse monoclonal L6 antibodies can lyse L6 antigen-positive human tumour cells in the presence of human complement. L6 antibody can inhibit the outgrowth of an L6 antigen-positive human tumour transplanted into nude mice. However, the L6 antibody only reacted weakly and infrequently with cells from normal tissues (Hellstrom et al., 1986b). This marker is therefore potentially important as a target for cancer therapy as its expression is markedly increased in a number of carcinomas. A phase I clinical study of patients with either recurrent breast, colon, lung or ovarian cancers showed that the mouse L6 antibody was well tolerated and was localised to the tumour *in vivo* (Goodman et al., 1990). Despite these early encouraging reports, no further reports of L6 clinical studies have appeared in the literature.

The L6 protein consists of 202 amino acids with two potential N-glycosylation sites. The L6 mRNA transcript is 1188 bp in length but a longer transcript of 1.8 kb is also detected by Northern analysis, suggesting alternate splicing. Southern blot analysis of a lung cancer and melanoma cell line using a L6 probe revealed no gross genomic rearrangements of the L6 gene (Marken et al., 1992). The mouse L6 protein shares 78% amino acid homology with its human counterpart (Marken et al., 1994).

1.4.9 SAS

Amplification of the chromosome region 12q13-14 occurs frequently in malignant fibrous histiocytoma (MFH), liposarcoma and other soft tissue sarcomas. SAS (sarcoma amplified sequence) was cloned from a MFH tumour specimen as a novel transcribed sequence from a 12q13-14 DNA amplification unit (Meltzer et al., 1991). Amplification of the SAS gene was detected by Southern blot analysis in 32% of MFH cases and in two liposarcomas (Smith et al., 1992). The SAS gene is frequently co-amplified with the CDK4 gene in malignant gliomas and these two genes map less than 10 kb from each other (Reifenberger et al., 1996). Marked overexpression of SAS is also seen in osteosarcoma and neuroblastoma cell lines (Jankowski et al., 1994).

The SAS gene codes for a 210 amino acid polypeptide with four potential N-linked glycosylation sites. Two SAS mRNA species are transcribed, 1.7 kb and 0.9 kb, presumed to be the result of alternative splicing (Jankowski et al., 1994). The SAS gene consists of six exons with the entire gene covering only 3.2 kb of DNA (Jankowski et al., 1995).

1.4.10 CO-029

The tumour-associated antigen CO-029 is another member of the transmembrane 4 superfamily. The CO-029 cDNA was cloned from the human colorectal carcinoma cell line, Sw948 by immunoselection of cDNA clones using the monoclonal antibody CO-029. The 1.15 kb mRNA transcript of CO-029 was detected in colorectal and rectal carcinoma cell lines. The CO-029 protein has one

potential N-glycosylation site and consists of 237 amino acids (Szala et al., 1990). The 32 kDa protein is expressed on gastric, colon, rectal and pancreatic carcinomas, but not on the corresponding normal tissues (Sela et al., 1989).

1.4.11 A15/TALLA-1/CCG-B7

The A15 gene has been cloned independently on three separate occasions as A15, TALLA-1 and CCG-B7. The A15 cDNA clone was isolated from the immature human T cell line HPB-ALL and is expressed on immature but not mature T cells (Emi et al., 1993). The TALLA-1 gene was cloned from the T-cell acute lymphoblastic leukemia (T-ALL) cell line Molt-4 and designated TALLA-1 (from T-ALL-associated antigen). TALLA-1 is expressed mainly in the brain, skeletal muscle and spleen and also in neuroblastoma cell lines (Takagi et al., 1995). The CCG-B7 cDNA clone was isolated from a brain cDNA library using an oligonucleotide probe containing ten repeats of the CCG trinucleotide as a probe in a study searching for novel genes expressed in the human brain which contain triplet repeats. The CCG-B7 clone contained seven uninterrupted triplet repeats of CCG nucleotides. A single CCG triplet is conserved in almost all tetraspan proteins in the large extracellular domain of each tetraspan protein. The significance of finding seven uninterrupted triplet repeats in a tetraspan gene is not clear (Li et al., 1993).

The A15 gene transcribes a 2 kb mRNA and codes for a 244 amino acid protein (Emi et al., 1993). Southern blot analysis of the TALLA-1 gene in T-ALL cell lines showed no gross gene rearrangement of TALLA-1 (Takagi et al., 1995).

1.4.12 Uroplakin Ia

The bovine uroplakin Ia protein is a differentiation product of the asymmetric unit membrane of the bladder (Yu et al., 1994). The bovine uroplakin Ia gene transcribes a 1,363 bp mRNA which codes for a 258 amino acid protein, containing one potential N-linked glycosylation site. Uroplakin Ia has 39% amino acid homology with uroplakin Ib, the bovine homologue of TI1. Uroplakin Ia was discussed in further detail in Chapter 1.3.3.

1.4.13 Sm23

The Sm23 gene codes for an integral membrane protein of *Schistosoma mansoni*, a human trematode parasite. The Sm23 gene spans 2264 bp, consisting of 5 exons and 4 introns. (Lee et al., 1995). The Sm23 protein has one potential N-glycosylation site and contains 218 amino acids. Epitope mapping revealed that Sm23 protein is highly immunogenic in mice, especially in the large hydrophilic extracellular domain of the protein (Wright et al., 1990). Both B and T cell epitopes were recognised by Sm23 (Reynolds et al., 1992). There are species homologues of Sm23 in *Schistosoma japonicum* (Sj23) and *Schistosoma haematobium* (Sh23), both Sj23 and Sh23 also belong to the tetraspan family (Davern et al., 1991, Inal et al., 1995). The existence of tetraspan proteins in such a diverse species from humans as the *Schistosoma* parasite suggests an important role for the tetraspans.

1.4.14 PETA-3/SFA-1/CD151

The PETA-3 (platelet-endothelial cell tetraspan antigen-3) cDNA was cloned from the megakaryoblastic leukemia cell line, MO7e and codes for a 253 amino acid protein. Expression of PETA-3 mRNA was detected by Northern analysis in all tissues, except for brain (Fitter et al., 1995). PETA-3 may regulate platelet activation and mediator release as a component of the FcγRII signal transducing complex in platelets (Roberts et al., 1995).

PETA-3 was also independently cloned as SFA-1 (SF-HT-activated gene-1) from an adult T-cell leukemic cell line. The expression of SFA-1 (CD151) was induced by human T-cell leukemia virus type 1 in lymphoid cells (Hasegawa et al., 1996). Sequence comparison of the two cDNA clones, PETA-3 and SFA-1 revealed identical sequence except for a 62 bp deletion of the PETA-3 gene in the 5' untranslated region. The authors suggest that the SFA-1 and PETA-3 mRNA transcripts arise through alternative splicing (Hasegawa et al., 1997b). There is 93% amino acid homology between human SFA-1 and its mouse homologue (Hasegawa et al., 1997c).

A study by immunohistochemistry of the distribution of PETA-3 in a number of tissues revealed expression in endothelium, epithelium, Schwann cells and dendritic cells and in skeletal, smooth and cardiac muscle. The localisation of PETA-3 expression was compared to the localisation of expression of two other tetraspan proteins, CD9 and CD63 and the integrins α5β1 and β1. There was co-localisation of PETA-3 with CD9, CD63, α5β1 and β1 in many tissues, providing a

basis for these tetraspan/integrin complexes to occur. However, the distinct localisation of the tetraspan antigens in some tissues, PETA-3 in cardiac muscle, for example, implies a separate role for these molecules in some cell types. (Sincock et al., 1997).

1.4.15 IL-TMP

The intestinal and liver tetraspan protein (IL-TMP) was discovered in the search for proteins that regulate proliferation and/or differentiation of the crypt-villus axis by subtractive hybridisation of a cDNA library prepared from proliferating HT-29 cells using subtracted cDNA probes. The expression of IL-TMP rapidly increases as the non-dividing epithelial cells migrate from the proliferative compartment of the crypt-villus onto the villus. This protein appears to mediate density-associated inhibition of proliferation of the human cell line HT-29 (Wice et al., 1995).

1.4.16 late bloomer

The *Drosophila* late bloomer (lbl) gene was isolated in the search for molecules involved in growth cone guidance, target recognition and synapse formation. The lbl gene is expressed by motor neurons and transiently expressed along motor axons, including growth cones and presynaptic terminals. Late bloomer protein facilitates the formation of neuromuscular connections by promoting motor neuron synapse formation. The late bloomer gene codes for a 208 amino acid protein with four putative transmembrane domains and many of the conserved residues of the other tetraspan proteins (Kopczynski et al., 1996).

1.4.17 YKK8

Protein sequence alignments revealed that the 27.4 kDa YKK8 protein of the nematode, *Caenorhabditis elegans*, has homology with the tetraspan family of proteins. The 244 amino acid protein has the typical tetraspan conserved protein sequence motifs. The genomic structure of YKK8 has the same intron/exon boundary structure as the other tetraspan genes (Tomlinson et al., 1996b).

1.4.18 Sj25/TM4

Another gene from the parasite *Schistosoma japonicum* which belongs to the tetraspan family is the Sj25/TM4 gene. The gene is expressed in the larval and adult stages of the *S. japonicum* parasites. Sj25/TM4 codes for a protein with 225 amino acids and is a different protein from Sj23 mentioned above (Fan et al., 1997).

1.4.19 NAG-2

A recently isolated member of the tetraspan family is the NAG-2 (novel antigen-2) gene. The NAG-2 protein was discovered in a study designed to further characterise the protein constituents present in multi-component tetraspan complexes. A series of monoclonal antibodies were raised against proteins co-immunoprecipitated with CD81 from MDAMB-435 breast cancer cells. One of the proteins recognised by the antibodies was the NAG-2 protein. The NAG-2 protein is detected by immunohistochemistry most strongly on fibroblasts, endothelial cells, follicular dendritic cell and mesothelial cells. The 1.5 kb mRNA transcript of NAG-2 is present in all human tissues except the brain. A larger 6.5

kb mRNA transcript is also detected in heart and skeletal muscle. The highest level of expression detected by Northern analysis was found in spleen, colon and the pancreas. The protein sequence of NAG-2 consists of 238 amino acids and contains two potential N-glycosylation sites. There is 95% amino acid homology between the NAG-2 proteins of human and mouse. Immunoprecipitation studies confirmed that NAG-2 interacts with the tetraspan proteins CD9 and CD81 as well as the $\alpha3\beta1$ and $\alpha6\beta1$ integrins (Tachibana et al., 1997).

1.4.20 TM4SF5

A screening for genes differentially expressed in pancreatic cancer has identified a putative novel tetraspan gene, TM4SF5, that is highly expressed in pancreatic cancer. The TM4SF5 protein consists of 197 amino acids and has the structural features consistent with other tetraspan proteins. TM4SF5 was found to be overexpressed in pancreatic cancers as compared to normal pancreatic tissue (Muller-Pillasch et al., 1998).

1.4.21 Tspan 1-6

The most recently reported tetraspan genes are designated Tspan1-6. These six new members were identified by the generation of a tetraspan consensus sequence used to search EST (expressed sequence tags) databases. Close examination of the sequences revealed that Tspan-4 had already been identified as NAG-2 (1.4.19) while the Tspan-6 sequence had been deposited in GenBank as T245 (Todd et al., 1998).

1.4.22 Associations of tetraspan proteins

Many of the tetraspan proteins are structurally or functionally associated with either other tetraspans or other membrane proteins. Uroplakin Ia and uroplakin Ib form protein plaques in association with uroplakin II and III on the bladder asymmetric unit membrane (Wu et al., 1995). Multi-component tetraspan-HLA-DR complexes consisting of CD37, CD53, TAPA-1 and CD82, along with DR antigens have been immunoprecipitated using tetraspan and DR monoclonal antibodies from B cell lines (Angelisova et al., 1994). CD81 forms a complement receptor complex with CD19 and CR2 on B cells (Fearon et al., 1995). CD9 interacts with the heparin-binding EGF-like growth factor through its heparin-binding domain to potentiate juxtacrine growth factor activity in CHO cells (Sakuma et al., 1997). Other tetraspan proteins cross-link with various other proteins, for example, CD9 can associate with a 14.5 kD diphtheria toxin receptor resulting in enhanced diphtheria ligand binding (Mitamura et al., 1992). The functional significance of these associations is unclear. It has been hypothesised that the tetraspans may act as transmembrane adaptor proteins, that organise the distribution and function of other cell surface molecules and their associated signalling proteins (Hemler et al., 1996).

A number of studies have reported associations between tetraspan proteins and integrins. Integrins are a large family of heterodimeric transmembrane glycoproteins, consisting of an α and a β subunit. Integrins are widely expressed cell-surface receptors able to mediate cell-matrix and cell-cell adhesion (reviewed in Giancotti et al., 1994). Some tetraspan proteins are able to specifically associate with subsets of integrins. CD81, CD63, CD9 and CD82 all form complexes with the

42

integrins $\alpha 4\beta 1$, $\alpha 3\beta 1$ and $\alpha 6\beta 1$ (Berditchevski et al., 1996, Radford et al., 1996, Rubinstein et al., 1996). The $\beta 1$ chain is a common element with all of the above tetraspan interactions, but there are other $\beta 1$ integrin complexes ($\alpha 2\beta 1$ and $\alpha 5\beta 1$) which do not associate with the abovementioned tetraspans (Hemler et al., 1996). The CD81 tetraspan also complexes with the $\alpha 4\beta 7$ integrin on the B cell line RPMI 866 (Mannion et al., 1996). There has been considerable speculation on the functions of these integrin/tetraspan complexes. Association with tetraspans may regulate integrin adhesive functions and/or these integrin/tetraspan complexes may be associated with cell motility. Tetraspans may regulate the signalling and movement of integrins (Hemler et al., 1996). There are still many unanswered questions regarding the specific biochemical consequences of the interactions between integrins and tetraspans, especially in relation to tumour cell metastasis and the functions of motility and adhesion.

Although the precise function of the tetraspan family members has not been determined, there is a strong association between the TM4SF proteins and cell cycle regulation. More specifically, it is likely that these proteins are involved in the regulation of cell adhesion, motility and proliferation. The correlation between overexpression or loss of expression of these proteins and the incidence of cancer is indicative of the important cell cycle control properties these proteins are likely to possess. The discovery of the existence of tetraspan proteins in species as diverse as *Drosophila*, *Schistosoma* and *C. elegans* as well as humans and other mammals suggests an important biological role for the tetraspans, as these proteins have been conserved throughout evolution.

1.5 BLADDER CANCER

1.5.1 Classification of bladder cancer

Ninety percent of bladder cancers originate in the urothelium and are termed "transitional cell carcinomas" (Skinner 1977). Bladder cancer is staged according to the tumour, metastasis and node (TMN) classification (Figure 1.6). The primary tumour is staged at Ta if it is a non-invasive tumour. Tis is the classification for a carcinoma *in situ*. T1 cancers are lesions which invade the lamina propria. T2 tumours invade superficial muscle, while T3 tumours invade deeper muscle. Tumours classified as T4 invade other organs, for example, prostate, uterus, vagina, pelvic wall or abdominal wall (Prout 1977). At the time of presentation, 70-80% of bladder neoplasms are superficial bladder tumours (stages, Ta, Tis and T1). The remaining 20-30% of lesions are invasive (T2, T3 and T4) or metastatic (M+, N+). The grading system of bladder cancer is that used by the World Health Organisation. There are three recognised grades of transitional cell carcinoma; GI, which classifies well differentiated tumours; GII, moderately differentiated and GIII, poorly differentiated. Low grade tumours (grade I and II) may or may not invade the lamina propria and rarely invade the musculature. However, the higher grade tumours (grade III) are more aggressive and often show muscle invasion. Although 70-80% of transitional cell bladder carcinomas are initially non-invasive, 20-30% of these will in time, progress to an invasive cancer with metastatic potential and eventually lead to the death of the patient (Fleshner et al., 1996). Approximately half of the remaining 20-30% of bladder cancers which present with invasive disease will have metastatic disease at the time of diagnosis (Lamm et al., 1996). The current treatment of those patients with clinically-localised invasive bladder cancer is radical surgery. The 5

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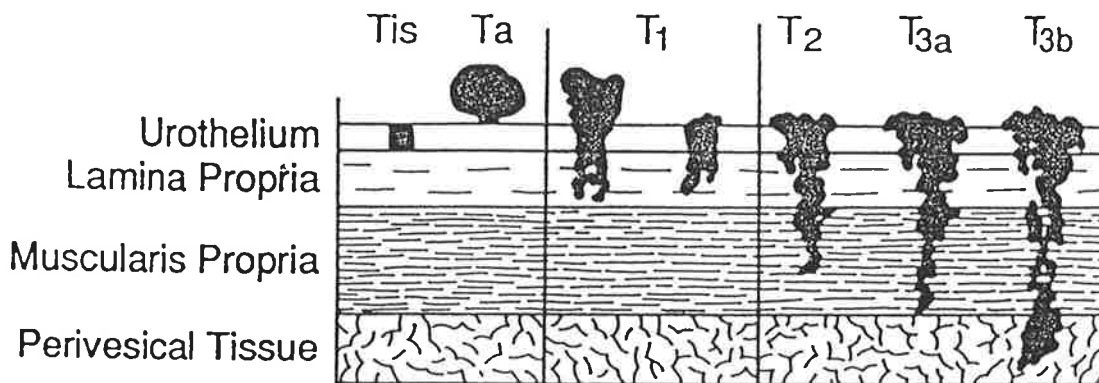
Figure 1.6
Diagram of the staging of bladder tumours. Tis is the classification for a carcinoma *in situ*. The Ta tumour is non-invasive and the T1 cancers invade the lamina propria. T2 tumours invade superficial muscle, while T3 tumours invade deeper muscle.

(This figure was taken from Cordon-Cardo et al., 1997).

year survival rate or overall cure is proportional to the pathological stage but is approximately 50%, with patients dying from metastatic disease. It is therefore presumed that micrometastases undetectable at the time of initial cystectomy are responsible for these therapeutic failures. Development of prognostic indicators based on tumour characteristics would allow early implementation of other therapies, with the hope of improving both disease free survival and cure rates for all bladder cancers.

1.5.2 Cytogenetic Studies of Bladder cancer

It has been postulated that two independent genetic events are required to inactivate a gene to result in cancer formation. The Knudson 'two-hit hypothesis' was originally devised to explain the familial and sporadic forms of retinoblastoma (Knudson 1971). For familial retinoblastoma, the 'first-hit' is inherited, so it is present in every cell of the body. The 'second-hit' is acquired and is present only in the tumour. For sporadic tumours, inactivation of both alleles of a gene occurs somatically. The second 'hit' often involves loss of heterozygosity, suggesting the region of allelic loss harbours a tumour suppressor gene. Cytogenetic analyses of bladder tumours have revealed some gross chromosomal alterations, often involving loss of a chromosome arm or even the whole chromosome. At a locus at which loss of heterozygosity frequently occurs, there may be a tumour suppressor gene, whose inactivation may be crucial to the pathogenesis of bladder cancer. Loss of heterozygosity could explain a decrease in expression of a tumour suppressor gene, but there is also likely to be a mutation in the same gene on the other allele which would result in total inactivation of the tumour suppressor gene. Initially, whole chromosomes and chromosome

regions were investigated in a number of cytogenetic studies to discover potential sites of tumour suppressor genes or markers of bladder cancer.

Cytogenetic deletions of regions of chromosome 9 can be detected in more than 50% of bladder tumours (Cairns et al., 1993). A study by Keen et al., (1994) refined the deletions of chromosome 9 in bladder tumours to two distinct regions, 9p21 and 9q13-9q34.1. Some of the bladder tumours cells were monosomic for chromosome 9, but others had only subchromosomal deletions, allowing delineation of the important areas of chromosome 9 in regard to bladder cancer. Other studies have shown deletions on chromosome 1, 7 and 11 in bladder tumours, as well as confirming the presence of deletions in chromosome 9 (Hopman et al., 1991, Waldman et al., 1991, Ruppert et al., 1993). There is some correlation between the specific chromosome region deleted and the pathology of the bladder cancer. For example, Presti et al., (1991) found a significant relationship between vascular invasion of the bladder cancer and the loss of chromosome 17p. Waldman et al., (1991) found an association between the polysomic nature of chromosome 7 and high grade bladder cancer. Olumi et al., (1990) found that allelic loss on chromosome 9q is present in both low and high grade bladder tumours, while allelic loss of 11p and 17p occurred in high grade tumours. It may be that the occurrence of many genetic lesions are required for the formation of a malignant bladder tumour (Olumi et al., 1990). In summary, there is nonrandom allelic loss of chromosomes associated with bladder cancer and some preliminary evidence of a number of genetic alterations which must occur for a metastatic tumour to eventuate.

1.5.3 Molecular Alterations of Oncogenes in Bladder Tumours

Ras

The frequency of point mutations of the ras oncogene in bladder tumours was determined to be approximately 10%, by using restriction endonuclease analysis and also direct sequencing of the first two exons of ras (Fuijita et al., 1985). However a more sensitive PCR-based assay revealed that approximately 40% of bladder tumours harboured H-ras codon 12 mutations, involving a substitution of valine for glycine resulting from a G-to-A mutation. Activation of the ras proto-oncogene to its oncogenic form occurs via a point mutation at one of three codon hotspots, codons 12, 13 or 61 (Czerniak et al., 1990). A recent study using single-strand conformation polymorphism (SSCP) analysis, detected mutations in exon 1 of H-ras in DNA isolated from urine sediments from 44% of bladder cancer patients. Codons 12 and 13 are located in exon 1 of H-ras (Fitzgerald et al., 1995). The authors suggested that using SSCP to identify ras mutations in urine sediments could be used as a clinical tool for detecting the presence of abnormal urothelial cells. Continued follow-up of those patients with ras mutations in early-stage tumours would determine if the mutations are prognostic for tumour progression or recurrence.

c-myc

Overexpression of the c-myc oncogene occurs in bladder carcinoma, although it is not associated with a particular tumour stage or grade. However, none of the bladder tumours in stage Ta, had increased levels of expression of c-myc (Kubota et al., 1995). A more recent study found no amplification or rearrangement of the c-myc gene in 24 primary bladder tumours. There was a significant enhancement

of c-myc expression in 90% of the bladder tumours, but the expression was not correlated with tumour grade or progression (Onodera et al., 1997). Therefore, c-myc overexpression may merely be a marker of tumour progression with no prognostic significance.

MDM2

The MDM2 oncogene binds to p53 and acts as a negative regulator of the cell cycle by inhibiting the transcriptional activation function of p53 (Oliner et al., 1992). MDM2 abnormalities were characterised in a study of 87 patients with bladder cancer. The MDM2 protein was overexpressed in 32% of patients, but only one of these cases showed amplification of the MDM2 gene. A correlation was found between MDM2-positive tumours and low grade and stage tumours. Sixty-two percent of papillary superficial tumours were MDM2-positive compared to only 24% of muscle invasive lesions (Lianes et al., 1994).

c-erbB-2

The c-erbB-2 gene encodes a transmembrane glycoprotein which has significant sequence homology and structural organisation to the epidermal growth factor receptor. In a study to evaluate the usefulness of the c-erbB-2 gene as a marker for bladder tumour recurrence and progression, amplification of c-erbB-2 was observed in sixteen of 89 patients with recurring disease. Of the sixteen patients with c-erbB-2 amplification, 14 of these patients had evidence of progressive disease, therefore indicating a strong and significant association between c-erbB-2 gene amplification and progressive disease. Nevertheless, there was no correlation between c-erbB-2 protein overexpression and disease progression

(Underwood et al., 1995). These results indicate that there is not a correlation between gene amplification and protein overexpression, which suggests the statistical correlations may not be relevant to actual disease phenotype. Perhaps there is another gene closely located to c-erb-2 which is also amplified which is important for disease progression. Strong staining of c-erbB-2 by immunohistochemistry was found in 20% of bladder tumours and weaker staining in 14% (Mellon et al., 1996). A study focussing on the recurrence of tumour after resection of primary superficial bladder cancer found a low prevalence of c-erbB-2 expression in the recurrence by immunohistochemistry, which was not significantly associated with early tumour recurrence (Tetu et al., 1996). These studies of the c-erbB-2 oncoprotein found no correlation between increased expression in bladder tumours and tumour grade or stage, therefore suggesting limited prognostic significance.

Epidermal Growth Factor Receptor

The epidermal growth factor receptor (EGFR) is activated by binding to either epidermal growth factor or transforming growth factor α. Overexpression of EGFR protein was found, by immunohistochemical staining, on bladder tumours which were more likely to result in, recurrence, progression or death. Increased EGFR staining was observed in 48% of bladder cancers and was associated with both high-grade and stage bladder cancer (Neal et al., 1990). Another study found overexpression of EGFR in 36% of bladder cancers but could find no evidence of DNA amplification or rearrangements (Wood et al., 1992).

Autocrine Motility Factor

Detection of the autocrine motility factor (AMF) in urine has been investigated as a potential marker of bladder cancer. AMF is a cytokine that induces protrusion of pseudopods in target tumour cells, therefore stimulating motility in human tumour cells. A statistical correlation was observed between the level of AMF in the urine and the degree of tumour invasiveness (Guirguis et al., 1988). A recent study investigated the autocrine motility factor receptor (AMFR) as a possible urine marker for bladder cancer. All muscle-invasive transitional cell carcinomas were positive for AMFR. Eighty percent of patients with superficial tumours tested positive for AMFR in their urine, which correlated with tumour grade. However, twenty-five percent of control urines tested positive (Korman et al., 1996). Therefore, the validity of this test comes into question because of the high false positive rate.

1.5.4 Molecular Alterations of Tumour Suppressor Genes in Bladder Tumours

Retinoblastoma

Two independent, immunohistochemical studies determined that altered expression of the retinoblastoma (Rb) protein is a prognostic indicator in bladder cancer. Using immunohistochemical techniques, Logothetis et al., (1992) studied Rb expression in 43 cases of locally advanced bladder cancer using paraffin-embedded archival primary tumour tissue and Cordon-Cardo et al., (1992) investigated 48 cases primary bladder tumours using fresh, frozen tissue. Both sets of results concurred that patients with retinoblastoma-negative tumours have a greater frequency of muscle invasion and a significantly decreased

survival rate when compared with retinoblastoma-positive tumours of patients from the same pathologic stage.

p53

The wildtype p53 gene codes for a tumour suppressor protein that has a significant role in cell cycle control and the prevention of carcinogenesis (Levine et al., 1991). The mutant p53 gene products have a half-life four to twenty times longer than the wildtype protein (Finlay et al., 1988). This observation allows the use of immunohistochemistry to detect mutant p53 protein, as the wildtype p53 protein is normally present at very low levels and is therefore undetectable by immunohistochemistry. Sarkis et al., (1993) found that 58% of T1 bladder carcinomas, which are superficial and only invade the lamina propria, overexpressed p53 protein. These T1 bladder cancers that exhibited nuclear overexpression of p53 protein, ie. mutant p53, had a higher probability of disease progression. A later study by the same investigators aimed to examine the potential prognostic role of overexpression of p53 in muscle-invasive bladder cancer treated with neoadjuvant chemotherapy. They found that those patients whose tumours overexpressed p53 protein were 3 times more likely to die of bladder cancer than those patients with normal p53 expression levels (Sakris et al., 1995).

p16 and p15

The p16 and p15 genes are localised on human chromosome 9p21, a region commonly deleted in bladder cancer. The p16 and p15 genes code for proteins which bind to and inhibit cyclin-dependent kinases. In a study of 110 bladder

tumours, the overall frequency of deletions and rearrangements was approximately 18% for both the p16 and p15 genes. The alterations of p16 correlated with low grade and stage bladder cancers, while there also was a significant association between p15 gene alterations and low stage tumours. Ta and T1, but not Tis, lesions showed deletions of either p16 or p15 (Orlow et al., 1995). Another study confirmed that p16 is the major deletion target at 9p21. All bladder tumours found to have small defined deletions of 9p21 had homozygous deletion of p16 and 58% of tumours with monosomy 9 and 10% of tumours with no chromosome 9 cytogenetic abnormalities also had homozygous deletion of p16. p15 was also found to be deleted in many cases, but no tumours had deletions of p15 without also having deletions of p16. However, there were deletions of p16 in tumours in the absence of p15 deletions. Therefore, the p16 gene appears to be the major target for deletion at chromosome 9p21 (Williamson et al., 1995). These observations suggest that p16 and p15 mutations early in carcinogenesis may provide a selective growth advantage for the affected cell. The association of p16 and p15 mutations with low grade and stage tumours, Ta and T1, but not Tis, suggests that another distinct molecular pathway may exist for bladder tumour progression.

Gelsolin

The gelsolin gene is a putative tumour suppressor gene which encodes a calcium binding and actin-regulatory protein. In 77% of bladder tumours and all 6 bladder cancer cell lines, gelsolin expression was either decreased or absent. However, there was no correlation between tumour grade or stage and the level of gelsolin

expression. Transfection of exogenous gelsolin into bladder cancer cell lines reduced its tumorigenicity *in vivo* (Tanaka et al., 1995).

1.5.5 Transforming Growth Factor β in Bladder Tumours

Coombs et al., (1993) investigated the relationship between the expression of TGFβ and transitional cell carcinoma. Northern analysis revealed marked reduction or even total loss of expression of TGFβ1 mRNA which correlated with the progression of transitional cell carcinoma. Loss of TGFβ expression could, therefore, be a potential marker for advanced disease or prognosis in bladder cancer.

An independent study by Miyamoto et al., (1995) used a quantitative RT-PCR-based assay to look at the levels of expression of TGFβ mRNA in human bladder cancer. This study revealed that TGFβ expression in bladder cancer was higher than that found in normal bladder epithelium. Higher levels of TGFβ transcript were observed in low and intermediate grade (Grade 1 and 2) tumours than in high grade (Grade 3) tumours. Superficial (pTa and pT1) tumours had higher levels of TGFβ, than invasive (pT2 or higher) tumours. These results suggest that enhanced expression of TGFβ is specific to low grade and stage bladder cancer and that TGFβ could provide a new relevant tumour marker for determining tumour progression. Studies concentrating on growth factors in invasive bladder cancer cell lines show a loss of responsiveness to the negative growth regulatory effects of TGFβ (DeBoer et al., 1997). These observations imply that TGFβ has a key role

to play in controlling cellular homeostasis by inhibiting cell proliferation in normal bladder urothelium. The studies by Coombs et al., (1993) and Miyamoto et al., (1995) both show low levels of expression of TGFβ in advanced tumours, however the study by Coombs et al., (1993) suggested a correlation between tumour progression and decreased TGFβ expression. Miyamoto et al., (1995) suggested that there is an initial increase of TGFβ expression in early-stage bladder cancer which decreases as the tumours become invasive. Both studies have investigated expression levels of TGFβ mRNA and not protein levels.

1.5.6 Molecular pathways in bladder cancer progression

Bladder carcinoma, like many other solid tumours, is expected to arise through a number of genetic changes that lead to tumour formation. Bladder cancer progression is most likely to be through a combination of activation of proto-oncogenes and the inactivation of tumour suppressor genes which enable the bladder epithelial cell to escape from the control of the cell cycle and progress from a superficial tumour to a metastatic tumour. It has been proposed that bladder carcinogenesis may proceed through two distinct molecular pathways (Figure 1.7). Noninvasive transitional cell carcinomas of the bladder can have two distinct morphologies, the papillary carcinomas, Ta or the carcinomas *in situ*, Tis. The papillary tumours are often multifocal and occasionally progress to more aggressive tumours, while the carcinomas *in situ* are flat tumours which often progress to more invasive tumours. The existence of these two distinct pathologies of noninvasive TCC suggests that each have different genetic abnormalities. A number of observations regarding gene mutations and the stage
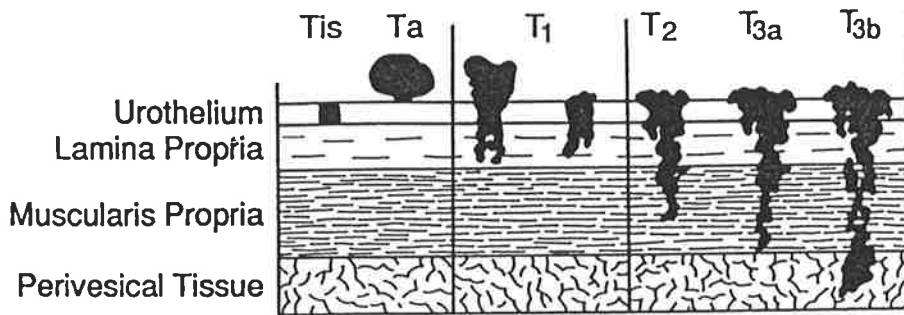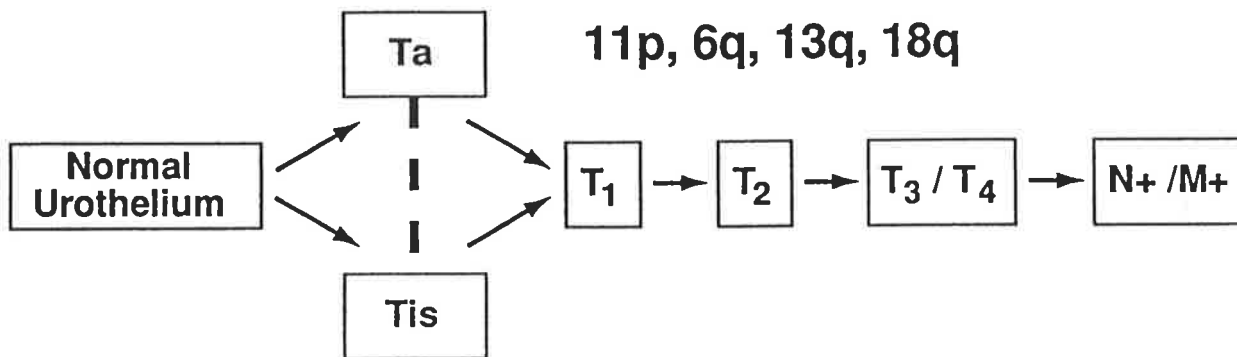
Figure 1.7
Schematic diagram of a genetic model of bladder cancer progression. It has been proposed that bladder carcinogenesis may proceed through two distinct molecular pathways. The non-invasive Ta and Tis tumours have different pathologies and chromosome abnormalities which may play a role in bladder tumour development.

(This figure was taken from Cordon-Cardo et al., 1997).

of bladder cancer at which they occur, have supported this hypothesis. One study of 60 TCC patients found 9q deletions in all superficial papillary tumours (Ta) and almost all tumours invading the lamina propria, (T1). Deletions of 3p, 5q and 17p (location of p53 gene) were associated with the transition from Ta to T1 tumours, with deletions observed in T1 but not Ta tumours (Dalbagni et al., 1993). A larger study of 216 bladder tumours, found loss of heterozygosity of chromosome 9 in 34% of Ta tumours but only 12% of Tis lesions. p53 gene mutations were observed in only 3% of Ta tumours but 65% of Tis. In some patients the carcinoma *in situ* (Tis) and the secondary tumour had different genetic alterations suggesting divergent pathways. The timing of the genetic alterations of either chromosome 9 (involving p16 and p15) or p53 may play a role in the progressive potential of a noninvasive tumour (Spruck et al., 1994). These observations support the hypothesis that bladder carcinogenesis may develop from two distinct molecular pathways which characterise early tumorigenic events occurring in the normal urothelium, which may result in the production of either papillary (Ta) or flat (Tis) tumour lesions.

1.6   TI1/UPKIb AND BLADDER CANCER

It is possible that the TI1/UPKIb gene functions as a tumour suppressor gene in bladder cancer. Perhaps its role as a cell-surface protein on the bladder membrane prevents tumour cell invasion and subsequent metastasis. The loss of the TI1/UPKIb gene may be involved in the multi-step progression of gene inactivation which results in the development of bladder cancer. A cancer cell is often incapable of undergoing complete differentiation and it is the most advanced cancers which are the most poorly differentiated. Therefore, loss of

TI1/UPKIb, a differentiation product of the bladder may be a key factor resulting in the dedifferentiated tissue seen in advanced bladder carcinomas.

1.7 SUMMARY

Cell growth is regulated by both stimulatory and inhibitory factors which interact to maintain tissue homeostasis. The growth inhibitors may act to control cell growth via growth arrest and differentiation. The TI1/UPKIb gene appears to be involved in both growth arrest and differentiation, being preferentially expressed during growth arrest and regulated by TGFβ. The uroplakin Ib gene, the bovine homologue of mink TI1, is a bladder-specific differentiation product of the asymmetric unit membrane. It is most likely that other mammalian species also have bladder-specific expression of TI1/UPKIb (Wu et al., 1994). The TI1/UPKIb gene is a member of the tetraspan family of cell-surface proteins which appear to be involved in cell regulation. Many of these family members have altered expression in cancer, confirming their role in normal cellular growth control.

In conclusion, it is known that the TI1/UPKIb gene is present in the mink and bovine genomes. The TI1/UPKIb gene is growth arrest-specific, produced as a differentiation product in the bovine bladder AUM and is a member of the tetraspan family of proteins. This review of the literature leaves many unanswered questions about the human TI1/UPKIb gene, its normal function, role in the pathogenesis of bladder cancer and potential tumour-suppressive activity.

1.8 AIMS OF THE THESIS

1. To confirm the existence of the human homologue of the bovine uroplakin Ib and mink TI1 genes and to clone and characterise the gene.

2. To determine the chromosomal location of the human uroplakin 1B gene.

3. To determine if the human bladder expresses the uroplakin 1B gene.

4. To compare the levels of expression of UPK1B in normal bladder and bladder tumour and, if necessary, investigate the molecular mechanisms of any altered expression.

5. To investigate the normal function of the uroplakin 1B gene by transfection analysis of the CCL64 cell line and the T24 and 5637 bladder cancer cell lines.

CHAPTER 2

GENERAL MATERIALS

AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

All chemicals were of analytical or molecular biology grade and except for the following, were obtained from Sigma (USA).

Progen (Queensland, Australia): agarose and low melting point agarose

Promega (Madison, WI, USA): IPTG and X-gal

Pharmacia (Uppsala, Sweden): Sephadex G-50

Merck (Victoria, Australia): phenol, chloroform, isopropanol, formaldehyde, glycerol, acetic acid, ethanol, hydrochloric acid and methanol

ICN (California, USA): Dulbecco's modified Eagle's medium powder

Gibco BRL (New York, USA): fetal calf serum, trypsin-EDTA

Bresatec (South Australia): Radiolabelled $\alpha^{32}P$ nucleotides

BioRad (Hercules, USA): acrylamide

2.1.2 Enzymes

All restriction enzymes and calf intestinal alkaline phosphatase were supplied by New England BioLabs (Beverly, USA). Proteinase K and RNaseA were supplied by Boehringer Mannheim (Germany). The T4 DNA ligase, Taq polymerase, AMV reverse transcriptase and RNasin were supplied by Promega (Madison, WI, USA).

2.1.3 Kits and plasmid vectors

Kits and plasmid vectors were obtained as follows:

Gigaprime random labelling kit (Bresatec, South Australia)

Qiagen plasmid midi kit (Qiagen, Germany)

pRc/CMV vector (Invitrogen, San Diego, USA)

Tet-On Gene Expression system (Clontech, California, USA)

pGEM-T cloning vector kit and Wizard PCR Prep purification system (Promega, Madison, WI, USA)

mink TI1/pBluescript (kindly provided by Professor L. Philipson, EMBL, Germany)

2.1.4 Antibiotics

G418 (geneticin), penicillin and streptomycin were supplied by Gibco BRL (NY, USA), ampicillin was supplied by Boehringer Mannheim (Germany) and hygromycin was supplied by Sigma (USA).

2.1.5 Cell lines

The TCC-SUP, ScaBER, J82 and T24 bladder carcinoma cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA), as well as the mink lung epithelial cell line, CCL64 and the mouse NIH3T3 fibroblast cell line. The 5637 bladder carcinoma cell line was kindly provided by Dr. D. Leavesley, Hanson Centre, South Australia.

2.1.6 Clinical samples

Normal urothelial tissue, bladder carcinoma tissue and venous blood were collected, with informed consent, from patients undergoing surgery in the Department of Urology, The Queen Elizabeth Hospital, Woodville, South Australia. The collection of clinical samples was approved by the University of Adelaide and The Queen Elizabeth Hospital ethics committees. Venous blood was collected from Red Cross donors in South Australia as normal controls.

2.1.7 Miscellaneous reagents

These reagents were supplied by the following companies:

GeneScreen Plus membrane (DuPont, NEN, Boston, USA)

Hyperfilm (Amersham, Buckinghamshire, England)

DNA molecular weight markers (Bresatec, South Australia)

herring sperm DNA (Boehringer Mannheim, Germany)

bacto-tryptone, bactoagar and yeast extract (Difco, Detroit, USA)

Hetastarch (DuPont-Pharma, Delaware, USA)

3MM filter paper (Whatman, Maidstone, England)

2.1.8 Bacterial strains

The *E.coli* bacterial strain JM109 (recA1, supE44, endA1, hsdR17, gyrA96, relA1, thi (lac-proAB)) was used for all bacterial transformation studies.

2.1.9 Solutions

DEPC-treated water

One ml of diethylpyrocarbonate (DEPC) was added to one litre of milliQ filtered water and mixed well. The DEPC-water was incubated overnight at $37^{\circ}C$, autoclaved and stored at $4^{\circ}C$.

DMEM media
Per litre:

1 pkt DMEM powder

3.7 g $NaHCO_3$

4.76 g HEPES

Ten ml of penicillin/streptomycin solution (5000 units/ml penicillin G sodium and 5 μg/ml streptomycin sulphate in 0.8% saline) (Gibco BRL, USA) was also added. The solution was made up to one litre with milliQ water, the pH adjusted to 7.4 and the solution was filter sterilised.

6 x DNA loading buffer
100 mM EDTA, pH 8

0.25% bromophenol blue

30% sucrose

formamide loading buffer
95% deionised formamide

50 mM Tris-HCl (pH8.3)

1 mM EDTA

0.1% bromophenol blue

0.1% xylene cyanol

10 x HEBS   5 g HEPES

                                   8 g NaCl

                                   0.32 g KCl

                                   0.125 g $Na_2HPO_4.2H_2O$

                                   1 g glucose

The reagents were dissolved in milliQ water, made up to a total volume of 100

ml and filter sterilised.

Luria broth   Per litre:

                                   10 g bactotryptone

                                   5 g bactoyeast extract

                                   10 g NaCl

10 x MOPS buffer   41.8 g MOPS free acid

                                   4.1 g sodium acetate

                                   20 ml EDTA, pH 8

The MOPS free acid and sodium acetate were dissolved in 900 ml $DEPC-H_2O$ and

the pH adjusted to 7.0 with 6 M NaOH (DEPC-treated). The EDTA was added, the

solution made up to 1 L with $DEPC-H_2O$ and stored in a foil-wrapped bottle at 4^oC.

10 x PBS   30 mM KCl

                                   15 mM KH_2PO_4

                                   1.4 M NaCl

                                   80 mM $Na_2HPO_4,$

plasmid mini-prep solution I   50 mM glucose

25 mM TrisCl (pH 8)

10 mM EDTA (pH 8)

plasmid mini-prep solution II   0.2 M NaOH

1 % SDS

Pre-hybridisation solution   50% deionised formamide

1 M NaCl

10% dextran sulphate

1% SDS

400µg/ml sheared herring sperm DNA

2 x RNA loading buffer   500 µl deionised formamide

100 µl 10 x MOPS buffer

167 µl 37% formaldehyde

100 µl glycerol

The above reagents were combined with a few grains of bromophenol blue and 3

µl of 10 mg/ml ethidium bromide and made up to 1 ml with DEPC-water.

RNA lysis buffer   4 M guanidine thiocyanate

25 mM sodium citrate

100 mM β-mercaptoethanol

0.5% lauryl sarcosine

0.1% antifoam A

Sephadex solution

Sterile milliQ water was added to 10 g of Sephadex powder to make a total volume of 160 ml. The solution was thoroughly mixed and then the Sephadex allowed to settle. The milliQ water was then replaced with fresh milliQ water and the solution stored at 4°C.

20 x SSC (pH 7.0)   Per litre:

175.3 g NaCl

88.2 g sodium citrate

10 x SSCP (pH 6.0)   1.2 M NaCl

0.15 M Na citrate

0.1 M Na_2HPO_4

0.1M NaH_2PO_4

STE buffer   0.1 M NaCl

10 mM TrisCl (pH 8)

1 mM EDTA (pH 8)

50 x TAE buffer	Per litre:
	242 g Tris base
	57.1 ml glacial acetic acid
	100 ml 0.5M EDTA, pH 8

5 x TBE buffer	Per litre:
	54 g Tris base
	27.5 g boric acid
	20 ml 0.5 M EDTA, pH 8

TE buffer	10 mM TrisCl, pH 8
	1 mM EDTA, pH 8

2.1.10 Computer programs

The GeneJockey (BioSoft, Cambridge, UK) software program was used to align and compare homology between two sequences. DNA Strider version 1.0 (Commissariat a l'Energie Atomique, France) was used to locate restriction enzymes sites of a particular sequence. The MacPlasmap program version 1.82 (University of Utah, Salt Lake City, USA) was used to draw schematic diagrams of plasmid vectors. The Amplify program version 1.0 (University of Wisconsin, Madison, USA) was used to design PCR primers. The multialign and prettybox alignment programs (Genetics Computer Group, Wisconsin, USA) were used to align and display multiple sequences.

2.2 METHODS

2.2.1 Isolation of genomic DNA

<u>Isolation of genomic DNA from tissue</u>

Approximately 100 mg of tissue was homogenised in STE buffer and then centrifuged at 1500 rpm for 5 min in a 10 ml centrifuge tube. The pellet was resuspended in 4.5 ml of STE buffer plus 500 µl of 10% SDS, 50 µl of 10 mg/ml proteinase K and 10 µl of 10 mg/ml RNase A and incubated at 37°C overnight. After the addition of one volume of phenol (buffered in TE) the tube was centrifuged at 1500 rpm for 10 min. The upper aqueous phase was removed, placed in a fresh tube with one volume of 24:1 chloroform:isoamyl alcohol and centrifuged again. Two volumes of 100% ethanol and 1/10 volume of sodium acetate (pH 5.2) were added to the upper phase and stored at -20°C for at least 1 hr. The DNA was precipitated in an Eppendorf centrifuge at 15 000 rpm for 10 min, the pellet washed with 70% ethanol and the spin repeated. The DNA was air-dried and resuspended in 100 µl sterile milliQ water.

<u>Isolation of genomic DNA from blood</u>

To extract DNA from venous blood, 1 ml of 6% Hetastarch in saline was added to 10 ml of heparinised blood, mixed and incubated at 37°C for approximately 30 min. The Hetastarch precipitates out the red blood cells. The upper layer, containing white blood cells, was removed and centrifuged with 5 ml of PBS at 1500 rpm for 5 min. The pellet was washed with 9 ml of PBS and 1 ml of sterile milliQ water to lyse any remaining red cells. After centrifugation at 1500 rpm for

5 min, the pellet was suspended in 4.5 ml of STE buffer plus 500 μl of 10% SDS, 50 μl of 10 mg/ml proteinase K and 10 μl of 10 mg/ml RNase A and incubated overnight at 37 °C. The DNA was then extracted as described above.

Isolation of genomic DNA from cell lines

Adherent cells were trypsinised from the cell culture flask as described in 2.2.18. The cells were pelleted at 1500 rpm for 5 min and resuspended in 900 μl of STE buffer plus 100 μl of 10% SDS, 50 μl of 10 mg/ml proteinase K and 10 μl of 10 mg/ml RNase A and incubated overnight at 37°C. To remove cellular debris, one volume of 3 M NaCl was added and the tube placed on ice for 15 min. After centrifugation at 4000 rpm for 15 min, the supernatant was removed and 2 volumes of 100% ethanol added to the supernatant. The tube was incubated at -20°C for at least one hour and then the DNA precipitated, washed in 70% ethanol and dissolved in water as described above.

2.2.2 Isolation of total cellular RNA

Isolation of total RNA from tissue

Approximately 100 mg of tissue was ground, through mesh placed in a petri dish, in 4 ml RNA lysis buffer until no solid tissue remained. Four hundred μl of 2 M sodium acetate, pH 4.0, 4 ml of phenol (buffered in DEPC-water) and 800 μl of chloroform was added to the tissue/lysis buffer mixture. The mixture was vortexed vigorously for 30 seconds and placed on ice for 15 min. After centrifugation for 15 minutes at 4000 rpm at 4°C, the top layer was transferred to a

fresh tube, 1 volume isopropanol added and the mixture incubated at -20°C for at least one hour. The RNA was precipitated by centrifugation at 4000 rpm for 30 min at 4°C. The pellet was resuspended in 500 µl lysis buffer, transferred to an Eppendorf tube and centrifuged at 12000 g for 5 min. The supernatant was placed in a fresh Eppendorf tube with 2 volumes of ice-cold 100% ethanol and incubated at -20°C for at least one hour. The RNA sample was centrifuged at 12 000 g for 10 min, washed with 70% ethanol, air-dried and resuspended in DEPC-water.

Isolation of total RNA from cell lines

To isolate RNA from a 25 cm² flask, the flask was first rinsed with DEPC-PBS. After complete removal of the DEPC-PBS, 4 ml of lysis buffer was added and the flask incubated on ice for 5 min. The lysate was transferred to a 10 ml tube and 4 ml phenol, 400 µl 2 M sodium acetate, pH 4.0 and 800 µl chloroform were added. The RNA was then extracted as described above.

2.2.3 Electrophoresis

Analysis of DNA

Gels for detecting genomic DNA, PCR products, plasmid DNA and restriction fragments were made using 1%-2% w/v agarose in 1 x TAE buffer. For a 100 ml gel, 30 µl of 1 mg/ml ethidium bromide was added directly to the gel before pouring. Each DNA sample was mixed with 1/6 volume of 6 x DNA loading buffer before loading on a gel. Five hundred µg of a DNA marker, either SPP1/EcoRI or pUC19/HpaII (Bresatec, Australia) was also loaded on the agarose

gels. The DNA was electrophoresed, in a BioRad Mini-sub cell electrophoresis tank, at approximately 100 V in 1 X TAE buffer for 45 - 60 min, depending on the size of the DNA fragments. DNA was visualised using a UV transilluminator (Ultra-Lum) and photographed using 667 Polaroid film (Kodak, Australia).

Analysis of RNA

RNA gels consisted of 1% w/v agarose in 10 ml of 10 x MOPS buffer, 16 ml formaldehyde and made up to 100 ml with DEPC-water. RNA samples were mixed with an equal volume of 2 x RNA loading buffer, heated to 68 °C for 2 min and chilled on ice. The RNA was then loaded into the wells of the gel and electrophoresed in 1 x MOPS buffer in a BioRad Mini-sub cell electrophoresis tank at 80 V for approximately 1 hr.

Polyacrylamide gels for microsatellite analysis

PCR products of the microsatellite markers, D3S1292 and D3S1278 (Chapter 6) were resolved on 6% polyacrylamide gels containing 7M urea. To make the acrylamide mixture, 75 ml of 40% acyrylamide/bis acrylamide (19:1) was added to 50 ml of 10 x TBE plus 230 g urea. The mixture was made up to 500 ml with milliQ water and filtered. To prepare a polyacrylamide gel, 420 µl of 10% ammonium persulphate (APS) and 70 µl of TEMED (N,N,N'N-Tetramethylethylenediamine) was added to 70 ml of the above acrylamide mixture. The gel was pre-electrophoresed for 30 min at 40 W. The samples were denatured at 95 °C for 5 min, loaded on the gel in formamide loading buffer and the gel run at 40 W in 1 x TBE for 2 - 3 hr. The gel was then fixed in 10%

methanol, 10% acetic acid for 15 min, transferred to Whatman 3MM paper, vacuum-dried for 45 - 60 min and placed at -70 °C with autoradiography film to develop.

2.2.4 Southern transfer

Ten μg of genomic DNA was digested with 40 - 60 units of restriction enzyme (New England BioLabs) in 1 x restriction buffer plus 100 μg/ml BSA if required and the total volume made up to 100 μl with sterile milliQ water. The DNA was digested overnight at the temperature recommended by the manufacturer, usually 37°C. The digested DNA was electrophoresed overnight at 40 V in a 20 x 20 cm, 1% agarose gel in 1 x TAE. The DNA Southern gel was acid-nicked in 0.25 M HCl for 10 min, rinsed with distilled water and placed in the transfer solution, 0.4 M NaOH. The transfer was set up as follows: a stack of interleaved paper towels, three pieces of Whatman 3MM filter paper cut to the same size of the gel pre-soaked in 0.4 M NaOH, the membrane (Genescreen, DuPont), pre-soaked in 0.4 M NaOH, the gel, three more pieces of Whatman and four Roar paper towels (Kimberly-Clark, Australia) also soaked in the transfer solution. The top of the transfer set-up was kept moist at all times and the transfer allowed to continue for 2-3 hr. After the transfer, the membrane was rinsed in 2 x SSC, the DNA cross-linked to the membrane with a UV Stratalinker 1800 (Stratagene, La Jolla, USA) and stored between Whatman 3MM filter paper in a sealed plastic bag.

2.2.5 Northern transfer

The Northern gels consisted of 3 g agarose (1%), 30 ml of 10 x MOPS, 218 ml DEPC water and 52 ml formaldehyde (16.6M). Approximately 10 µg of RNA was heated to 68°C for 2 min with an equal volume of 2 x RNA loading buffer immediately prior to loading on the gel. The gel was then run overnight in 1 x MOPS at 40 V and then placed in milliQ water for 20 min to remove excess formaldehyde. After the RNA was visualised with UV and a photograph taken, the gel was placed in transfer buffer, 10 x SSC. The transfer was set up as described for the Southern blot in 2.2.4, except the transfer was carried out 10 x SSC, rather than 0.4 M NaOH. After transfer, the membrane was photographed and the positions of the 28S and 18S rRNA marked in pencil. The RNA was cross-linked using a UV Stratalinker 1800 (Stratagene, La Jolla, USA) and the membrane stored between Whatman 3MM paper in a sealed plastic bag.

2.2.6 Southern and Northern hybridisation

Pre-hybridisation

A total of 10 ml pre-hybridisation solution was prepared by mixing 1 ml of 10% SDS, 5 ml of 50% deionised formamide, 2.5 ml of 5 M NaCl, 2.5 ml of 50% dextran sulphate and 400 µl of 10 mg/ml denatured herring sperm DNA. The pre-hybridisation solution was added to the membrane in a hybridisation bottle in a Hybaid Micro-4 hybridisation oven (Middlesex, UK) and allowed to incubate for at least 3 hr at 42°C.

Hybridisation

The probe of interest was random-labelled with either α^{32}P-dATP or α^{32}P-dCTP using the Gigaprime kit (Bresatec, South Australia). Six µl of 150-200 ng of denatured probe was added to 6 µl decanucleotide solution, 6 µl nucleotide cocktail buffer A, 1 µl (5 units) of Klenow enzyme and 5 µl (50 µCi) α^{32}P-dATP and allowed to incubate at 37°C for 15 - 30 min. Unincorporated radionucleotides were removed using a Sephadex G-50 column, prepared from a 2 ml sterile syringe which had the plunger removed and sterile cotton wool placed at the bottom of the syringe. The Sephadex G-50 slurry was added to almost fill the column and 180 µl of 2 mM β-mercaptoethanol and 20 µl of 10 mg/ml herring sperm added on top of the column. The column was placed into a 10 ml tube which was in turn placed into a 50 ml tube for centrifugation at 400 rpm for 4 min. The column was placed in a fresh 10 ml tube and the labelled probe mixture plus 200 µl of 2 mM β-mercaptoethanol was loaded onto the column, which was then centrifuged within a 50 ml tube at 800 rpm for 8 min. The labelled probe was eluted from the column and the unincorporated nucleotides remained on the column. The probe was then denatured at 95°C for 5 min, chilled on ice and added to the Hyb-aid bottle containing the membrane and pre-hybridisation solution. Hybridisation was carried out overnight at 42°C.

Membrane washing

After overnight hybridisation, the radioactive mixture was removed from the Hyb-aid bottle. The first wash of the membrane was in 2 x SSC at 42°C for 30 min

and the radioactivity on the membrane was monitored by a hand-held beta Geiger counter. The membrane was then washed until background radioactivity was minimal. Subsequent washes included 1 x SSC/0.1% SDS at 60°C and 0.1 x SSC/0.1% SDS at 65°C and, if required, 0.1 x SSC/0.1% SDS at 68°C. The membrane was covered in plastic wrap and exposed at -80°C to autoradiographic film (Hyperfilm, Amersham) in a film cassette with double intensifying screens. The film was developed using an automatic x-ray developer in the Radiology Department, The Queen Elizabeth Hospital.

Stripping DNA and RNA probes

To remove radioactively-labelled DNA and RNA probes from Southern and Northern blots respectively, solutions of 0.1 x SCC/0.1% SDS were boiled and added to membranes on a rocking platform for 30 min. The procedure was repeated and the membrane checked for radioactivity by the hand-held Geiger counter. After all radioactivity had been removed, the membrane was rinsed in 2 x SSC, blotted dry and stored between Whatman 3MM paper in sealed plastic bags at room temperature.

2.2.7 Polymerase Chain Reaction (PCR)

Polymerase chain reactions (PCR) were carried out in a volume of 50 µl with 2 µl of the template DNA (50 ng) plus 50 ng of each primer. The PCR mixture also included 40 mM dNTPS, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM MgCl$_2$ and 1 unit Taq polymerase (Promega Corporation, Madison, WI).

Negative control tubes for PCR consisted of all reagents except for DNA, 2 µl of sterile milliQ water was added to make the reaction up to 50 µl. Each PCR amplification tube was layered with one drop of mineral oil to prevent evaporation. PCR cycling conditions consisted of an initial denaturation at $94^{o}C$ for 5 min, followed by 35 cycles of denaturation at $94^{o}C$ for 1 min, annealing (dependent on the Tm of the primers) for 1 min and extension $72^{o}C$ for 1 min, followed by a final $72^{o}C$ extension for 5 min. All PCR amplifications were carried out for 35 cycles as described above unless otherwise stated, usually the only variable was the annealing temperature, which was dependent on the melting temperature of the primer. The melting temperature (Tm) was calculated by assigning $4^{o}C$ for each G or C nucleotide in the primer and $2^{o}C$ for each A or T nucleotide. The PCR thermal cyclers used were ARN Electronics model LTV10DNA water-cooled thermal cycler (Adelaide, Australia) and the Eppendorf Mastercycler 5330 refrigerated cycler (Hamburg, Germany). The following table (Table 2.1) lists each primer used in this thesis, its nucleotide sequence and the company that synthesised the primer.

Table 2.1: Primer sequence details

Primer Name	Primer Sequence	Supplied by
5′CMV	5 ′GAACCCACTGCTTAACTGGCTTAT 3′	OPERON[1]
3′CMV	5′ TGATCAGCGAGCTCTAGCATTTAG 3′	OPERON
3′ORF	5′ TTCTTAATANTCAATTCTGCTCC 3′	OPERON
TM3	5′ GAAGTGGCATCTTGTATCACAGCA 3′	OPERON
ECD	5′TCCATTGGTCATCATTGTTTGGAG 3′	OPERON
ECD2	5′ TGCCAGTCTGACGGGCCATT 3′	Gibco BRL[2]
TM2	5′ GGCATCGTAGGCATCATGAAG 3′	Gibco BRL
NH1B	5′ AATCCCGACAATGGCGAAAGA 3′	Gibco BRL
pGEM-5′	5′ GTAAAACGACGGCCAGT 3′	Gibco BRL
pGEM-3′	5′ CAGCTATGACCATGATTACG 3′	Gibco BRL
TGFβ-5′	5′ GAGACGGATCTCTCTCCGA 3′	Gibco BRL
TGFβ-3′	5′ CTCTGCTTGAACTTGTCATAG 3′	Gibco BRL
RC-TM3	5′ TGCTGTGATACAAGATGCCACTTC 3′	Gibco BRL
R-ECD	5′ CTCCAAACAATGATGACCAATGGA 3′	Gibco BRL
TM1	5′ GCAGAGTGCATCTTCTTCGTA 3′	Gibco BRL
RTM1	5′ TACGAAGAAGATGCACACAGC 3′	Gibco BRL
5′ TET	5′ AGAGCTGCTTAATGAGGTCGG 3′	Gibco BRL
3′ TET	5′ CCATTGCGATGACTTAGTAAAGC 3′	Gibco BRL
5′ TRE	5′ TCGTTTAGTGAACCGTCAGATCG 3′	Gibco BRL

Table 2.1 continued

Primer Name	Primer Sequence	Supplied by
3' TRE	5' GCATTCTAGTTGTGGTTTGTCC 3'	Gibco BRL
UPK1B-1	5' CATGCTCATTTACATGTGTGC 3'	Bresatec[3]
hTI1A	5' CAAATGCTGCAGGGACCTC 3'	Bresatec
D3S1278-CA	5' GGACACATGCTCCTGGAA 3'	Bresatec
D3S1278-GT	5' TGCACTACAGGGCAGTTG 3'	Bresatec
D3S1292-CA	5' TGGCTTCATCACCAGACC 3'	Bresatec
D3S1292-GT	5' CAGATTCAAGAGGCACTCCA 3'	Bresatec

[1]OPERON = OPERON Technologies, California, USA

[2]Gibco BRL = Gibco BRL, Victoria, Australia

[3]Bresatec = Bresatec, South Australia, Australia

2.2.8 Reverse-transcription PCR

First-strand cDNA was synthesised using 1 µg of RNA plus 500 ng oligo (dT), 15 units AMV reverse transcriptase, 20 units rRNasin, 10 mM dNTPs, 250 mM Tris-HCl (pH8.3), 250 mM KCl, 50 mM $MgCl_2$, 50 mM DTT and 2.5 mM spermidine (Promega, Madison, WI, USA) in a total volume of 20 µl. The reverse-transcription mixture was incubated at 42°C for 30 min followed by 95 °C for 5 min. Two µl of the reverse transcription was used as template for the subsequent PCR as described in 2.2.7.

2.2.9 Purification of PCR products and restriction fragments

The Wizard PCR Preps DNA Purification system (Promega, Madison, WI, USA) was used to purify both PCR products and DNA restriction fragments. The relevant PCR product or restriction fragment was electrophoresed on a low-melting point agarose gel and then excised under UV light using a sterile scalpel. The agarose slice was then incubated at 70°C until the agarose had completely melted. Following the instructions recommended by the manufacturer and using the Wizard Minicolumn, the DNA was purified and subsequently eluted in 40 μl sterile milliQ water. The yield of the purified PCR product or restriction fragment was determined by agarose gel electrophoresis by comparison to DNA molecular markers (2.2.3).

2.2.10 Cloning of PCR products

PCR products purified by Wizard PCR Preps (2.2.9) were cloned into either the pGEM-T or pGEM-T-Easy vector (Promega, Madison, WI, USA) according to the manufacturers instructions. Both vectors have been prepared so that there are single 3′ thymidine overhangs at the *EcoRV* insertion site at nucleotide 51, to allow cloning of PCR products which contain a 5′-adenosine overhang added on by Taq polymerase. The pGEM-T-Easy vector has a more extensive selection of restriction sites in its polylinker and was used to clone the mouse genomic Upk1b PCR product (Chapter 4.3.5). All other PCR products were cloned into the pGEM-T vector. Colonies containing recombinant pGEM plasmids were identified by blue/white colour screening, as cloning of an insert into pGEM disrupts the coding sequence of the lacZ gene, preventing β-galactosidase activity.

Transformed bacteria were plated out on Luria agar plates containing 50 µg/ml of ampicillin, 0.5 mM IPTG and 80 µg/ml X-Gal. The addition of ampicillin to the plates selects for bacteria containing the pGEM plasmid and the IPTG induces β-galactosidase activity which in turn metabolises X-Gal, producing blue-coloured colonies. Therefore, clones containing PCR products produce white colonies as there is no β-galactosidase enzyme activity to metabolise X-Gal. Further details on bacterial transformation are described in 2.2.14.

2.2.11 Restriction digests of plasmids

To determine the orientation of an insert cloned into a plasmid, approximately two µg of plasmid DNA was mixed with 10 units of restriction enzyme (New England BioLabs), 1 x restriction buffer, made up to 30 µl with distilled water and digested for 1 - 2 hr at the temperature recommended by the manufacturer. Bovine serum albumin (BSA) at a concentration of 100 µg/ml was also added if required. Restriction fragment sizes were determined by comparison to DNA markers by agarose gel electrophoresis as described in 2.2.3. To prepare plasmid vectors for insertion of a fragment, 10 µg of plasmid DNA was digested with 40 units of restriction enzyme in a total volume of 100 µl.

2.2.12 Cloning of genomic and cDNA fragments into plasmid vectors

Ten µg of the plasmid vector was digested as described in 2.2.11 with either one or two restriction enzymes to linearise the plasmid or create "sticky" ends

respectively. Plasmids that were linearised were treated with calf intestinal alkaline phosphatase (CIP) which removes the 5′ phosphate residue thereby preventing self-ligation of the plasmid. Following the initial restriction digest, 2 μl of 10 units/μl CIP was added directly to the digest and incubated at 37 $^{\circ}$C for 1 hr, then at 55 $^{\circ}$C for 45 min and finally at 65 $^{\circ}$C for 5 min to inactivate the enzyme. Some of the cDNA inserts to be cloned into the vectors may also need to be excised from another plasmid using appropriate restriction enzymes. Both the linearised plasmid vectors and the inserts were run on low-melting point agarose gels and purified through Wizard minicolumns (2.2.9). The ligation mixture consisted of plasmid vector and insert in molar ratios of 1:1, 3:1 or 1:3, 1 x ligation buffer and 20 units of T4 ligase in a total volume of 20 μl and was placed at 4°C overnight. The ligations included a negative control of the linearised plasmid vector only, thus any colonies formed after transformation into bacteria result from self-ligated plasmid.

2.2.13 Preparation of competent cells

A loop of cells from a frozen glycerol stock of *E.coli* JM109 bacterial cells was streaked onto an Luria agar plate and incubated at 37°C overnight. A single colony was picked, suspended in 10 ml Luria broth and propagated in a shaking incubator overnight at 37°C. One and a half ml of the overnight culture was transferred into 20 ml of fresh Luria broth and grown for approximately 90 min. The culture was chilled on ice for 20 min and centrifuged for 10 min at 4000 rpm at 4°C. The supernatant was decanted and the pellet resuspended in 10 ml ice-

cold 0.1 M CaCl$_2$. After the centrifugation was repeated, the pellet was resuspended in 2.5 ml ice-cold 0.1 M CaCl$_2$ and the cells left on ice for 1 hr. Cells not required immediately were mixed with an equal volume of 80% glycerol and stored in cryotubes at -80 ^{o}C.

2.2.14 Transformation of plasmids into competent cells

Five μl of ligation mixture was added to 200 μl of ice-cold competent bacteria cells in a 1.5 ml Eppendorf tube and placed on ice for 30 min. Controls included bacterial cells only which were plated on Luria agar plates with and without ampicillin to control for ampicillin activity. Competent bacterial cells without transformed plasmids will only grow on Luria plates without ampicillin. To determine transformation efficiency, an intact plasmid was also transformed into competent cells. To transform the plasmid, the cells were heat shocked at 42°C for 2 min and then placed immediately on ice. One ml of Luria broth was added to the cells and the contents of the tube placed in a 10 ml tube and incubated in a shaking 37°C incubator for 40 min. The bacterial cells were pelleted by centrifugation at 12 000 g for 1 min and then resuspended in approximately 100 μl of Luria broth. The suspension was then spread onto Luria agar plates containing 50 μg/ml plates and incubated overnight at 37°C. For transformation of PCR products cloned into pGEM, 0.5 mM IPTG and 80 μg/ml X-Gal were also added to the plates to allow for blue/white colour screening.

2.2.15 Plasmid mini-preps

Two ml of Luria broth containing 2 μl of 50 mg/ml ampicillin was spiked with a bacterial colony from a transformation experiment and grown overnight in a shaking incubator at $37^{o}C$. Five hundred microlitres of this culture was stored at $4^{o}C$ for later use and the remaining 1.5 ml centrifuged for 1 min at 12,000 g. The resultant pellet was resuspended in 100 μl of plasmid mini-prep solution I (2.1.9) and 200 μl of plasmid mini-prep solution II (2.1.9) was added. After 5 min at room temperature, 125 μl of ice-cold 3 M sodium acetate, pH 5.2 was added, the solution mixed by inversion and placed on ice for 5 min. The tube was centrifuged for 5 min at 12 000 g, the supernatant removed to a new tube, 10 μl of 10 mg/ml RNase A added and the mixture incubated at $37^{o}C$ for 30 min. Four hundred μl each of both phenol and chloroform were added to the tube and the aqueous layer removed to a fresh tube after centrifugation for 5 min at 12 000 g. Two volumes of 100 % ethanol were added to the tube which was stored at $-20^{o}C$ for 20 min. The plasmid DNA was precipitated by centrifugation at 12 000 g for 10 min, the pellet washed in 70% ethanol and the spin repeated. The plasmid DNA was air-dried and dissolved in 40 μl sterile milliQ water.

2.2.16 Large-scale plasmid purification

One hundred microlitres of the plasmid mini-prep culture (2.2.15) was added to 50 ml of Luria broth with 50 μg/ml ampicillin and incubated at $37^{o}C$ overnight. Two ml of the overnight culture was mixed with 2 ml of 80% glycerol in

cryotubes and stored at -80°C. The remaining culture was centrifuged at 4000 rpm for 10 minutes to pellet the bacterial cells. Purification of the plasmids was then carried out using the Qiagen midi-prep plasmid kit (Qiagen, Hilden, Germany). The purified plasmid was dissolved in 150 µl sterile milliQ water and the yield determined by agarose gel electrophoresis (2.2.3). Plasmid purified by the Qiagen midi-prep kit was used for sequence analysis, further cloning experiments and as probes for Northern and Southern analysis.

2.2.17 DNA sequencing

The sequencing of plasmids and PCR products was carried out by automated sequencing by the Flinders Sequencing Service, Flinders Medical Centre, Bedford Park, South Australia. The pGEM-T primers (Table 2.1) were used to prime the sequencing reactions of PCR products cloned into the pGEM-T vector. Other vector-specific primers (Table 2.1) were used for fragments cloned into the pRc/CMV and the pTRE vectors. Purified PCR products, not cloned, were sequenced using the original PCR primers to prime the sequencing reaction.

2.2.18 Maintenance of cell lines

All cell lines were propagated in Dulbecco's modified Eagle's medium, (DMEM) pH 7.4, supplemented with 10% fetal calf serum at 37°C in an atmosphere of 5% CO_2. Fresh media was added to the cells 2-3 times weekly. Cells were passaged when confluent, depending on growth rate. To passage adherent cells, the media was removed from the flask and the cells washed with sterile 1 x PBS. After

complete removal of PBS from the flask, 1 ml of trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA) was added to the cells and the flasks placed at 37 °C for 5 min to allow cells to detach from the plastic. The cell suspension was diluted 1:10 and placed in new flasks with fresh medium. To prepare frozen stocks, the cells were trypsinised, resuspended in 1 ml of ice-cold fetal calf serum plus 1 ml of ice-cold 20% dimethylsulfoxide in 1x PBS added dropwise. The cells were stored at -80°C for 24 hours and then transferred to liquid nitrogen for long-term storage.

2.2.19 Eukaryotic cell transfection

Cells were seeded at 4 X 10^5 cells per small flask (25cm^2) and grown until approximately 50% confluent. At this time, the media (DMEM plus 10% fetal calf serum) in the flask was replaced with fresh media and the cells were transfected approximately two hours later. To prepare the plasmid DNA for transfection, 0.675 ml of 2 x HEBS (pH 7.1) was added to a 10 ml tube containing 10 μg plasmid plus 10 μg herring sperm DNA as carrier DNA. The solution was aerated by pipetting air through a 1 ml plastic pipette while 0.675 ml of 0.25 M $CaCl_2$ was added dropwise. After being allowed to stand for 30 - 40 min at room temperature, the DNA mixture was added to the flask, while gently rocking the flask. The flasks were incubated at 37°C in 5% CO_2. The media was changed approximately 16 hr after transfection. Two days after the transfection, the selection marker G418 (400 μg/ml) was added to the cells. Experiments were set up in duplicate and included an empty vector as a negative control.

CHAPTER 3

CLONING AND CHARACTERISATION OF THE HUMAN UROPLAKIN 1B GENE

3.1 INTRODUCTION

The uroplakin 1B gene has previously been cloned in only two species; mink and cow. The cDNA sequences of both the mink homologue, TI1 and the bovine homologue, UPKIb, have been characterised and putative protein sequences predicted. There is 90% homology between the cDNA sequences corresponding to the open reading frames (ORF) of the mink TI1 and the bovine UPKIb genes. The predicted amino acid identity between the mink TI1 and bovine UPKIb proteins is 93% (Kallin et al., 1991, Yu et al., 1994). Although there is high cDNA sequence homology within the ORF between mink TI1 and bovine UPKIb, the degree of homology within the 5' and 3' untranslated regions is very low.

In order to study the human homologue of the mink and bovine uroplakin genes, it was necessary to clone human-specific uroplakin 1B sequences. Cloning of genomic human UPK1B sequences would enable characterisation of the human uroplakin 1B gene in terms of its chromosomal location by *in situ* hybridisation and its gene structure by detecting rearrangements and polymorphisms by Southern blot analysis. Human UPK1B cDNA sequences are required to be cloned into expression vectors for functional analysis of UPK1B activity by transfection studies and also used as probes for Northern blot analysis to detect mRNA expression. Cloning of genomic and cDNA fragments will allow not only a comparison of sequence identity of human UPK1B to its bovine and mink homologues, but also to other tetraspan genes.

Also described within this chapter, is the search for an intragenic restriction fragment length polymorphism (RFLP) of the human UPK1B gene. An RFLP is

an inherited difference in a restriction enzyme's recognition of a particular sequence. These differences may be a single base change which results in a restriction enzyme no longer recognising the sequence. Therefore, different size fragments are produced by cleavage with the relevant restriction enzyme depending on whether the individual's DNA has the base change. An RFLP does not result in a functional alteration to the protein and is often found in an intron of a gene, rather than in the coding sequence. Highly polymorphic RFLPs are particularly useful for allelic loss studies as a high percentage of individuals in the population will be heterozygous for the polymorphism, that is, one allele will contain the restriction enzyme site and the other allele will not. An RFLP can be used to differentiate between the two alleles of the UPK1B gene to enable allelic loss studies in bladder cancer as described later in Chapter 6. The observed *Taq*I polymorphism is analysed in terms of its frequency and its location within the uroplakin 1B gene structure.

3.2 METHODS

3.2.1 Cloning of human UPK1B cDNA

Total RNA was isolated from normal human ureter and renal pelvis tissue (2.2.2) and RT-PCR analysis (2.2.8) was performed to synthesise first-strand cDNA. The 5′ region of the UPK1B gene was amplified in the subsequent PCR using NH1B and ECD2 primers (Table 2.1) with an annealing temperature of 58°C (2.2.7). The 3′ region of the UPK1B gene was amplified using TM3 and 3′ORF primers (Table 2.1) with an annealing temperature of 55°C (2.2.7). Both the NH1B-ECD2 and the TM3-3′ORF PCR products were cloned into pGEM-T vectors

using the pGEM-T kit (Promega) as described in 2.2.10. The plasmids were transformed into competent *E.coli* cells (2.2.14) and ampicillin-resistant colonies picked and grown in Luria broth plus ampicillin overnight at 37°C. Plasmids were isolated using the mini-prep procedure (2.2.15) and positive colonies were selected by PCR amplification with either NH1B-ECD2 or TM3-3'ORF primer combinations. Positive colonies were propagated and large-scale plasmid preparations were performed using the Qiagen midi-prep plasmid kit (2.2.16). The identity of the plasmids were verified as UPK1B-specific by sequencing (2.2.17) using pGEM-T-specific primers (Table 2.1) and alignment with mink TI1 and bovine UPKIb cDNA sequences using GeneJockey.

3.2.2 Ligation of the human UPK1B cDNA PCR products

The NH1B-ECD2/pGEM-T construct was first digested with the *Eco*NI and *Sac*II enzymes (2.2.11). The larger NH1B-*Eco*NI/pGEM fragment was purified from the smaller *Eco*NI-ECD2-*Sac*II fragment (Figure 3.2) by gel electrophoresis in low melting-point agarose, followed by Wizard Prep purification (2.2.9). The TM3-3'ORF/pGEM construct was digested with *Eco*NI and *Sac*II enzymes and the smaller *Eco*NI-3'ORF fragment purified as described above. The NH1B-*Eco*NI/pGEM and the *Eco*NI-3'ORF-*Sac*II fragments were ligated using T4 ligase (2.2.12) and the plasmids transformed into competent *E.coli* cells (2.2.14). Colonies were picked and grown in Luria broth and ampicillin overnight at 37°C and mini-preparations of the plasmid were isolated (2.2.15). A NH1B-3'ORF/pGEM plasmid was selected by two independent PCR reactions using the TM3 and ECD2 primers or the NH1B and ECD2 primers at an annealing

temperature of 55°C (2.2.7) and confirmed with double digests using *Eco*NI/*Sac*II and *Sac*II/*Sac*I (2.2.11). After a large-scale preparation of the plasmid was isolated using a Qiagen midi-prep plasmid kit (2.2.16), the plasmid was sequenced (2.2.17) and compared with mink and cow uroplakin cDNA sequences using the GeneJockey program.

3.2.3 Cloning of human UPK1B genomic DNA

Using normal human genomic DNA as a template, the TM3 and ECD primers (Table 2.1) directed the amplification of a 784 bp human UPK1B genomic fragment, using an annealing temperature of 60°C (2.2.7). The PCR product was cloned into the pGEM-T vector (2.2.10) and the plasmids transformed into competent *E.coli* cells (2.2.14). PCR analysis, using the TM3 and ECD primers, allowed identification of a positive UPK1B/pGEM plasmid which was isolated using the large-scale plasmid preparation Qiagen kit (2.2.16). The UPK1B/pGEM plasmid was sequenced (2.2.17) using pGEM-T-specific primers and compared with mink and cow uroplakin cDNA sequences using the GeneJockey program. A 778 bp human UPK1B genomic probe was isolated by digesting the UPK1B/pGEM construct with the *Kpn*I and *Acc*I enzymes (2.2.11), followed by gel-purification of the probe from low-melting point agarose using Wizard Prep (2.2.9).

3.2.4 Detection of polymorphisms by Southern analysis

Genomic DNA was isolated from human peripheral blood leucocytes (2.2.1) and digested separately with the following enzymes *Pst*I, *Xba*I, *Hind*III, *Pvu*II, *Bgl*II,

*Bam*HI and *Taq*I. The digested DNAs were electrophoresed on 1% agarose gels and transferred to nylon membranes (2.2.4). The individual Southern blots were hybridised with a 778 bp human UPK1B genomic probe, labelled by random-priming with α^{32}P-dATP (2.2.6). After the *Taq*I-digested Southern blot was stripped (2.2.6), it was also hybridised with the 796 bp human UPK1B cDNA probe (NH1B-3'ORF/pGEM), also labelled by random priming with α^{32}P-dATP (2.2.6).

3.2.5 Cloning of genomic DNA using a modified inverse-PCR method

Genomic DNA was isolated (2.2.1) from an individual homozygote for the 2.5 kb allele of the UPK1B *Taq*I polymorphism (described in Chapter 3.3.3). The DNA was digested with the *Taq*I enzyme (2.2.4) and ligated overnight using T4 DNA ligase (2.2.12) to form circular *Taq*I-ligated DNA molecules. Using the solution containing the ligated *Taq*I molecules as template, the primers RC-TM3 and R-ECD (reverse primers of TM3 and ECD) (Table 2.1) directed the amplification of a 1.7 kb PCR product at an annealing temperature of 60°C for 40 cycles (2.2.7). The 1.7 kb UPK1B PCR product was cloned into the pGEM-T vector (2.2.10) and transformed into competent cells (2.2.14). Positive colonies were identified by PCR using the original RC-TM3 and R-ECD primers. Large-scale plasmid purification was carried out using the Qiagen kit (2.2.16). The 1.7 kb UPK1B/pGEM plasmid was sequenced (2.2.17) using pGEM-T-specific primers and the sequence was aligned with the mink TI1 and bovine UPKIb cDNA sequences using GeneJockey. To allow sequencing of the remaining 700 bp of unknown sequence in the centre of the PCR product, PCR primers, hTI1A and UPK1B-1 (Table 2.1) were designed from the original sequencing of the 1.7 kb

UPK1B/pGEM plasmid (Figure 3.13). Using the 1.7 kb UPK1B/pGEM plasmid as template and an annealing temperature of 55°C, the hTI1A and UPK1B-1 primers directed the amplification of an approximately-sized 700 bp PCR product (2.2.7). The PCR product was purified from low-melting point agarose using Wizard Prep purification (2.2.9) and sequenced using the hTI1A and UPK1B-1 primers (2.2.17).

3.3 RESULTS

3.3.1 Cloning of the human UPK1B ORF cDNA

The cDNA corresponding to the putative open reading frame of the human UPK1B protein was cloned using PCR-based strategies. The template cDNA was obtained by reverse transcription of RNA isolated from normal tissue samples of renal pelvis or ureter from individuals undergoing surgery for reasons other than for transitional cell carcinoma of bladder. The renal pelvis and ureter also contain urothelial cells, the same cell type as the bladder. The degree of homology between the mink TI1 and bovine UPKIb cDNA corresponding to their respective protein open reading frames is high (90%), but is much lower in the 5′ and 3′ untranslated regions. Therefore, it was difficult to design primers to amplify the entire open reading frame of human UPK1B in a single PCR reaction. Attempts to obtain true, non-artefact PCR products, when using primers with less than 100% homology between mink and bovine UPKIb, were not successful because a number of non-specific bands of varying sizes were amplified in each PCR. The most compliant contiguous sequence of approximately 20 nucleotides, NH1B, at the 5 ′end of the cDNA ORF contained four nucleotides which differed

between the mink and cow sequences. The primer, NH1B, spanning the start codon was synthesised to have the same nucleotide sequence as the mink cDNA sequence. A primer, 3'ORF, spanning the stop codon was designed with a "N" nucleotide at nucleotide 15 of the primer because there was one nucleotide difference between mink and cow sequences (Table 3.1).

Table 3.1 Primer homology between mink TI1 and bovine UPKIb cDNA

NH1B (85%)	mink:	AATCCCGACA**ATG**GCGAAAGATGACTCC
	bovine:	AATCCTGAAG**ATG**GCCAAAGACGACTCC
3'ORF (95%)	mink:	TGGAGCAGAATTGAATAT**TAA**GAA
	bovine:	TGGAGCAGAATTGACTAT**TAA**GAA

Nucleotides which differ between mink and cow are underlined. The bold ATG in the NH1B primer represents the start codon and the bold TAA in the 3'ORF primer represents the stop codon.

To obtain a human UPK1B cDNA product comprising the 5' region of the UPK1B ORF, it was necessary to combine a 5' primer (NH1B) which was 85% homologous between mink and cow sequences with an internal 3' primer (ECD2) which was 100% homologous between the two species. The assumption was made that the highly conserved ECD2 primer sequence would also be completely conserved in the human UPK1B gene. A 100% homologous internal primer, TM3, in conjunction with the 3' ORF primer with 95% homology between mink and cow sequences also amplified a specific human UPK1B cDNA product comprising the 3' region of the UPK1B ORF (Figure 3.1). The two PCR products, NH1B-ECD2 and TM3-3'ORF, containing overlapping sequences, were separately cloned into the pGEM-T vector. Sequence analysis of the NH1B-ECD2/pGEM and

Figure 3.1

A. Schematic diagram of the PCR primers

The human UPK1B ORF cDNA was cloned by PCR amplification of the two halves of the UPK1B ORF by use of the two PCR primer pairs, NH1B-ECD2 and TM3 -3'ORF. The presence of an unique restriction site, *Eco*NI, found between the two overlapping fragments, NH1B-ECD2 and TM3-3'ORF was used to ligate together the two fragments. The numbering corresponds to the bovine UPKIb cDNA sequence.

B. NH1B-ECD2 PCR product

RNA was isolated from normal urothelium and reverse-transcribed using oligo(dT). Using the NH1B and ECD2 primers, a 516 bp UPK1B cDNA PCR product was amplified, then run on a 1.5% agarose gel and stained with ethidium bromide.

Lanes 1: pUC19/*Hpa*II marker
Lanes 2 &3: PCR products amplified from two separate samples of normal human urothelial tissue
Lanes 4 & 5: negative controls (no DNA)
Lane 6: Positive control (mink TI1 cDNA/pBluescript)

C. TM3-3'ORF PCR product

RNA was isolated from normal urothelium and reverse-transcribed using oligo(dT). Amplification was directed by the TM3 and 3'ORF primers and the 482 bp PCR product run on a 1.5% agarose gel and stained with ethidium bromide.

Lane 1: SPP1/*Eco*RI marker
Lane 2: Negative control (no DNA)
Lane 3: PCR product from normal human urothelial tissue.

A

NH1B
51

516 bp

ECD2
567

TM3 365

482 bp

3'ORF 847

62

ATG

UPK1B ORF

844

TAA
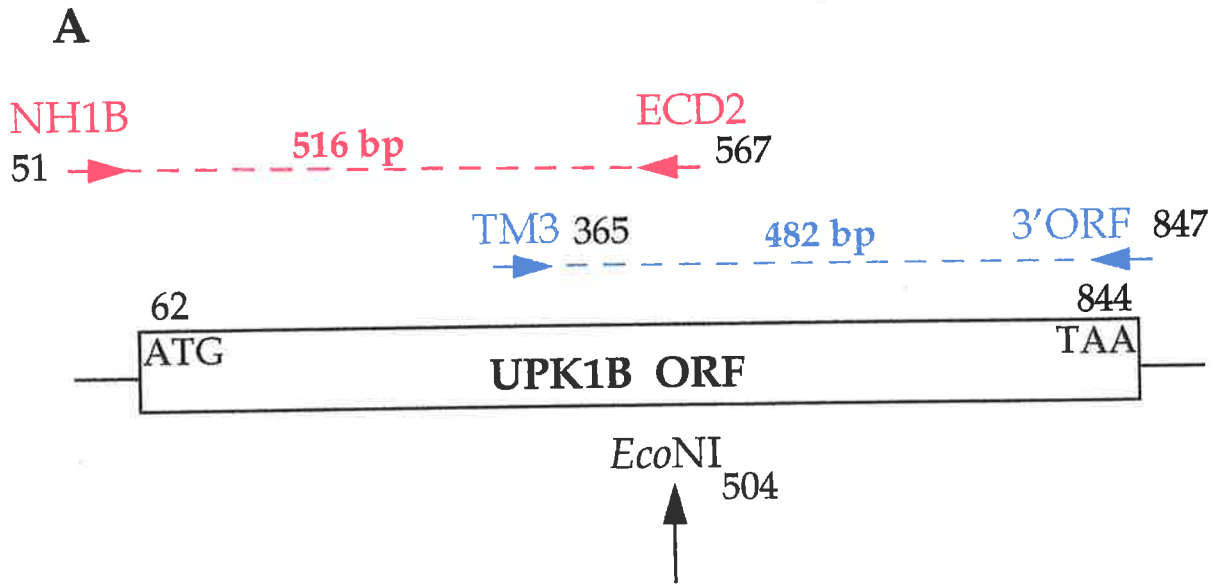
*Eco*NI
504

B

1 2 3 4 5 6

516 bp

C

1 2 3

482 bp

TM3-3'ORF/pGEM constructs (Appendix I) revealed that both were cloned in the reverse orientation to the 5' pGEM-T primer.

A unique *Eco*NI restriction enzyme site located between the TM3 and ECD2 primers allowed the joining of the two human UPK1B cDNA fragments by employing the following strategy (Figure 3.2). The NH1B-ECD2/pGEM-T construct was first digested with the *Eco*NI and *Sac*II enzymes. The *Sac*II enzyme site is located in the multiple cloning site of the pGEM-T vector, downstream of the ECD2 primer site. The resultant larger fragment containing UPK1B cDNA from the NH1B to *Eco*NI sites plus the pGEM-T vector was separated from the smaller *Eco*NI-ECD2-*Sac*II region of cDNA. The TM3-3'ORF/pGEM construct was also digested with *Eco*NI and *Sac*II enzymes and the smaller *Eco*NI-3'ORF fragment isolated. The NH1B-*Eco*NI/pGEM and the *Eco*NI-3'ORF-*Sac*II fragments were ligated (Figure 3.2) and then transformed into competent cells for propagation of plasmids. PCR analysis of the putative NH1B-3'ORF/pGEM plasmid using either NH1B and ECD2 primers or TM3 and ECD2 primers confirmed the correct sized products had been cloned (Figure 3.3a). Digestion of the NH1B-3'ORF/pGEM plasmid with *Eco*NI/*Sac*II and *Sac*II/*Sac*I enzyme combinations ensured that the two cDNA fragments had been ligated and were also in the correct orientation (Figure 3.3b). The 796 bp ligated cDNA, NH1B-3'ORF, was sequenced using pGEM-specific primers (Appendix II) and confirmed as human UPK1B cDNA because of its high degree of sequence identity with mink TI1 and bovine uroplakin Ib cDNA. The human UPK1B ORF cDNA sequence has 91% homology with bovine UPKIb ORF cDNA and 92% homology

Figure 3.2

Schematic diagram of the joining of the two partial UPK1B cDNA fragments, NH1B-ECD2 and TM3-3'ORF.

A.

The NH1B-ECD2/pGEM plasmid (10 µg) was digested with *Eco*NI and *Sac*II for 2 hr at 37°C, removing the short section of DNA from the *Eco*NI site, in the NH1B-ECD2 insert, to the ECD2 primer.

B.

The TM3-3'ORF/pGEM plasmid (10 µg) was also digested with *Eco*NI and *Sac*II at 37 °C for 2 hr, releasing the DNA from the *Eco*NI site to the 3'ORF primer.

C.

The *Eco*NI-3'ORF-*Sac*II fragment was ligated into the open NH1B-*Eco*NI plasmid by directional ligation of the *Eco*NI and *Sac*II "sticky "ends with T4 ligase overnight at 4 °C.

NH1B-ECD2/pGEM
- f1 ori
- AmpR
- *Sac*II
- ECD2
- *Eco*NI
- NH1B
- *Sac* I
- ori

A *Eco*NI/*Sac*II

TM3-3'ORF/pGEM
- f1 ori
- AmpR
- *Sac*II
- 3'ORF
- *Eco*NI
- TM3
- *Sac*I
- ori

B *Eco*NI/*Sac*II

NH1B-- /pGEM
- f1 ori
- AmpR
- *Sac*II
- *Eco*NI
- NH1B
- *Sac* I
- ori

*Eco*NI-3'ORF-*Sac*II

LIGATE

NH1B-3'ORF/pGEM **C**
- f1 ori
- AmpR
- *Sac*II
- 3'ORF
- *Eco*NI
- NH1B
- *Sac*I
- ori
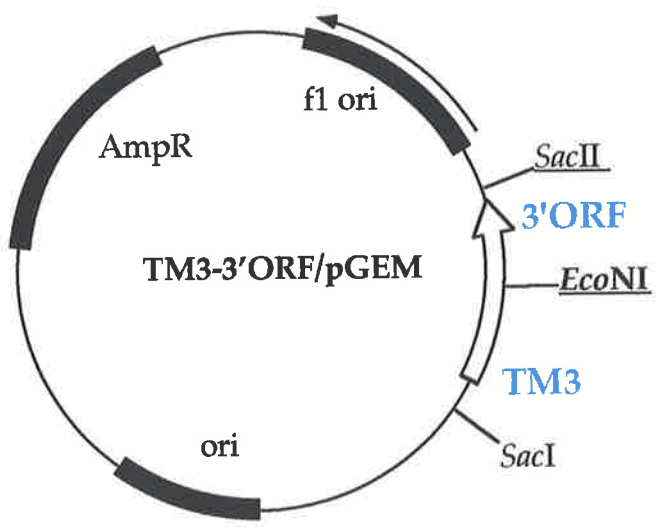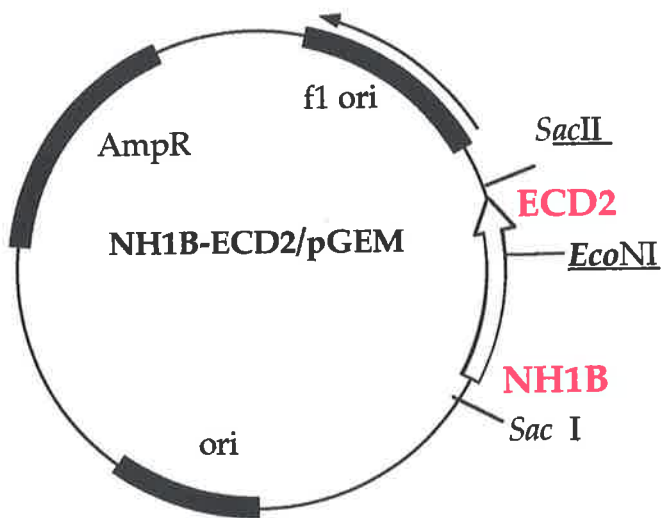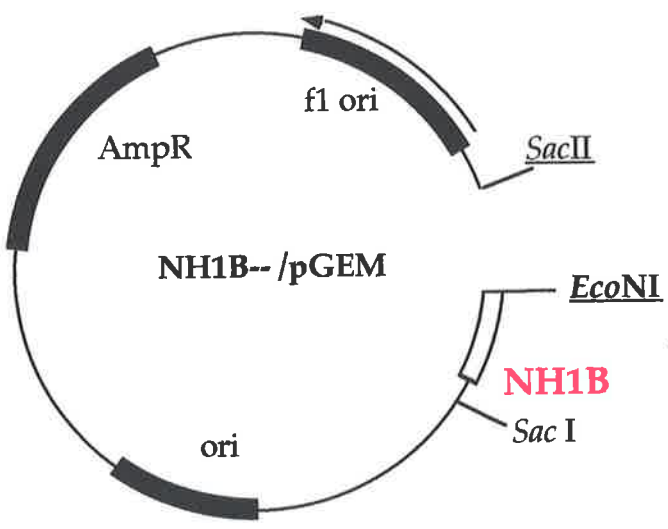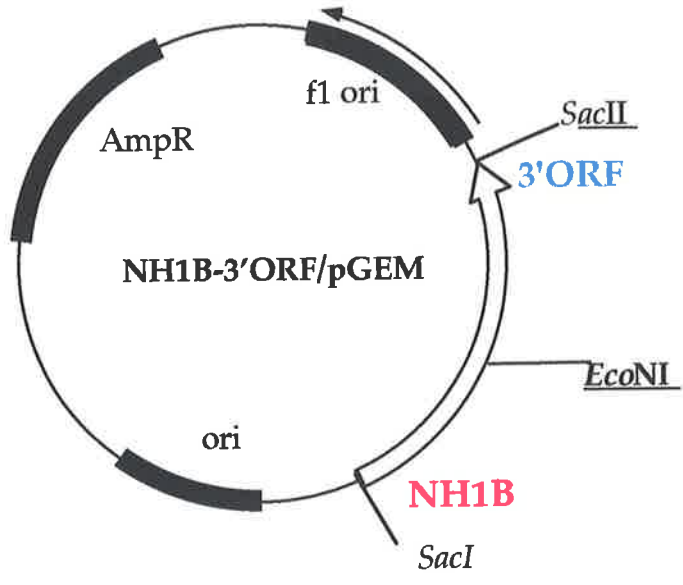
Figure 3.3a

A.

Diagrammatic representation of the NH1B-ECD2 and TM3-ECD2 primer combinations used to confirm the cloning of the UPK1B cDNA open reading frame. The numbers represent positioning of the primers for the human uroplakin 1B cDNA.

B.

PCR amplification of UPK1B cDNA sequence using NH1B-ECD2 and TM3-ECD2 primers from the putative NH1B-3'ORF/pGEM plasmid. The PCR products were run on a 1.5% agarose gel and stained with ethidium bromide.

Lane 1:   pUC19/*Hpa*II marker
Lane 2:   NH1B-3'ORF/pGEM (NH1B-ECD2 primers)
Lane 3:   NH1B-3'ORF/pGEM (TM3-ECD2 primers)
Lane 4:   Negative control (no DNA)

A

NH1B   ECD2
1   516 bp
                        TM3   202 bp ECD2
11   314   516   793
ATG   NH1B-3'ORF/pGEM   TAA

B   1   2   3   4

516 bp
202 bp
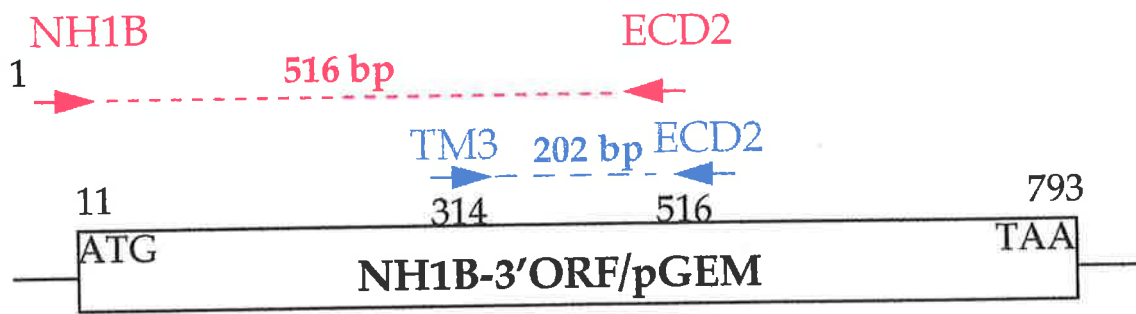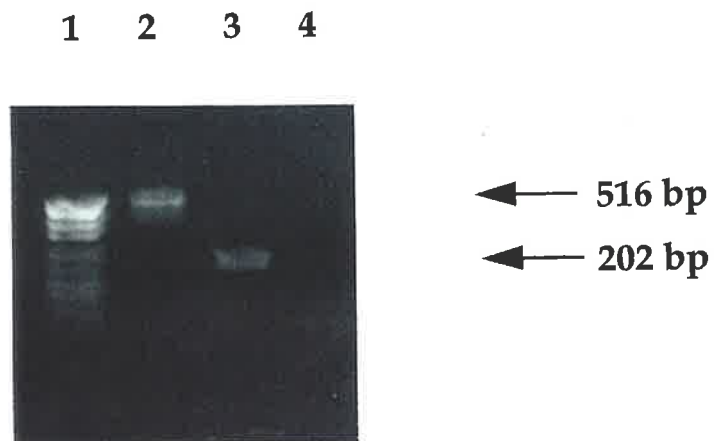
Figure 3.3b

C.

Schematic diagram of the NH1B-3'ORF/pGEM and the NH1B-ECD2/pGEM plasmids showing the positions of the *Sac*II, *Eco*NI and *Sac*I restriction enzyme sites, in relation to the human UPK1B cDNA sequence.

D.

Agarose gel electrophoresis of the double digests of the NH1B-3'ORF/pGEM plasmid and the NH1B-ECD2 plasmid as a control. Two µg of each plasmid was digested with the different enzyme pairs for 2 hours at 37°C. The restriction fragments were separated on 2% agarose gels.

For the NH1B-3'ORF/pGEM plasmid, the expected size insert released from the pGEM plasmid by the *Sac*II/*Eco*NI digest was 345 bp and for *Sac*II plus *Sac*I, 845 bp. Digestion of the NH1B-ECD2/pGEM plasmid was used as a control with a 67 bp product released from the pGEM plasmid for the *Sac*II/*Eco*NI digest and a 567 bp fragment for the *Sac*II/*Sac*I digest. The 67 bp fragment is too small to be seen on the gel.

The numbering in green represents the positions of the enzyme sites in the pGEM plasmid. The NH1B-3'ORF and NH1B-ECD2 inserts were cloned into the pGEM plasmid at nucleotide 51.

Lane 1:   pUC19/*Hpa*II marker
Lane 2:   NH1B-ECD2/pGEM - *Sac*II/*Eco*NI
Lane 3&4:   NH1B-3'ORF/pGEM - *Sac*II/*Eco*NI
Lane 5:   NH1B-ECD2/pGEM - *Sac*II/*Sac*I
Lane 6&7:   NH1B-3'ORF/pGEM - *Sac*II/ *Sac*I
Lane 8:   SPP1/*Eco*RI marker.

C

NH1B-3'ORF/pGEM

f1 ori
AmpR
46 *Sac* II
51 3'ORF
796
455 *Eco*NI
ori
51
1 NH1B
94
*Sac*I

NH1B-ECD2/pGEM

f1 ori
AmpR
46 *Sac* II
516
51 ECD2
455 *Eco*NI
ori
51
1 NH1B
94
*Sac*I
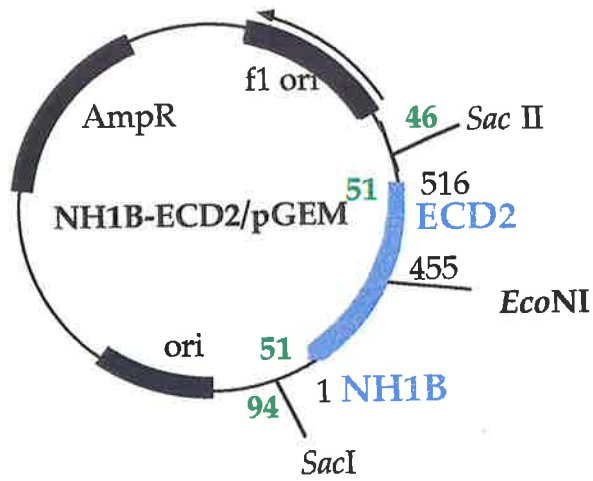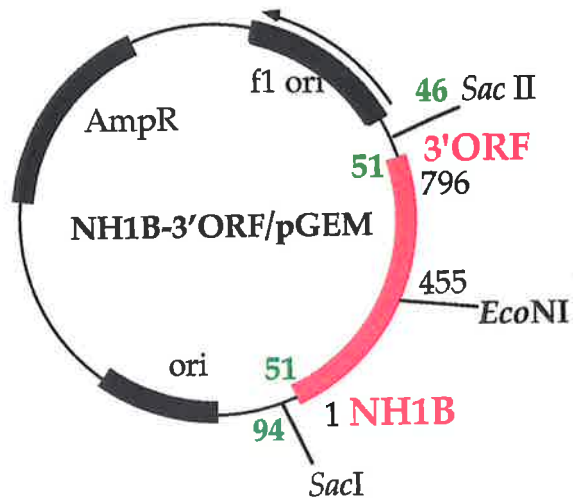
D

1 2 3 4 5 6 7 8
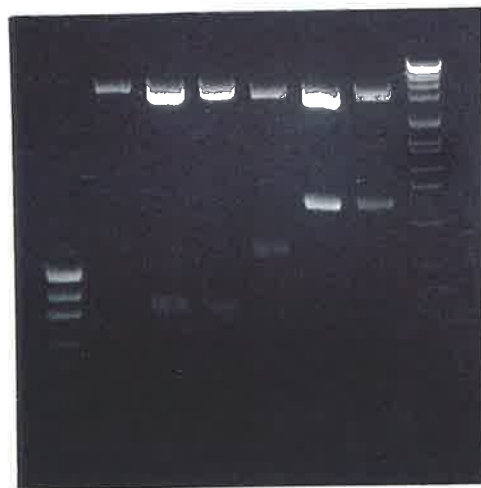
pGEM plasmid

845 bp
567 bp
345 bp

with mink TI1 ORF cDNA as calculated by alignment of the three sequences by the "multialign" program (Figure 3.4) (GenBank Accession Number: AF042331).

The open reading frame coding for the putative protein sequence of UPK1B was determined to begin from the ATG codon present at nucleotide 11 of the cDNA sequence and end after the stop codon, TAA, at nucleotide 793. The position of the start and stop codons are conserved in both mink TI1 and bovine UPKIb sequences. The putative human UPK1B protein is 260 amino acids in length, comparable to the 260 amino acid residues of both mink TI1 and bovine UPKIb (Kallin et al., 1991, Yu et al., 1994). The human UPK1B protein has 92% amino acid homology with bovine UPKIb and 93% amino acid homology with mink TI1 (Figure 3.5). The human UPK1B putative protein sequence has one potential N-linked glycosylation recognition site (NNS) at amino acid residues 131 to 133. Human UPK1B shares many of the consensus motifs observed in other tetraspan molecules (Wright et al., 1994), including three motifs in the large extracellular domain that contain four highly conserved cysteine residues, <u>CC</u>G, PRQ<u>C</u> and EA<u>C</u> (Figure 3.6). The human UPK1B protein also contains a highly conserved asparagine residue (N) in the first transmembrane domain and a highly conserved glutamic acid residue (E) in transmembrane domain 3, as do both the mink TI1 and bovine UPKIb proteins (Kallin et al., 1991, Yu et al., 1994). None of the human, mink or bovine uroplakin proteins contain either a glutamic acid or glutamine residue which is normally conserved in other tetraspans in transmembrane domain 4. Alignment of the human UPK1B protein with the other tetraspan proteins reveals homology especially to the four-membrane spanning domain structure of the tetraspans (Figure 3.7).

Figure 3.4

Alignment of the human uroplakin 1B, bovine uroplakin Ib and mink TI1 cDNA sequences using the "multialign" program (Genetics Computer Group, 1994). The asterisk represents a nucleotide conserved in all three species. A dot represents a nucleotide conserved in two out of the three homologues. If neither an asterisk or a dot is found beneath an nucleotide, no conservation of the nucleotide is observed between any of the species.

The numbering of the human UPK1B cDNA is from the start of the cloned partial cDNA, beginning with the first nucleotide of the NH1B primer. The numbering of the bovine UPKIb cDNA and mink TI1 corresponds to their respective transcription start sites (Yu et al. 1994, Kallin et al. 1991).

The start codon, ATG and the stop codon, TAA are in bold.

```
                    10        20        30        40        50        60
                     .         .         .         .         .         .
   1 AATCCCGACAATGGCGAAAGACAACTCAACTGTTCGTTGCTTCCAGGGCCTGCTGATTTT human
  51 AATCCTGAAGATGGCCAAAGACGACTCCACTGTTCGTTGCTTCCAGGGCCTGCTGATTTT bovine
  58 AATCCCGACAATGGCGAAAGATGACTCCTCTGTTCGTTGCTTCCAGGGCCTGCTGATTTT mink
     ****.**..*****.*****..****..************************* *

  61 TGGAAATGTGATTATTGGTTGTTGCGGCATTGCCCTGACTGCGGAGTGCATCTTCTTTGT human
 111 TGGAAATGTGATTATCGGTATGTGCAGCATCGCCCTGATGGCAGAGTGCATCTTCTTTGT bovine
 118 TGGAAATGTGATTGTTGGTATGTGCGGCATCGCCCTGACCGCAGAGTGCATCTTCTTCGT mink
     *************.*.***...***.****.*******. ** .************** **

 121 ATCTGACCAACACAGCCTCTACCCACTGCTTGAAGCCACCGACAACGATGACATCTATGG human
 171 ATCAGACCAAAACAGCCTCTACCCACTGCTTGAAGCCACCAACAATGACGACATCTATGC bovine
 178 ATCTGACCAGCACAGCCTCTACCCATTGCTTGAAGCCACCGACAACGATGACATCTACGG mink
     ***.*****..*************** .*************.****.**.********.*.

 181 GGCTGCCTGGATCGGCATATTTGTGGGCATCTGCCTCTTCTGCCTGTCTGTTCTAGGCAT human
 231 GGCAGCCTGGATTGGCATGTCTGTTGGCATCTGCCTCTTCTGCCTCTCTGTCCTGGGCAT bovine
 238 GGCAGCCTGGATTGGCATGTTTGTCGGCATCTGCCTCTTCTGTCTGTCCGTTCTAGGCAT mink
     ***.********.*****.*.*** .*****************.**.**.**.**.*****

 241 TGTAGGCATCATGAAGTCCAGCAGGAAAATTCTTCTGGCGTATTTCATTCTGATGTTTAT human
 291 CGTAGGCATCATGAAGTCCAACAGGAAAATTCTTCTGGTGTATTTCATCCTGATGTTTAT bovine
 298 TGTAGGCATCATGAAGTCCAACAGGAAAATTCTTCTGGCGTATTTCATTCTGATGTTTAT mink
     .*****************.*****************.********.***********

 301 AGTATATGCCTTTGAAGTGGCATCTTGTATCACAGCAGCAACACAACGAGACTTTTTCAC human
 351 TGTATATGCTTTTGAAGTGGCATCTTGTATCACAGCAGCAACACAACGAGACTTTTTCAC bovine
 358 AGTATATGGCTTTGAAGTGGCATCTTGTATCACAGCAGCAACACAACGAGACTTCTTCAC mink
     .*******..*************************************************.*****

 361 ACCCAACCTCTTCCTGAAGCAGATGCTAGAGAGGTACCAAAACAACAGCCCTCCAAACAA human
 411 ACCCAACCTCTTCCTGAAGCAGATGCTGGAGAGATACCAAAACAACAGTCCTCCAAACAA bovine
 418 GCCCAACCTCTTCCTGAAGCAGATGCTGGAGAGGTACCAAAACAATAGCCCTCCAAACAA mink
     .**********************************.*****.*.***********.**.***********

 421 TGATGACCAGTGGAAAAACAATGGAGTCACCAAAACCTGGGACAGGCTCATGCTCCAGGA human
 471 TGATGACCAATGGAAAAACAATGGAGTCACCAAGACCTGGGACAGACTTATGCTCCAGGA bovine
 478 TGATGACCAATGGAAAAATAATGGAGTCACCAAGACTTGGGACAGACTCATGCTCCAGGA mink
     *********.*******.***************.**.********.**.***********

 481 CAATTGCTGTGGCGTAAATGGTCCATCAGACTGGCAAAAATACACATCTGCCTTCCGGAC human
 531 CAATTGCTGTGGTGTAAATGGCCCGTCAGACTGGCAGAAATACACCTCTGCCTTCCGGAC bovine
 538 CCACTGCTGTGGTGTCAATGGCCCGTCAGACTGGCAGAGATACACATCTGCCTTCCGGAC mink
     *.*.********.**.*****.**.***********.*.******.************

 541 TGAGAATAATGATGCTGACTATCCCTGGCCTCGTCAATGCTGTGTTATGAACAATCTTAA human
 591 TGAGAACAGCGATGCTGACTACCCCTGGCCTCGTCAATGCTGTGTTATGAACAGCCTTAA bovine
 598 TGCGAATAATGATGCCGACTATCCCTGGCCTCGTCAGTGCTGTGTGATGAACAGTCTGAA mink
     **.***.*.*.*****.*****.**************.********.*****.*..**.**
```

```
601  AGAACCTCTCAACCTGGAGGCTTGTAAACTAGGCGTGCCTGGTTTTTATCACAATCAGGG  human
651  AGAACCTCTCAACCTGGACGCCTGCAAATTAGGAGTGCCTGGATACTACCATAGTCATGG  bovine
658  AGAACCTCTCAATGTGGAGGCCTGCAAGCTAGGAGTGCCCGGGTACTATCACAAAGAGGG  mink
     ***********..****.**.**.**..****.*****.**.* *..**.**.*...*.**


661  CTGCTATGAACTGATCTCTGGTCCAATGAACCGACACGCCTGGGGGGGTTGCCTGGTTTGG  human
711  CTGCTATGAGCTGATCTCTGGACCAATGAACCGACATGCCTGGGGAGTTGCATGGTTTGG  bovine
718  GTGCTATGAACTCATCTCTGGACCCATGAACCGACACGCCTGGGGGGGTTGCCTGGTTTGG  mink
     .*******.**.*********.**.***********.*******.*****.********


721  ATTTGCCATTCTCTGCTGGACTTTTTGGGTTCTCCTGGGTACCATGTTCTACTGGAGCAG  human
771  ATTTGCCATTCTCTGTTGGACTTTCTGGGTTCTCCTGGGTACCATGTTCTACTGGAGCAG  bovine
778  ATTTGCCATTCTCTGCTGGACATTTTGGGTTCTCCTGGGTACCATGTTCTACTGGAGCAG  mink
     ***************.*****.**.***********************************


781  AATTGAATAT**TAA**GAA                              human
831  AATTGACTAT**TAA**GAA                              bovine
838  AATTGAATAT**TAA**GAA                              mink
     ******.*********
```

Figure 3.5

Alignment of the putative amino acid sequences of human uroplakin 1B, bovine uroplakin Ib and mink TI1 by the "multialign" program (Genetics Computer Group, 1994). An asterisk represents conservation of an amino acid betwen all species. A dot represents conservation of the amino acid between two of the three homologues. If neither an asterisk or dot is present, the amino acid is not conserved between any of the three species.

```
          10        20        30        40        50        60
           .         .         .         .         .         .
  1 MAKDDSTVRCFQGLLIFGNVIIGMCSIALMAECIFFVSDQNSLYPLLEATNNDDIYAAAW BOVINE UPK1b
  1 MAKDNSTVRCFQGLLIFGNVIIGCCGIALTAECIFFVSDQHSLYPLLEATDNDDIYGAAW HUMAN UPK1B
  1 MAKDDSSVRCFQGLLIFGNVIVGMCGIALTAECIFFVSDQHSLYPLLEATDNDDIYGAAW MINK TI1
    ****.*.************** .*.*.***.*********.*********.*****.***


             .         .         .         .         .         .
 61 IGMSVGICLFCLSVLGIVGIMKSNRKILLVYFILMFIVYAFEVASCITAATQRDFFTPNL BOVINE UPK1b
 61 IGIFVGICLFCLSVLGIVGIMKSSRKILLAYFILMFIVYAFEVASCITAATQRDFFTPNL HUMAN UPK1B ‚
 61 IGMFVGICLFCLSVLGIVGIMKSNRKILLAYFILMFIVYGFEVASCITAATQRDFFTPNL MINK TI1
    **..*****************.*****.*********.*****.**************


             .         .         .         .         .         .
121 FLKQMLERYQNNSPPNNDDQWKNNGVTKTWDRLMLQDNCCGVNGPSDWQKYTSAFRTENS BOVINE UPK1b
121 FLKQMLERYQNNSPPNNDDQWKNNGVTKTWDRLMLQDNCCGVNGPSDWQKYTSAFRTENN HUMAN UPK1B
121 FLKQMLERYQNNSPPNNDDQWKNNGVTKTWDRLMLQDHCCGVNGPSDWQRYTSAFRTANN MINK TI1
    *************************************.***********.*******.*.


             .         .         .         .         .         .
181 DADYPWPRQCCVMNSLKEPLNLDACKLGVPGYYHSHGCYELISGPMNRHAWGVAWFGFAI BOVINE UPK1b
181 DADYPWPRQCCVMNNLKEPLNLEACKLGVPGFYHNQGCYELISGPMNRHAWGVAWFGFAI HUMAN UPK1B
181 DADYPWPRQCCVMNSLKEPLNVEACKLGVPGYYHKEGCYELISGPMNRHAWGVAWFGFAI MINK TI1
    **************.******..********.**   ********************


             .         .         .         .         .         .
241 LCWTFWVLLGTMFYWSRIDY                           BOVINE UPK1b
241 LCWTFWVLLGTMFYWSRIEY                           HUMAN UPK1B
241 LCWTFWVLLGTMFYWSRIEY                           MINK TI1
    ******************.*
```

1 MAKDNSTVRCFQGLLIFG<u>N</u>VIIGCCGIALTAECIFFVSDQHSLYPLLEAT
    NH2   TM1   small ECD

DNDDLYGAAWIGIFVGICLFCLSVLGIVGIMKSSRKILLAYFILMFIVYA
                    TM2   TM3

F<u>E</u>VASCITAATQRDFFTPNLFLKQMLERYQ**<u>NNS</u>**PPNNDDQWKNNGVTKTW
                              large ECD

DRLMLQDN**<u>CCG</u>**VNGPSDWQKYTSAFRTENNDADYPW**<u>PRQC</u>**CVMNNLKEPL

NL**<u>EAC</u>**KLGVPGFYHNQGCYELISGPMNRHAWGVAWFGFAILCWTFWVLLG
                                                  TM4

TMFYWSRIEY 260
        COOH

Figure 3.6
Conservation of tetraspan motifs in the human UPK1B protein.
The predicted protein sequence of human UPK1B and its putative domain structure are shown in the above diagram with each domain represented by a different colour. There are a number of protein motifs conserved in the tetraspan family (Wright and Tomlinson 1994). The conserved motifs found in the human UPK1B protein are shown in bold and underlined. The asparagine residue (N) in the first transmembrane domain (TM1) is highly conserved in tetraspans, as is the glutamic acid (E) in transmembrane domain 3 (TM3). The three motifs in the large extracellular domain (ECD) that contain four highly conserved cysteine residues CCG, PRQC and EAC are also conserved in the human UPK1B protein. There is a potential N-linked glycosylation recognition site (NNS) at amino acid residues 131-133 in the large ECD. The amino (NH2) and carboxy (COOH) termini are both cytoplasmic domains.

Figure 3.7

Alignment of the human UPK1B protein with fourteen tetraspan proteins using "multialign" and "prettybox" programs (2.1.10). Amino acids highly conserved between the tetraspan proteins are depicted by dark shading and the less conserved residues are lightly shaded. The conserved amino acid residues include an asparagine (N) residue at amino acid 19 (UPK1B sequence) and a glutamic acid residue (E) at amino acid 102. The three cysteine-containing motifs, CCG, PRQC and EAC are conserved between many of the tetraspans including UPK1B at residues, 159, 187 and 203 respectively.

```
a15          .........METK PVITCLKTLL IIYSFVFWIT GVILLAVGVW G...KLTLGT YISLIAE...  48
cd37         ......MSAQE SCLSLIKYFL FVFNLFFFVL GSLIFCFGIW I...LIDKTS FVSEVGL..A  50
cd53         ........MGM SSLKLLKYVL FFFNLLFWIC GCCILGFGIX L...LIHNN. .FGVLFH..N  46
cd63         ......MAVE GGMKCVKFLL YVLLLAFCAC AVGLIAVGVG A...QLVLS. .QTIIQG...  46
cd9          ......MPVK GGTKCIKYLL FGFNFIFWLA GIAVLAIGLW LRFDSQTKSI FEQETNN..N  52
co-029       .........MA GVSACIKYSM FTFNFLFWLC GILILALAIW VR.VSNDS. .QAIFGSED V  48
il-tmp       ......MCT GGCARCLGGT ITPLAFFGFL ANILLFFPGG ....KVIDD. .NDHLSQ..E  45
kai1         ......MGS ACIKVTKYSL FLFNLIFFIL GAVILGFGVW I...LADKSS FISVLQT..S  48
16           ......MCY GKCARCIGHS LVGLALLCIA ANILLYFPNG E...TKYAS. .ENHLSR..F  46
peta-3       MGEFNEKKTT CGTVCLKYLL FTYNCCFWLA GLAVMAVGIW T...LALKSD YISLLAS...  54
sas          ......MVC GGFACSKNAL CALNVVYMLV SLLLIGVAAW G......KG. .LGLVSS...  42
sm23         ....MATLG TGMRCLKSCV FVLNIICLLC SLVLIGAGAV V..EVKFSQ YGDNLHK...  49
tapa-1       ......MGVE GCTKCIKYLL FVFNFVFWLA GGVILGVALW LRHDPQTTNL LYLELGDKPA  54
upk1a        MASAAAATTE KGSPVVVGLL VMGNIIILLS GLALFAETVW VTADQYRIYP LMGVSGK...  57
humanupk1b   .....MAKDN STVRCFQGLL IFGNVIIGCC GIALTAECIF F...VSDQHS LYPLLEATD   51

a15          ..NSTNAPYV LIGTGTTIVV FGLFGCFATC RGSPWMLKLY AMFLSLVFLA ELVAGISGRV  106
cd37         FVPLQIWSKV LAISGIFTMG IALLGCVGAL KELRCLLGLY FGMLLLLFAT QTLGILIST   110
cd53         LPSLTL.GNV FVIVGSIIMV VAFLGCMGSI KENKCLLMSF FILLLIILLA EVTLAILLFV  105
cd63         ATPGSLLPVV IIAVGVFLFL VAFVGCCGAC KENYCLMITF AIFLSLIMLV EVAAAIAGYV  106
cd9          NSSFYTGVYI LIGAGALMML VGFLGCCGAV QESQCMLGLF FGFLLVIFAI EMAAAIWGYS  112
co-029       GSSSYVAVDI LIAVGAVIMI LGFLGCCGAV KESRCMLLLF FIGLLLLILL QVATGILGAV  108
il-tmp       IWFFG..GIL GSGVLMIFPA LVFLGLK.NN DCCGCCGNEG .CGKRFAMFT STIFAVVGEL  101
kai1         SSSLRMGAYV FIGVGAVTML MGFLGCIGAV NEVRCLLGLY FAFLLLILIA QVTAGALFYF  108
16           VWFFS..GIV GGGLLMLLPA FVFIGLE.QD DCCGCCGHEN .CGKRCAMLS SVLAALIGIA  102
peta-3       .GTYLATAYI LVVAGTVVMV TGVLGCCATF KERRNLLRLY FILLLIIFLL ETIAGILAYA  113
sas          ...IHIIGG VIAVGVFLLL IAVAGLVGAV NHHQVLLFFY MIILGLVFIF QFVISCSCLA  98
sm23         ..VWQAAPIA IIVVGVIILI VSFLGCCGAI KENVCMLYMY AFFLVVLDIA ELAAAIVAVV  107
tapa-1       PNTFYVGIYI LIAVGAVMMF VGFLGCYGAI QESQCLLGTF FTCLVILFAC EVAAGIWGFV  114
upk1a        .DDVFAGAWI AIFCGFSFFV VASFGVGAAL CRRRSMILTY LILMLIIYIF ECASCITSYT  116
humanupk1b   NDDIYGAAWI GIFVGICLFC LSVLGIVGIM KSSRKILLAY FILMFIVYAF EVASXITAAT  111

a15          FRH.....EI KDTFLRTYTD AMQTYNGNDE ....RSRAVD HVQRSLSCCG VQ.NYTNWST  156
cd37         QRA.....QL ERSLRDVVEK TIQKYGTNPE ETA.AEESWD YVQFQLRCCG WH.YPQDWFQ  163
cd53         YEQ.....KL NEYVAKGLTD SIHRYHSDNS .T...KAAWD SIQSFLQCCG IN.GTSDWTS  155
cd63         FRD.....KV MSEFNNNFRQ QMENYPKNN. ...HTASILD RMQADFKCCG AA.NYTDWEK  156
cd9          HKD.....EV IKEVQEFYKD TYNKLKTKDE PQ..RETLK AIHYALNCCG LA.GGVEQF.   162
co-029       FKS.....KS DRIVNETLYE NTKLLSATGE SEKQFQEAII VFQEEFKCCG LVNGAADWGN  163
il-tmp       GAG.....YS FIISAISINK GPKCLMANS. ......TWG YPFH..DGDY LN.DEALWN.   144
kai1         NMG.....KL KQEMGGIVTE LIRDYNSSRE .DS.LQDAWD YVQAQVKCCG WV.SFYNWTD  160
16           GSG.....YC VIVAALGLAE GPLCLDSLG. ......QWN YTFASTEGQY LL.DTSTWS.   147
peta-3       YYQ.....QL NTELKENLKD TMTKRYHQPG .HEAVTSAVD QLQEFHCCG SN.NSQDWRD   166
sas          ......I NRSKQTDVIN ASWWVMSNK. ......TRD ELERSFDCCG LFNLTTLYQQ  141
sm23         YKD.....RI DSEIDALMTG ALDKPTKEIT ......EFMN LIQSSFHCCG AK.GPDDYRG  155
tapa-1       NKD.....QI AKDVKQFYDQ ALQQAVVDDD ANN.AKAVVK TFHETLDCCG SS.TLTALTT  167
upk1a        HRD.....YM VSNPSLITKQ MLTFYSADSN QGRELTRLWD RIMIEQECCG TS.GPMDWVN  170
humanupk1b   QRDFFTPNLF LKQMLERYQN NSPPNNDDQW KNNGVTKTWD RLMLQDNCCG VN.GPSDWQK  170
```

```
a15           SP......YF.  .LEHGIPPSC  CMNETDCNPQ  ..........  ..........  ........DLHNLTVA  187
cd37          VLILRGNGS.   .EAHRVPCSC  YNLSATNDST  ILDKVILPQL  SRLGHLARSR  HSADICAVPA.......  221
cd53          .........    ..GPPASC    PSDR......  ..........  ..........  ....CGINFN         165
cd63          IPSMS.....   ..KNRVPDSC  CINVTVG...  ..........  ..........  ........V          182
cd9           .........    ...ISDIC    PKKD......  ..........  ..........  .....CQSYN         185
co-029        N.......F    .QHYPELCAC  LDKQRP....  ..........  ..........  ........            151
il-tmp        .........    ...KCRE     PLN.......  EVKGEEDNSL  SVRKGFCEAP  GNR.......TQSGNHPE  207
kai1          NAELMNR...   .PEVTYPCSC  EVKGEEDNSL  ..........  ..........  ........            154
16            .........    ....ECTE    PKH.......  ..........  ..........  ....RDHAS          199
peta-3        SEWIRSQEA.   .GGRVVPDSC  CKTVVALCGQ  ..........  ..........  ........            154
sas           D........    .YDFCTAIC   KSQS......  ..........  ..........  ........           165
sm23          N........    ...VPASC    KEEN......  ..........  ..........  ........I          181
tapa-1        S........    ..VLKNNLC   PSGSN.....  ..........  ..........  .......N           196
upk1a         FTSAFRATTP   EVVFPWPPLC  CRRTG.....  ..........  ..........  .......L           196
humanupk1b    YTSAFRTENN   DADYPWPRQC  CVMNN.....  ..........  ..........  ........

a15           ATKVNQKGCY  DLVTSF..ME  TNMGIIAG..  .VAFGIAFS.  .QLIGMLLA   CCLS...RFI  236
cd37          ESHIYREGCA  QGLQKW..LH  NNSNELYIGI  .ITICVCVI.  .EVLGMSFAA  LTLN..CQID  211
cd53          ...KVEGCY   AKARLW..LR  KNVLLVVIAA  .AALGIAFV.  .EVLGIVFAA  CCLV...KSI  231
cd63          EKATHKEGCV  EKIGGW..LR  NKFHIIGA..  .ISFGLAVI.  .MIFGMTFFS  MILC...CAI  221
cd9           LETFTVKSCP  DAIKEV..FD  NKFHIIGA..  .VGIGIAVV.  .EILGLVES   MVLY....CQ  233
co-029        GKQVYKETCI  SFIKDF..LA  KNLIIVIG..  .IQMVLCAI.  .QVVNGLLG   TLCGDCQCCG  194
il-tmp        ...VVPWNL   T......LF   SILLVVGG..  .VGVGVAII.  .ELLGMVLS   ICLCRHVHSE  259
kai1          DWPVYQEGCM  EKVQAW..LQ  ENLGIILG..  .IEFILCLI.  .QVINGVLG   GICG..FCCS  195
16            ...IVEWNV   S......LF   SILLALGG..  .VGIGIACV.  .QVFGMIRT   CCLY...RSL  248
peta-3        NIYKVEGGCI  TKLETF..IQ  EHLRVIGA..  .VGLFFSFT.  .EILGVWLA   MRKRN.QKDP  202
sas           ...PTCQMCG  EKFLKH..SD  EALKILGG..  .VAFGVCFF.  .QLLSIVIA   CCLG....RQ  211
sm23          ..LTYTEGCV  SVFGAF..LK  RNLVIVAC..  .AAIVVAVI.  .MIFEMTLS   MVLC...CGI  230
tapa-1        ISNLFKEDCH  QKIDD...S   GCFEHIGHAI  DSYTWGISWF  GFAILMWTLP  VMLI...AMY  253
upk1a         FIPVNEEGCR  LGHLD..LFTK  GCYELISGPM  NRHAWGVAWF  GFAILCWTEW  VLLG...TMF  253
humanupk1b    KEPLNLEACK  LGVPGFYHNQ  GCYELISGPM  ..........  ..........  ........

a15           TANQYEMV.  244
cd37          VYNRLARYR  281
cd53          KTSQTIG..  218
cd63          RSGYEVM..  238
cd9           RRNREMV..  228
co-029        IGNK.....  237
il-tmp        CCGGDGPV.  202
kai1          DYSKVPKY.  267
16            HQQQYDC..  202
peta-3        KLEHY....  253
sas           RANPSAFL.  210
sm23          IKEYENV..  218
tapa-1        RNSSVY...  236
upk1a         FYTTL....  258
humanupk1b    YWSRIEYE.  261
```

3.3.2 Cloning of human UPK1B genomic DNA

A PCR cloning strategy was employed to amplify a region of the human UPK1B gene. PCR primers were designed to anneal to regions of cDNA with 100% homology between mink TI1 and bovine UPKIb cDNA sequences with the assumption these regions would be most likely to be conserved in the human homologue. These primers, TM3 and ECD, were also designed to anneal to two putative adjacent exons, the third transmembrane domain and the large extracellular domain which would flank a putative intron. This intron location is conserved in other tetraspan members, CD53, CD63 and TAPA-1 (Wright et al., 1993). To date, no genomic sequence or structure for either the mink TI1 gene or the bovine UPKIb gene is available. The sequence of the TM3 and ECD primers was run through "FASTA" (Pearson et al., 1985) to ensure they would not anneal to any sequences from other tetraspan family genes. From genomic mink DNA, extracted from the CCL64 cell line, a fragment of approximately 800 bp in length was amplified using TM3 and ECD primers. A human genomic UPK1B PCR product of 784 bp was amplified using TM3 and ECD primers and cloned into the pGEM-T vector (Figure 3.8). Sequencing using pGEM-T vector-specific primers (Appendix III) confirmed the identification of this product as human UPK1B (GenBank Accession number AF067147) and not as any other member of the tetraspan family. This genomic sequence consists of 119 bp of total cDNA flanking a 665 bp intron. The positioning of the exon/intron boundaries was determined by the abrupt end of sequence homology between the 784 bp human genomic product and the human UPK1B cDNA sequence as well as the presence of consensus sequences observed at the exon/intron boundaries (Figure 3.9). The sequence at both the start and finish of the intron for TM3 and ECD match the

A.

TM3

314

665 bp

434

ECD

784 bp UPK1B genomic product

B

1 2 3 4
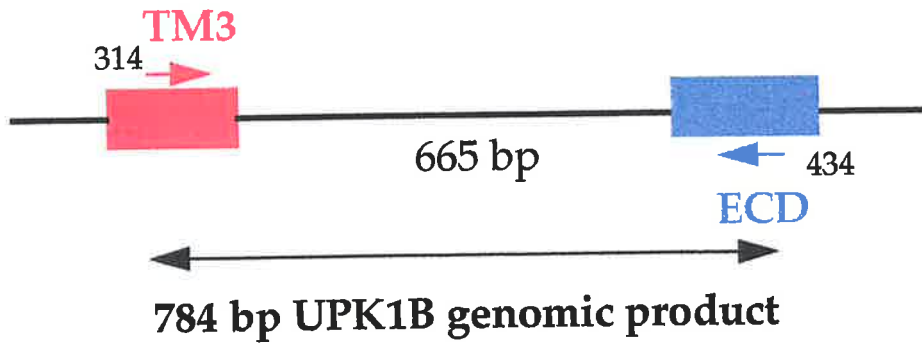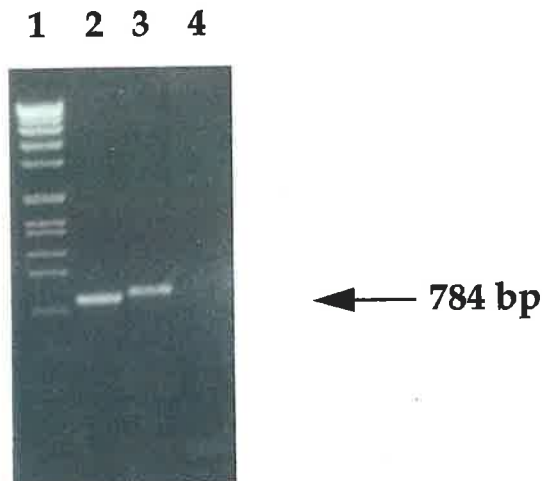
◄— **784 bp**

Figure 3.8
A.
Location of the TM3 and ECD primers in respect to the numbering of the human UPK1B cDNA sequence. The TM3 and ECD primers were used to amplify a 784 bp human genomic UPK1B PCR product. Exons are represented by boxes and the introns are represented by black lines.

B.
Agarose gel electrophoresis of the PCR products amplified from human genomic DNA and also the mink lung epithelial cell line, CCL64, using TM3 and ECD primers, run on a 1.2% gel.
Lane 1: SPP1/*Eco*RI marker
Lane 2: human genomic DNA
Lane 3: CCL64 cell line genomic DNA
Lane 4: negative control (no DNA)

Figure 3.9 Sequence of the human UPK1B 784 bp genomic PCR product

A.

Exon/intron boundaries were determined by aligning the 784 bp human UPK1B genomic sequence with region of the human UPK1B cDNA sequence. Nucleotides with 100% homology between the genomic and cDNA sequence are shown as the corresponding nucleotides. Regions of sequence with no homology are represented by dots. The TM3 exon is represented in red, with the TM3 primer in bold. The sequence representing the exon/intron boundary between the TM3 exon and intron is underlined. The ECD exon is represented in blue, with the ECD primer in bold. The sequence representing the exon/intron boundary between the ECD exon and intron is underlined.

B.

Exon/intron boundaries were also determined by comparison with consensus sequences (Mount et al. 1982). Those sequences not in agreement with the consensus sequence are in italics.

A

(TM3)

GAAGTGGCATCTTGTATCACAGCAGCAACACAACGAGACTTTGTGAGTACAACCTCAAAA genomic
GAAGTGGCATCTTGTATCACAGCAGCAACACAACGAGACTTT.................. cDNA

AGCAAAATAATATGAATTATCATTATTACAACAACCAACATGTATTAACCCCTTACTATG genomic
.... cDNA

TGCCCAGGCAATCCAATATGCACAGTAATAATACATCTATCATTTTACTTAATCCTCATA genomic
.... cDNA

AATCTATGAAATAGGCATTATCACTCTGGTCATTTGAGAAAGGAAAAAAAATGAGACAGG genomic
.... cDNA

AAGGTAAAATGACTTGTCTAAAGTGACATAGTTGGCAACTGAAAGAGCAAGAATTCTGAA genomic
.... cDNA

CCCAGATGGCCTGACTCCCAGGGCCCAAGTTTGTAATCCCCTGTGGGTTTCACCACTCTG genomic
.... cDNA

CCATGGCCATGGGCTTCCAATGGGAGGATCTCTTGTTCTTCCTCAAGCTAAGACTTGCTC genomic
.... cDNA

TCAATTCTACATCTTTCTTTCTCTCCCATTCTCTGTTTTCCTAGCCTAAGCCACCCTACA genomic
.... cDNA

GTATGTGTTCCCCTGGCCTCATAGTGATTTCTTTTAAGATGAGAGTCTCTTCTCTGCATG genomic
.... cDNA

GGGACTTATGCCAACCTCAGCCAGCTGTACTTGGTGCCTCTGAGTACAGTTTTGCTCCAG genomic
.... cDNA

GAGAGAATGTTCAGTGTCCCCAGGAAAAGACAGAGAAAAATATTTTCCTCTCCTTGAAAA genomic
.... cDNA

TTAGCGTGCTATCTCTCCCTCTTGTGTTGCCTCTTCCTACTATAGTTCACACCCAACCTC genomic
....*TTCACACCCAACCTC* cDNA

TTCCTGAAGCAGATGCTAGAGAGGTACCAAAACAACAGCC**CTCCAAACAATGATGACCAA** genomic
TTCCTGAAGCAGATGCTAGAGAGGTACCAAAACAACAGCCCTCCAAACAATGATGACCAA cDNA

TGGA (ECD) genomic
TGGA cDNA

B

TM3 exon/intron:	*T*	*T*	*T*	G	T	G	A	G	T
Donor consensus :	C/A	A	G	G	T	A/G	A	G	T

ECD intron/exon:		T	A	T	A	G	*T*
Acceptor consensus:		T/C	N	C/T	A	G	G

consensus sequence. However, the TM3 exon sequence does not contain the "C/A A G" sequence commonly seen at the exon/intron splice site, nor does the beginning of the exon of ECD start with a "G". The consensus sequences were determined from a compilation of donor and acceptor sequences by examining the frequency of a nucleotide at each position at the splice junctions and therefore there may be some differences for individual genes (Mount, 1982). A 778 bp human UPK1B genomic probe was isolated by digesting the 784 bp UPK1B/pGEM plasmid with *Kpn*I and *Acc*I.

3.3.3 Detection of a *Taq*I restriction fragment length polymorphism

To search for an RFLP in the human UPK1B gene, genomic DNA samples isolated from normal peripheral blood lymphocytes were digested with various restriction enzymes. These digested DNA samples were subjected to Southern analysis using the 778 bp human genomic UPK1B probe, isolated as described above (3.3.2). When the DNA was digested with the enzymes *Xba*I, *Pst*I, *Hind*III, *Pvu*II, *Bgl*II and *Bam*HI no polymorphism was detected, that is, all DNA samples analysed showed the same banding pattern (Figure 3.10). Hybridisation of the UPK1B probe on the *Xba*I, *Pst*I, *Hind*III, *Bgl*II and *Bam*HI Southerns produced bands sizes of 7.2 kb, 15 kb, 7.4 kb, 13.5 kb and 12 kb respectively. Two bands of sizes 2.8 kb and 3.5 kb were detected for all individuals when the *Pvu*II-digested Southern was probed with UPK1B. However, a polymorphism was observed when the DNA was digested with the *Taq*I restriction enzyme, as indicated by detection of two fragments of sizes 2.5 kb and 6.4 kb (Figure 3.11). Not all individuals showed the same banding pattern, suggesting the presence of a restriction fragment length polymorphism. To determine the heterozygosity rate of this RFLP, DNA was isolated from forty-
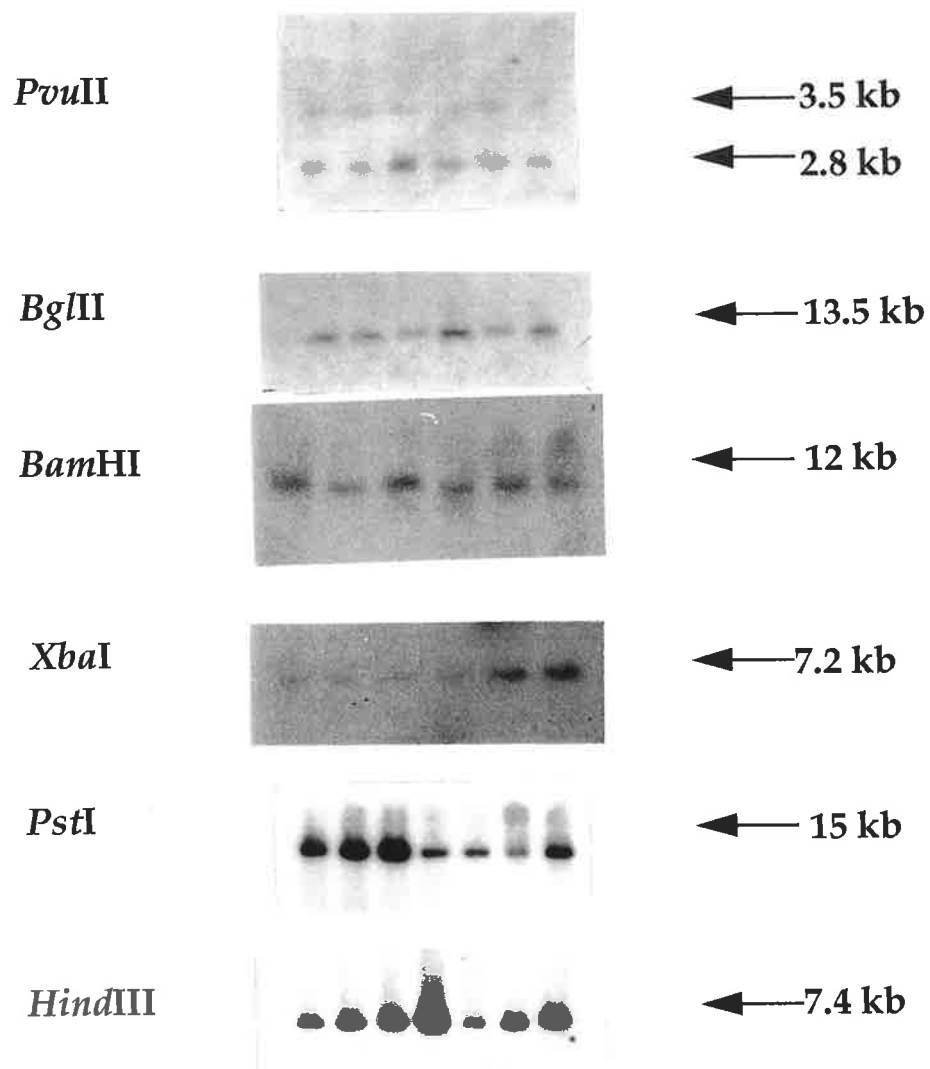
Figure 3.10
Genomic DNA was separately digested with *Pvu*II, *Bgl*II, *Bam*HI, *Xba*I, *Pst*I and *Hind*III and transferred to nylon membrane. Each individual Southern was probed with the 778 bp human genomic UPK1B fragment. No UPK1B polymorphism was detected for any of the enzymes.
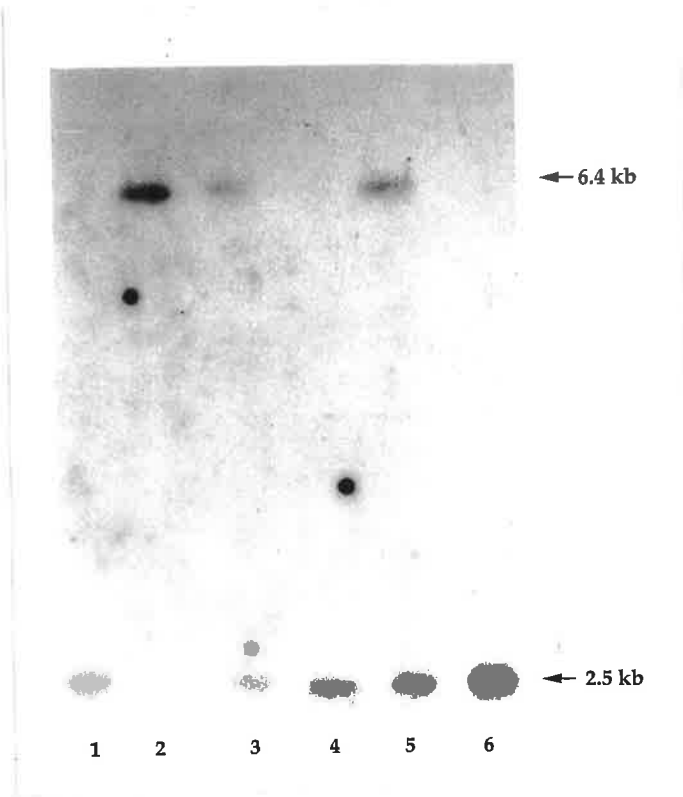
Figure 3.11

Autoradiograph of a Southern blot containing genomic DNA, from six unrelated individuals, digested with *Taq*I. The blot was hybridised with the 778 bp human UPK1B genomic probe.

Lane 1: homozygote 2.5/2.5
Lane 2: homozygote 6.4/6.4
Lane 3: heterozygote 6.4/2.5
Lane 4: homozygote 2.5/2.5
Lane 5: heterozygote 6.4/2.5
Lane 6: homozygote 2.5/2.5

(This figure was published in Finch et al., 1997).

nine individuals (ninety-eight chromosomes) from an unrelated Caucasian population of blood donors and analysed for the *Taq*I polymorphism (Table 3.2).

Table 3.2   Distribution of genotypes of the *Taq*I polymorphism

Genotype	6.4 / 6.4 kb (homozygote)	6.4 / 2.5 kb (heterozygote)	2.5 / 2.5 kb (homozygote)	Total
Number	1	14	34	49

Of the forty-nine individuals analysed for the *Taq*I polymorphism, fourteen were heterozygous (6.4/2.5), giving a heterozygosity rate of 29%. Allele frequencies were determined to be 0.84 and 0.16 for the 2.5 kb and 6.4 kb fragments respectively (Table 3.3).

Table 3.3   Allele frequencies of the 2.5 kb and 6.4 kb fragments

Allele	Number observed	Frequency
2. 5 kb	82	0.84
6.4 kb	16	0.16
Total	98	1

A family study suggested autosomal co-dominant segregation of this *Taq*I polymorphism (Figure 3.12). All three offspring had inherited the 2.5 kb allele from their mother and the 6.4 kb allele from their father. Studies of larger family pedigrees with multiple generations would be required to confirm Mendelian inheritance of the *Taq*I polymorphism in an autosomal co-dominant manner.
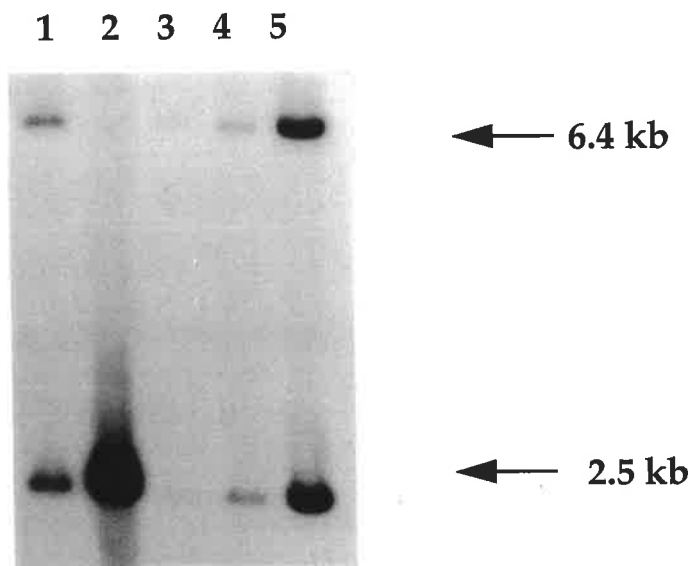
Figure 3.12
Autoradiograph of a Southern blot containing genomic DNA, from a family of individuals, digested with *Taq*I. The blot was hybridised with the 778 bp human UPK1B genomic probe.

Lane 1:   Father   -heterozygote 6.4/2.5
Lane 2:   Mother   -homozygote 2.5/2.5
Lane 3:   Son   -heterozygote 6.4/2.5
Lane 4:   Daughter   -heterozygote 6.4/2.5
Lane 5:   Daughter   -heterozygote 6.4/2.5

3.3.4 Cloning of the 2.5 kb UPK1B *Taq*I fragment

To determine further human UPK1B genomic sequence, an adaptation of the inverse-PCR method (Silver et al., 1989) was used to clone the *Taq*I-generated 2.5 kb genomic fragment of the human UPK1B gene. Primers were designed in the reverse orientation (on the opposite DNA strand) to the TM3 and ECD primers, originally used to produce the 778 bp probe. These primers were designated RC-TM3 and R-ECD; 'R' representing "reverse" and the 'C' representing "complementary". Normal genomic DNA isolated from an individual homozygous for the 2.5 kb *Taq*I allele was digested with the *Taq*I enzyme. The digested DNA was self-ligated using T4 ligase to form circular DNA molecules of *Taq*I fragments. The reverse primers, RC-TM3 and R-ECD were used to amplify the sequences immediately 5′ and 3′ of the 778 bp probe (Figure 3.13). The approximately-sized 1.7 kb PCR product was cloned into the pGEM-T vector and partially sequenced using pGEM-T-specific primers (Appendix IV). The sequence contained 500 bp located 5′ from the TM3 primer and 273 bp from the ECD primer to the *Taq*I site. Continuing beyond the 3′ *Taq*I site was 200 bp of sequence which began from the 5′ *Taq*I site, located approximately 1.4 kb upstream of the TM3 primer (Figure 3.13). To sequence the remaining 700 bp, primers were then designed from the initial partial sequencing of the 1.7 kb PCR *Taq*I product. These primers, designated hTI1A and UPK1B-1, were used to amplify the remaining 700 bp of unknown sequence from the pGEM plasmid containing the 1.7 kb region of the 2.5 kb *Taq*I fragment (Figure 3.14). The PCR product was then sequenced using the hTI1A and UPK1B-1 primers (Appendix V). Analysis of this sequence by GeneJockey did not reveal any homology with the cDNA sequence 5′ of the TM3

Figure 3.13 Amplification of the 1.7 kb UPK1B region from the 2.5 kb *Taq*I fragment.

A.The diagram displays the*Taq*I fragment of genomic DNA that was self-ligated and used as a template from which the 1.7 kb region of UPK1B genomic DNA was amplified.

B.The lower diagram represents of the linear UPK1B 2.5 kb DNA fragment. The boxes represent exons and the arrows represent the primers. The reverse primers, RC-TM3 and R-ECD amplify the DNA sequence up to the *Taq*I sites. The thick black line represents the original 778 bp probe used to detect the 2.5 kb *Taq*I fragment on Southern blots.

A

RC-TM3

R-ECD

778 bp

*Taq*I
~2.5 kb

273 bp

500 bp

*Taq*I

200 bp

700 bp

B

*Taq*I

TM3 exon

778 bp

ECD exon

*Taq*I

5′   200 bp   700 bp   500 bp

273 bp   3′

RC-TM3

R-ECD

A
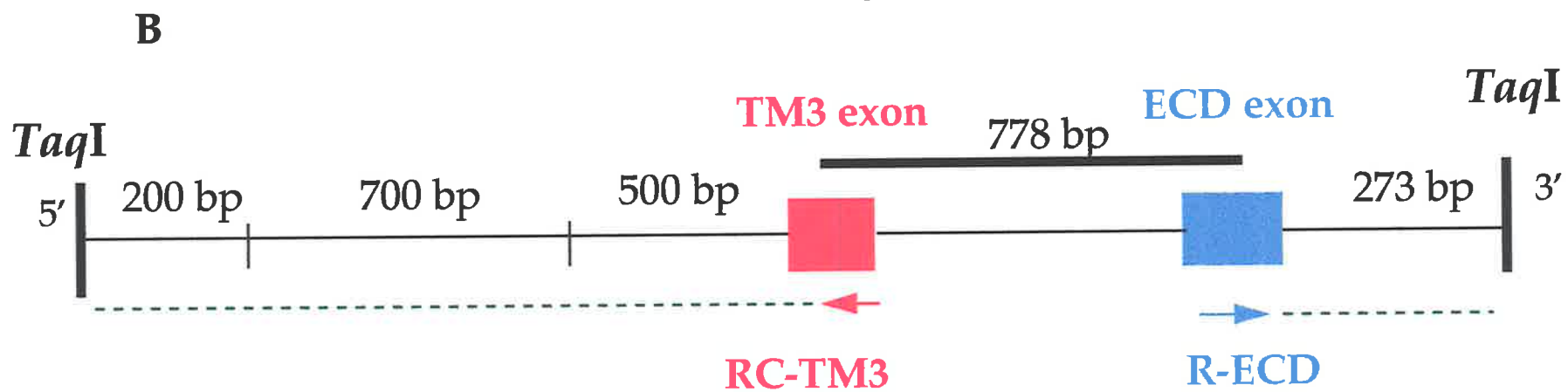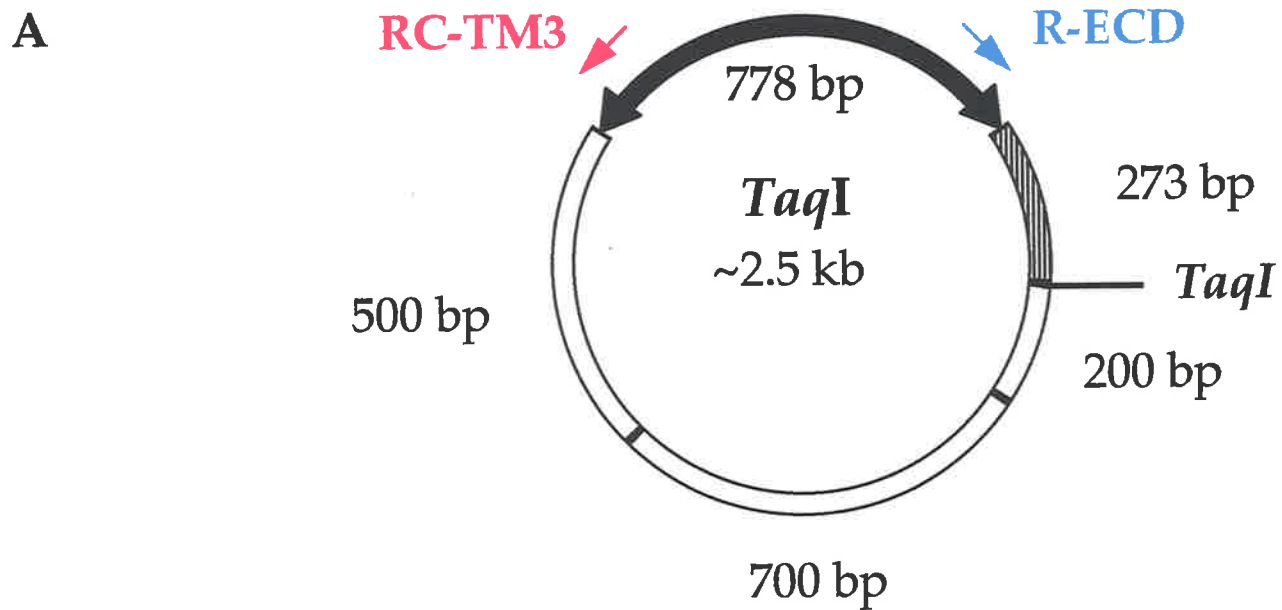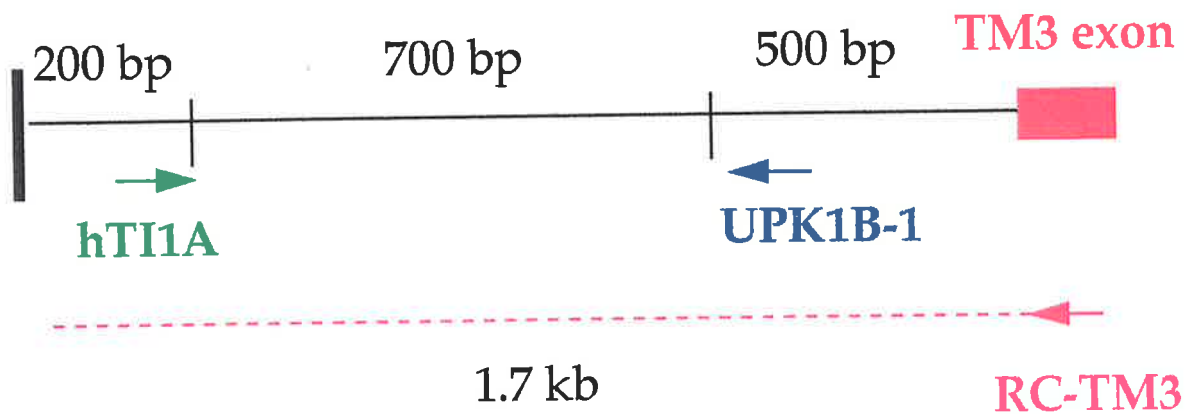
B
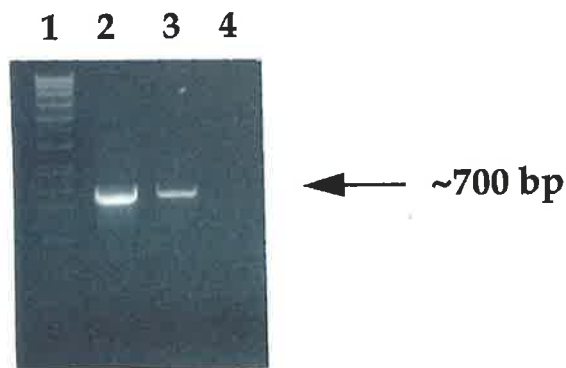
Figure 3.14

A.
Diagrammatic representation of the positioning of the hTI1A and UPK1B-1 primers, designed to allow amplification and subsequent sequencing of the unknown 700 bp UPK1B genomic sequence.

B.
Gel photo of the PCR amplification of the 700 bp human UPK1B genomic sequence from the 1.7 kb inv/pGEM plasmid using hTI1A and UPK1B-1 primers.

Lane 1: SPP1/*Eco*RI marker

Lane 2: 1.7 kb/pGEM plasmid

Lane 3: human genomic DNA

Lane 4: Negative control (no DNA)

exon, indicating that there is at least 1.4 kb of intronic sequence immediately 5′ of the TM3 exon.

3.3.5 Human UPK1B gene structure

From sequence comparisons of human UPK1B genomic and cDNA sequences, it was determined that the TM3 exon consists of 73 bp and the ECD exon is 122 bp in length. The TM3 exon most likely codes for the entire third transmembrane domain of the protein, which is approximately 26 amino acids in length. However, there are probably a number of ECD exons that code for the extracellular domain of the UPK1B protein which is approximately 118 amino acids in length.

Hybridisation of the entire open reading frame cDNA of UPK1B onto a Southern blot consisting of human genomic DNA digested with the *Taq*I enzyme, detected 5 different-sized bands (Figure 3.15). The sum of these bands equals approximately 17 kb, suggesting that the human UPK1B gene is at least 17 kb in length.

3.4   DISCUSSION

This chapter describes the isolation and cloning of human uroplakin 1B-specific sequences. These results confirm the existence of a human homologue of the mink TI1 and bovine UPKIb genes. The cDNA coding for the open reading frame of the human uroplakin 1B gene was cloned using a PCR-based strategy for a number of reasons. Firstly, no normal human bladder cDNA library is commercially available, therefore it was not possible to obtain a full length ORF
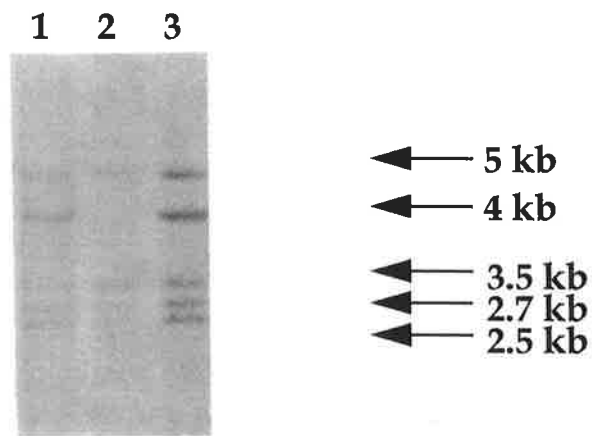
Figure 3.15
Genomic DNA (Lanes 1-3) was isolated from normal individuals, digested with
*Taq*I, run on a Southern gel and transferred to nylon membrane. The blot was
hybridised with the total UPK1B cDNA ORF. Five bands were detected of sizes
5, 4, 3.5, 2.7 and 2.5 kb. The sum of these bands is 17 kb, indicating the UPK1B
gene is at least 17 kb in length. The DNA used was from individuals homozygote
(2.5/2.5) for the *Taq*I polymorphism, therefore explaining the absence of the
6.4 kb band in any of the individuals.

clone by standard library screening techniques. Secondly, the difficulty in obtaining large amounts of normal human bladder tissue made it impossible to make our own human bladder cDNA library. Thirdly, 5′ and 3′ RACE (rapid amplification of cDNA ends) methods were excluded because it was not deemed necessary at this stage to obtain the full length cDNA sequence. Only the cDNA which coded for the protein would be required for functional transfection studies and as a probe for Northern analysis. Similarly, at the time of these experiments no expressed sequence tags (EST) were available for the human uroplakin 1B sequence, ascertained by using bovine UPKIb cDNA sequence to search for relevant ESTs. A PCR-based cloning strategy was therefore chosen to clone the cDNA coding for open reading frame of the protein. Comparisons of the human uroplakin 1B ORF cDNA with its bovine and mink homologues revealed homologies of 91% and 92% respectively. The putative human UPK1B protein is 260 amino acids in length and has homologies of 92% and 93% with the bovine UPKIb and mink TI1 proteins. Other tetraspan proteins, also exhibit high percentage identities between their respective mammalian homologues. For example, the human CD53 protein has 82% homology with mouse CD53 and 80% homology with rat CD53 (Wright et al., 1993). The human CD9 antigen has 89% amino acid homology with mouse CD9 and 83.5% homology with the bovine CD9 protein (Rubinstein et al., 1993b, Martin-Alonso et al., 1992).

A total of 2.5 kb of contiguous human uroplakin 1B genomic sequence was cloned. The total genomic size of human uroplakin 1B is predicted to be greater than 17 kb in length. The UPK1B cDNA probe used to hybridise to the genomic

Southern does not span the 5′ and 3′ untranslated regions so the size of the UPK1B gene is likely to be at least 17 kb in length. The sum of the fragments produced upon hybridisation of the UPK1B cDNA probe on the genomic Southern was used to estimate the size of the UPK1B gene. Other tetraspan genes have variable gene sizes including 20 kb (CD9) (Rubinstein et al., 1993a), 26 kb (CD53) (Korinek et al., 1993) and 80 kb (KAI1) (Dong et al., 1997). Characterisation of the 784 bp TM3-ECD genomic fragment revealed the presence of a 665 bp intron between the 73 bp TM3 exon and the 122 bp ECD exon. The positioning of an intron between the cDNA coding for the TM3 and ECD protein domains of UPK1B is also conserved in mouse TAPA-1, mouse CD53 and human CD63 tetraspan genes (Wright et al., 1993).

An intragenic *Taq*I RFLP was discovered within the UPK1B gene, most probably within an intron. The heterozygosity rate of the *Taq*I polymorphism was determined to be 29%, therefore the polymorphism is not highly informative. The discovery of this polymorphism adds to the characterisation of the human uroplakin 1B gene and adds to the building of a restriction enzyme map of the genomic structure. The polymorphism would be useful for allelic loss studies of the UPK1B gene in tumours, providing large numbers of matched normal and tumour samples were available as the polymorphism is only informative in 29% of individuals.

The results in this chapter confirm the existence of the human homologue of the mink TI1 and bovine uroplakin Ib gene. For the first time, human uroplakin 1B cDNA and genomic sequences have been cloned. The human uroplakin 1B gene

has high homology with its species homologues as well as showing considerable homology with motifs conserved in the tetraspan proteins. These results will enable further characterisation of this gene as described in the following chapters.

CHAPTER 4

CHROMOSOMAL LOCALISATION OF THE HUMAN AND MOUSE UROPLAKIN 1B GENES

4.1 INTRODUCTION

At the commencement of this study, the chromosomal location of the human UPK1B gene and the mink TI1 gene was unknown. However, the bovine homologue, UPKIb, had previously been localised to bovine chromosome 1 (Ryan et al., 1993). Human homologues of other genes found on bovine chromosome 1 including superoxide dismutase 1 (SOD1) and uridine monophosphate synthase (UMP), have been previously mapped to either human chromosome 21 or 3q (Barendse et al., 1993), suggesting that the human UPK1B gene would be localised to either of these chromosome regions.

The main aim of this study was to determine the chromosomal location of the human UPK1B gene. The mapping of the UPK1B gene to a particular human chromosome may provide insight into a potential role in human disease and, in particular, bladder cancer. A gene located in a region frequently deleted in a specific cancer may be implicated as a candidate tumour suppressor for that cancer. Also, chromosome regions often affected by allelic loss may harbour a tumour suppressor gene as is consistent with the Knudson (1971) 'two-hit' hypothesis (Chapter 1). The 'two-hits' may involve inactivation of both alleles of a suppressor gene by allelic loss or by a germ-line mutation followed by allelic loss. Therefore the identification of cytogenetic deletions associated with the occurrence of cancer are important in the search for tumour suppressor genes. The retinoblastoma gene, for example, was cloned following the discovery of deletions which consistently overlapped the chromosome sub-band 13q14 in some familial retinoblastomas. Further analysis using DNA probes spanning 13q14, which detected RFLPs, showed loss of heterozyosity of this chromosome

region in the retinoblastoma patients suggesting the retinoblastoma gene was located at this locus (Cavenee et al., 1983). Other cancer genes, including those responsible for Wilms tumour, familial adenomatous polyposis and neurofibromatosis type 1 (NF1), were discovered due to close examination of regions of large cytogenetic deletions or translocations (reviewed in Fearon, 1997). Identification of the chromosomal location of a gene also aids in the development of the genetic map of the order of genes for a particular chromosome. Regional localisation of the UPK1B gene might allow the application of chromosome-specific polymorphic markers for loss of heterozygosity studies.

The mapping of the mouse Upk1b gene to mouse chromosomes is also described in this chapter. A mouse Upk1b genomic fragment was cloned by PCR techniques with the same TM3 and ECD primers used to clone the human 784 bp UPK1B genomic fragment (Chapter 3.3.2). Strong sequence homology was observed between the cDNA sequences, present in the TM3-ECD genomic probes, of both human and mouse uroplakin 1B. Hybridisation of the mouse-specific Upk1b probe onto mouse chromosomes allowed the chromosomal location of Upk1b to be determined.

A direct approach for chromosome localisation of a particular gene is localisation by *in situ* hybridisation. This method involves the hybridisation of a nucleic acid probe directly onto slides which contain fixed chromosome spreads. The probe will hybridise to its complementary DNA sequence, allowing determination of the location of the gene (Buckle et al., 1986). *In situ* hybridisation experiments

usually involve one of two labelling and detection systems; either fluorescent *in situ* hybridisation (FISH) or radioactive *in situ* hybridisation (RISH). The FISH method is less sensitive and works only with probes of length greater than one kilobase (Lemieux et al., 1992), therefore the radioactive *in situ* approach, using tritium labelling was chosen for the current study because one of the human UPK1B genomic probes was only 778 bp in length and the mouse probe was only 727 bp.

4.2 METHODS

4.2.1 Preparation of human UPK1B probes

Two contiguous human UPK1B genomic probes, of lengths 778 bp and 1.4 kb, were used to map UPK1B. A 832 bp human UPK1B cDNA probe was also used to search for processed pseudogenes of UPK1B. The 778 bp human UPK1B genomic probe was isolated from the TM3-ECD/pGEM construct as described in 3.3.2. The 1.4 kb human UPK1B genomic probe was isolated from the 1.7 kb UPK1B/pGEM construct (3.3.4) by digestion with the *Sac*II and *Taq*I enzymes (2.2.11). The 832 bp human UPK1B cDNA probe was isolated from the UPK1B cDNA/pGEM plasmid (described in 3.3.1) by digestion with the *Sac*II and *Sac*I enzymes (2.2.11). All three probes were electrophoresed on a low-melting point agarose gel and purified by phenol-chloroform extraction. The agarose slice was melted at 70°C and 3 volumes of TE added. The DNA was extracted by the addition of 1 volume of phenol, then phenol/chloroform (1:1), followed by chloroform. The mixture was centrifuged at 12 000 g for 5 min between each step and the top aqueous layer retained. The DNA was then precipitated with 2.5 volumes of ethanol and 1/10

volume of 3M sodium acetate, pH 5.2; the resultant pellet washed in 70% ethanol and resuspended in 30 µl water.

4.2.2 Cloning of a mouse Upk1b genomic fragment

The PCR primers, TM3 and ECD (Table 2.1), were used to amplify a 693 bp mouse Upk1b fragment from genomic DNA from a C57BL mouse, at an annealing temperature of 60°C (2.2.7). The 693 bp mouse Upk1b PCR product was cloned into the pGEM-Easy vector (2.2.10), transformed into competent cells (2.2.14) and a mouse Upk1b/pGEM plasmid propagated using the Qiagen plasmid kit (2.2.16). The mouse Upk1b/pGEM plasmid was sequenced using pGEM-specific primers (2.2.17). To isolate a probe to use for mapping the mouse Upk1b gene, the Upk1b/pGEM plasmid was digested with the *Not*I enzyme (2.2.11) to release a 727 bp genomic fragment of Upk1b. The mouse Upk1b fragment was purified from low-melting point agarose, using phenol-chloroform extraction as described in 4.2.1.

4.2.3 Probe labelling by nick translation

The two human genomic UPK1B probes of length 778 bp and 1.4 kb, the 832 bp human cDNA UPK1B probe and the 727 bp mouse Upk1b probe were labelled using a nick translation kit (Amersham) and tritiated dATP, dCTP and dTTP (Amersham). 12.5 µCi of tritiated dATP, dCTP and dTTP were vacuum-dried in an Eppendorf tube. To the tube were added 200 ng probe (5 µl of 40 ng/µl), 1 µl cold 300 µM dGTP, 1.25 µl enzyme mix (Amersham) and 5.25 µl water and the

mixture was incubated at 15°C for 2 hr. A column was prepared by adding fine Sephadex G-50 to a pasteur pipette, plugged with sterile, non-absorbent cotton wool. The incorporated tritiated nucleotides were purified from the unincorporated nucleotides by fractionation through the Sephadex column. After the column was washed three times with TE, the nick translation reaction mixture was layered onto the column and fractions collected in separate Eppendorf tubes after an initial addition of 500 μl TE, 11 additions of 100 μl TE, 5 additions of 300 μl TE and a final addition of 600 μl TE. To determine which fractions contained the labelled probe, 2 μl of each fraction was added with 300 μl water to 3 ml scintillation fluid (Amersham) and the samples counted on a Beckman LS 2800 Scintillation counter. The DNA probe appears in the void volume of the column, while the unincorporated nucleotides are retarded by the Sephadex gel and appear in later fractions. The fraction of the radioactivity in the probe peak was calculated using the formula below.

$$\text{specific activity (CPM/μg)} = \frac{\text{total microcurie} \times \text{fraction incorporated} \times 2.22\times10^6}{\text{mass of probe in μg}}$$

a) $$\text{fraction incorporated} = \frac{\text{total counts in probe}}{\text{total counts in probe} + \text{unincorporated bases}}$$

b) 1 microcurie = 2.22×10^6 CPM

The radiolabelled probe fractions were ethanol-precipitated with 1/10th volume of 3M sodium acetate, pH 5.2, plus 2.5 volumes of ethanol and stored at -20°C.

4.2.4 Chromosome preparations from human lymphocytes

The following procedure is described in detail by Webb (1998). Briefly, lymphocytes from whole blood were cultured by standard methods (Buckle et al., 1986) using RPMI medium and the mitogens phytohaemagglutin (PHA) (Gibco BRL) at 2ml/100ml and pokeweed mitogen (Sigma) at 5 µg/ml. After 3 days, 200 µg/ml 5-bromodeoxyuridine (5-BrdU) was added to the cells. The 5-BrdU is usually incorporated into the pale G-bands and can cause the cells to arrest growth at the mid-S phase of DNA replication. After overnight incubation, the 5-BrdU was rinsed out using PBS and the cells resuspended in medium containing 10^{-5}M thymidine. The cells were cultured to allow completion of the mitotic cycle for 6.5 - 7 hours. The cells were then treated with colchicine for 15 min to disrupt the mitotic spindle and harvested by standard methods including hypotonic treatment and air-drying of the cells onto slides. The slides were stored at -20°C with a silica drying agent.

Preparation of mouse chromosomes was similar to human except that splenic lymphocytes were cultured with 3 µg/ml concanavalin A (Sigma) as the mitogen, and the time allowed for completion of the mitotic cycle after release of the 5-BrdU block was 4 - 4.5 hr.

4.2.5 Radioactive *in situ* hybridisation (RISH)

<u>RNase treatment of chromosomes preparations</u>

Slides were incubated in 100 µg/ml RNase in 2 x SSC at 37°C for 1 hr. The slides were then rinsed at room temperature in 4 changes of 2 x SSC for 2 min each.

The slides were dehydrated through an ethanol series of 35, 70, 95 and 100% ethanol for approximately 2 min each and then air dried.

Hybridisation

The chromosomal DNA was denatured by placing slides, preheated to 70°C, into 70% formamide/2 x SSC, pH 7.0, at 70°C for 2 min, with frequent agitation. The slides were cooled quickly in ice-cold 70% ethanol to prevent reannealing of the DNA and then dehydrated through an ethanol series of 80, 95 and 100% ethanol for 2 min each and allowed to air dry. The tritium-labelled probe (4.2.3) was precipitated from ethanol by centrifugation for 15 min at 12 000 g, dried in a vacuum desiccator and resuspended in water at approximately 5 ng/μl. The hybridisation mixture was made up in the following proportions: 5 parts of 20% dextran sulphate in deionised formamide, 2 parts of 10 x SSCP, 2 parts probe at a final concentration of 200 ng/ml and 1 part carrier DNA (salmon sperm) at 1000 x probe final concentration. The hybridisation mixture was denatured at 70°C for 10 min, cooled on ice and then 50 μl was added to each slide. Coverslips, washed in 70% ethanol, were placed on the slides and sealed with rubber cement which was allowed to dry. The slides were incubated in a moist hybridisation chamber at 37°C overnight.

Stringency washes

The rubber cement and the coverslips were removed and the slides washed in 3 changes of 50% deionised formamide/2 x SSC, pH 7.0 for 3 min each at 39°C. The

slides were then washed in 5 changes of 2 x SSC, dehydrated in an ethanol series of 35, 70, 95 and 100% ethanol and air dried.

Autoradiography, staining and scoring of slides

The slides were coated with Ilford L4 emulsion and exposed for a certain number of days depending on the specific activity of the probe. They were then developed for 5 min with 50% Kodak D19 developer at 20°C and immediately fixed with film-strength Hypam Fixer with hardener (Ilford, Cheshire, England). After 5 rinses in water, the slides were soaked in 10 μg/ml Hoescht 33258 in 2 x SSC for 30 min, then irradiated with long-wave UV (350 nm) for 1 hour and stained for 20 min with 12-15% Giemsa (BDH R66) V/V in phosphate buffer pH 6.8 (BDH) to detect GBG (G-banding with 5-BrdU and Giemsa Stain) - bands. Grains were initially scored onto a 550 band idiogram of the human chromosomes and also on more detailed idiograms of chromosome 3. Autoradiography, staining and scoring of the slides was carried out by Dr. Graham Webb.

4.3   RESULTS

4.3.1 Mapping of the 778 bp human UPK1B genomic probe

A 778 bp human genomic UPK1B fragment was cloned using PCR primers designed to amplify the intron located between exons coding for the third transmembrane domain and the large extracellular domain (Chapter 3.3.1). This partial genomic probe was used to determine the chromosomal location of the UPK1B gene. The 778 bp probe was hybridised *in-situ* to the chromosomes of three normal human subjects, one male and two female. The slides were exposed

for 75 days due to the low specific activity of the probe which was calculated at 3.37 x 10^7 CPM/µg, (a specific activity of 1.0 x 10^8 CPM/µg is considered a satisfactory label incorporation, Webb, 1998).

The UPK1B genomic probe was localised by *in situ* hybridisation to the proximal region of the long arm of chromosome 3 (3q), using the chromosomes of a normal male subject (Figure 4.1). A cluster of five tall columns, of 6-40 silver grains, were found over the proximal half of arm 3q. A maximum height of only 3 grains were seen over the other chromosomal regions, without any notable clustering to form minor peaks. In approximately 200 cells, 311 grains were scored over the whole karyotype with 96 (30.9%) grains found located over bands 3q13-q21 (Figure 4.2). The tallest column, of 40 grains, was located over sub-band 3q13.3 and the next tallest, of 24 grains, over the interface of this sub-band with band 3q21. Together, the two tallest columns contained 62.7% of the grains over the proximal half of arm 3q: 3cen. - q23.

After initially scoring over all chromosomes to establish the position of the major clusters of grains, an independent score at higher resolution was made to chromosome 3. Detailed scoring of chromosome 3 showed that the two tallest columns of grains were on either side of the interface between bands 3q13.3 and q21, with the taller column over the distal half of 3q13.3 (Figure 4.3). These two columns contained 59.3% of the grains over 3cen.-q23. Therefore, *UPK1B* is probably situated in bands 3q13.3-q21 with a possible point localisation near the interface of these two bands. Localisation of UPK1B to chromosome 3 was

Figure 4.1
A single metaphase chromosome spread hybridised with the 778 bp human UPK1B genomic probe. Signal grains are over 3q13.3-21 and are indicated by large arrows. Background grains are indicated by small arrows.
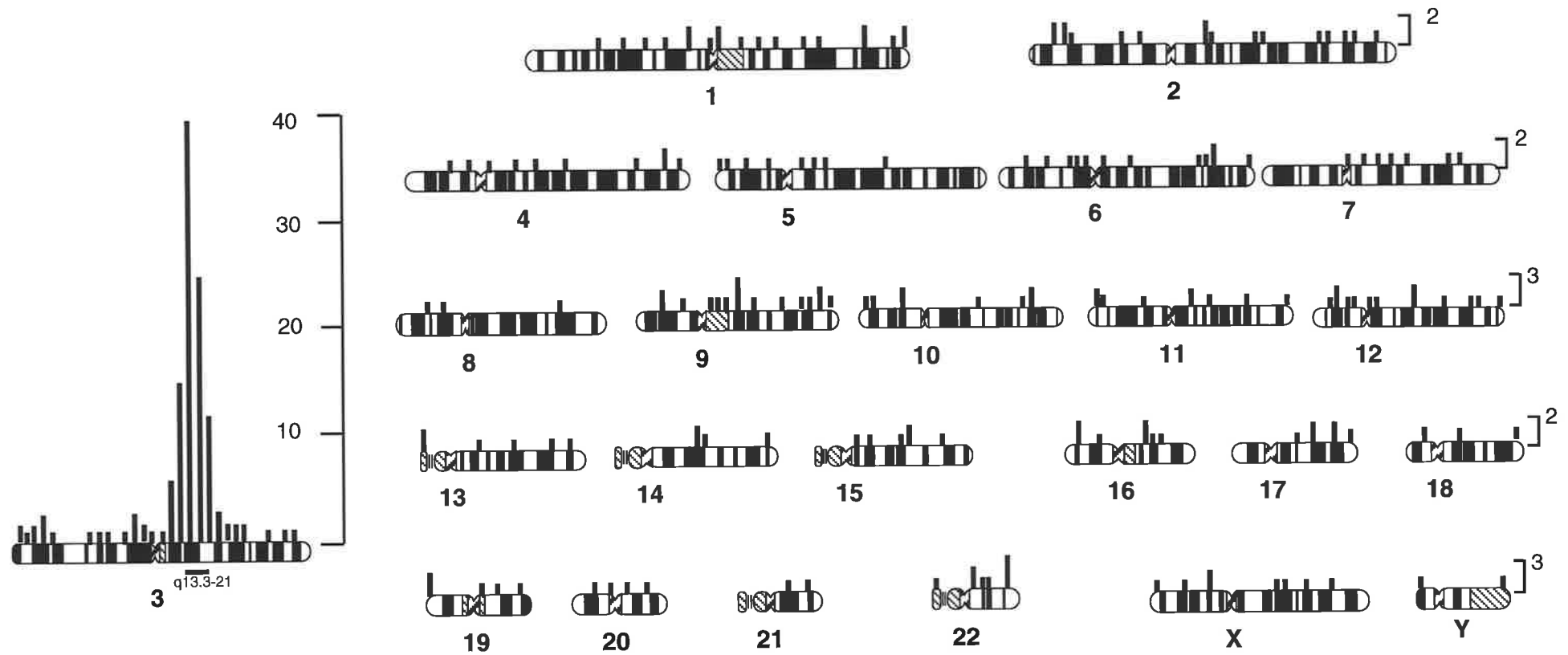
Figure 4.2
Mapping of the human uroplakin 1B gene to chromosome 3q13.3-q21 using the 778 bp human UPK1B genomic probe. Of 311 grains scored in approximately 200 cells, over the entire human chromosome complement, 30.9% of grains were found located over bands 3q13-q21. The tallest column, of 40 grains is located over sub-band 3q13.3 and the next tallest column, of 24 grains, is located over the interface of this sub-band with band 3q21.
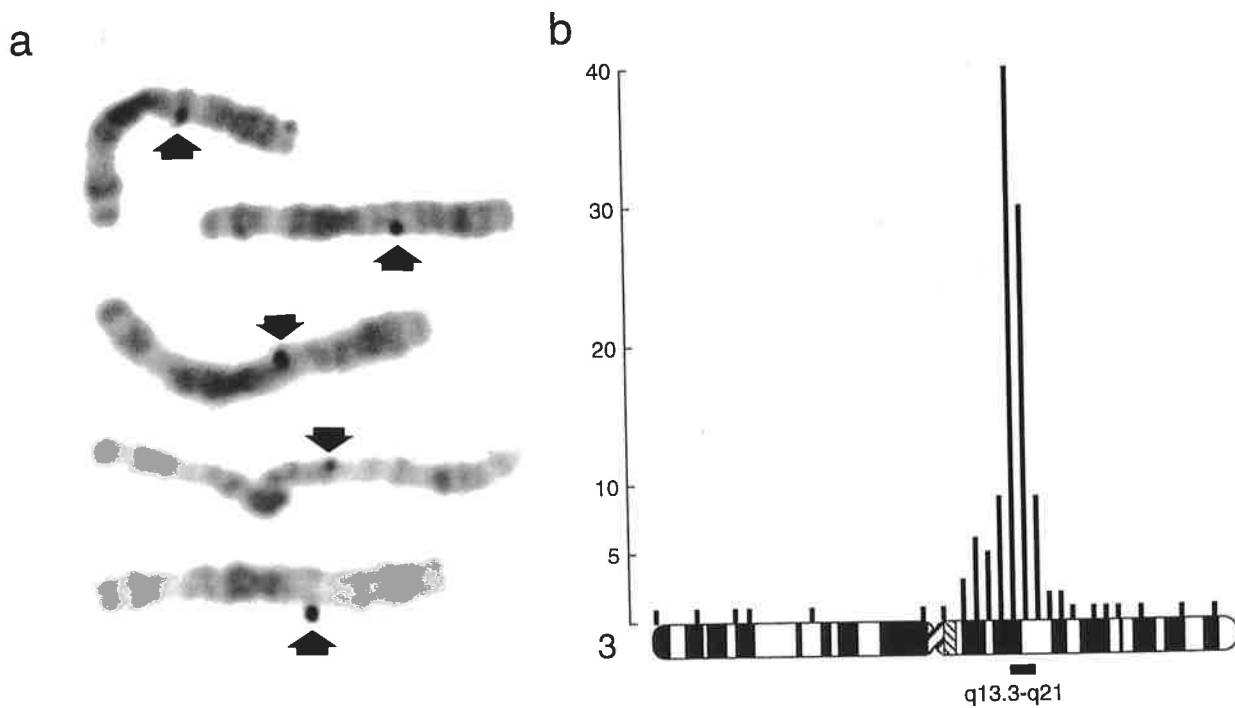
Figure 4.3

a.
Silver grains generated by *in situ* hybridisation of the tritium-labelled 778 bp genomic probe of human UPK1B. The grains are on chromosome 3 and the top two chromosomes are from the same cell.

b.
A detailed score of grains over chromosome 3. The two tallest columns were on either side of the interface between 3q13.3 and 3q21 and contain 59.3% of grains over 3cen-q23.

(This figure was published in Finch et al., 1997.)

qualitatively confirmed in chromosome preparations from the other two, female, subjects.

4.3.2 Mapping of the 1.4 kb human UPK1B genomic probe

A larger human genomic UPK1B fragment of 1.4 kb was also used as a probe for *in situ* hybridisation. To obtain the 1.4 kb probe, the 1.7 kb UPK1B/pGEM plasmid (Chapter 3) was digested with the *Sac*II and *Taq*I enzymes. The *Sac*II site is in the polylinker of the pGEM-T vector and the *Taq*I enzyme cuts within the 1.7 kb UPK1B insert in the 1.7 kb UPK1B/pGEM construct to remove non-contiguous UPK1B genomic DNA. The region of DNA contained in the 1.4 kb probe is found immediately 5′ of the 778 bp probe and encompasses part of the intron located between the second and third transmembrane domains (Figure 4.4). The 1.4 kb probe was tritium-labelled to a specific activity of 7.91×10^6 CPM/µg. Scoring of the slides hybridised with the tritium-labelled 1.4 kb UPK1B probe confirmed the localisation of the UPK1B gene to 3q13.3-21. Qualitative analysis of the slides suggested a single clustering of grains on chromosome 3q. Higher resolution scoring of the long arm of chromosome 3 showed that the highest two peaks contained 40 and 31 grains on bands 3q13.3 and 3q21 respectively (Figure 4.5). These two columns constituted 59% of all grains scored over 3cen.-q23. Localisation of the 1.4 kb and 778 bp human UPK1B genomic probes both show that the most likely location of the UPK1B gene is at 3q13.3-21.

113
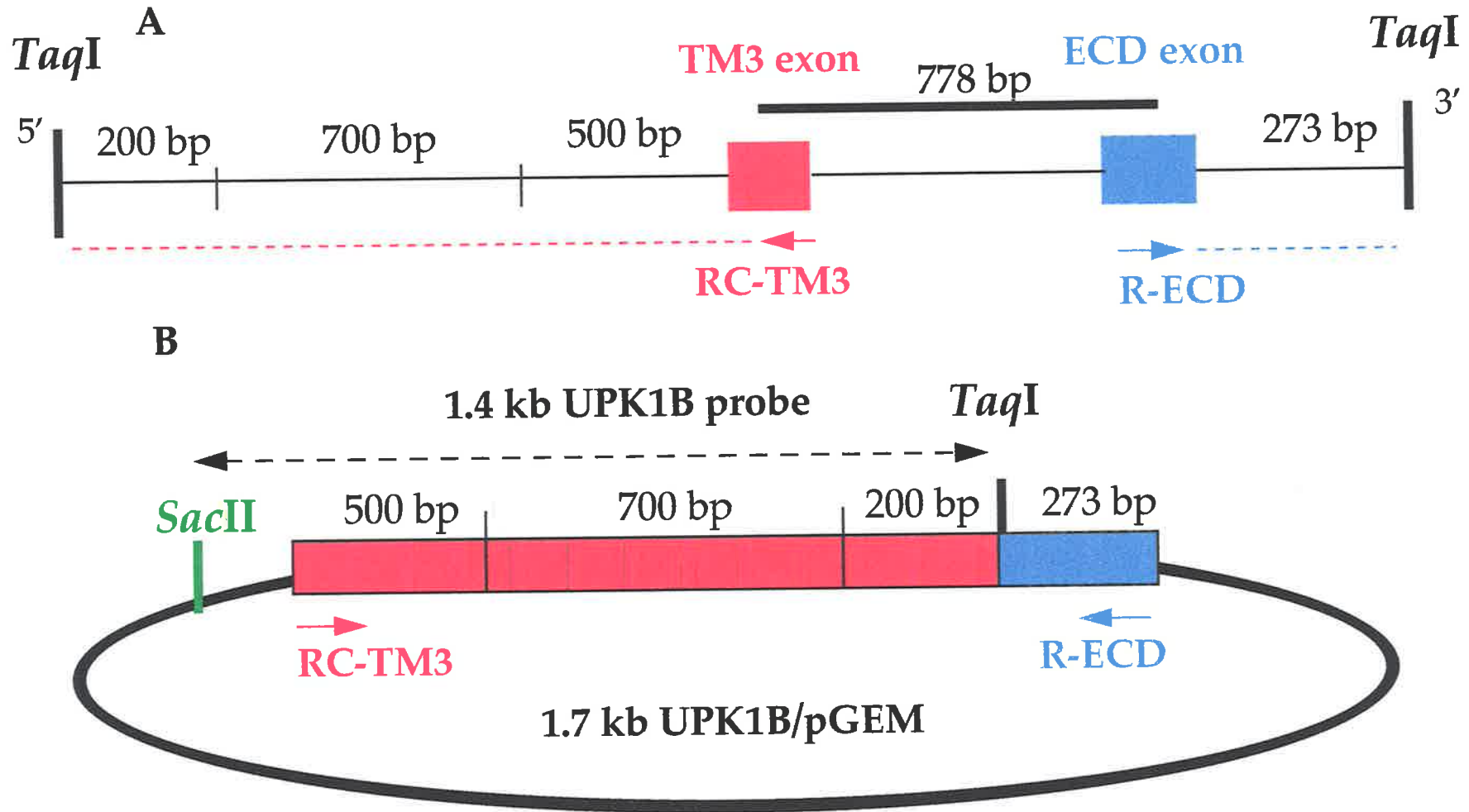
Figure 4.4
A. The RC-TM3 and R-ECD primers were used to amplify the 1.7 kb product, from the circularised *Taq*I fragment as described in Chapter 3 (3.3.4). The 1.7 kb UPK1B PCR product was cloned into pGEM.
B. The 1.4 kb probe was isolated by digestion of the 1.7 kb UPK1B/pGEM plasmid with *Sac*II and *Taq*I.
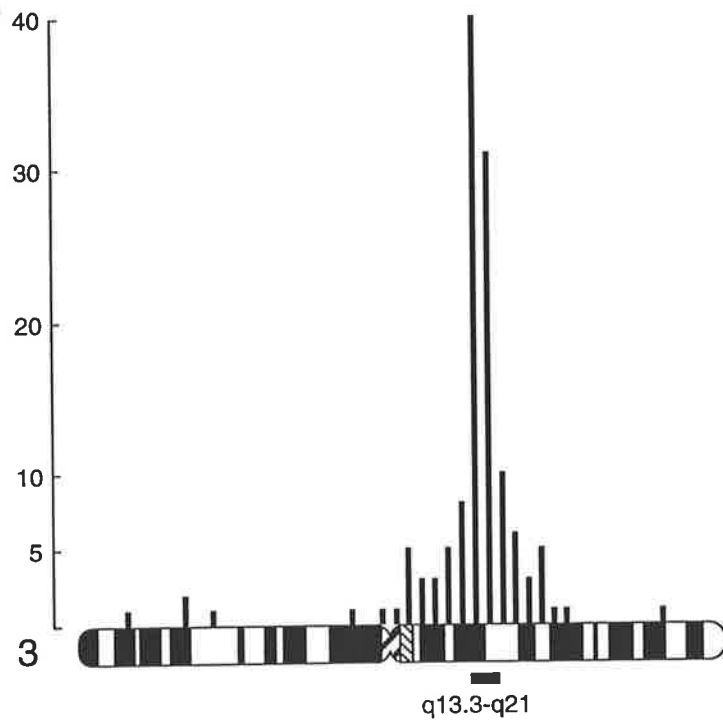
Figure 4.5
High resolution scoring over chromosome 3 using the tritium-labelled 1.4 kb UPK1B genomic probe. The two highest columns of 40 and 31 grains are over bands 3q13.3 and 3q21. These two columns constitute 59% of all grains scored over 3cen.-q23.

4.3.3 Exclusion of nonprocessed UPK1B pseudogenes

Nonprocessed pseudogenes retain the same genomic structure as the active gene, including introns, and are most likely to be found in tandem with the active gene (Sharp, 1983). Chromosomal localisation of the human UPK1B gene to 3q13.3-21, using genomic UPK1B probes, has not excluded the possibility of nonprocessed UPK1B pseudogenes. Southern analysis of normal genomic DNA digested with various enzymes and hybridised with the genomic UPK1B probes, would enable detection of any extra bands, indicating nonprocessed pseudogenes. Hybridisation of the 778 bp human UPK1B genomic probe on Southern blots digested with *Taq*I produced one band, 2.5 kb, for all individuals except for those who have the *Taq*I polymorphism found in this section of the UPK1B gene (Chapter 3.3.3). Hybridisation of the 778 bp UPK1B probe on a number of other Southerns, consisting of DNA digested with either *Xba*I, *Pst*I, *Hind*III, *Bgl*II or *Bam*HI also produces a single band of sizes 7.2 kb, 15 kb, 7.4 kb, 13.5 kb or 12 kb respectively (Chapter 3 -Fig. 3.10). However, the Southern consisting of DNA digested with *Pvu*II produces two bands, 2.8 kb and 3.5 kb when probed with the 778 bp UPK1B genomic fragment (Chapter 3 -Fig. 3.10).

The 1.4 kb probe detected only one band when it was hybridised to Southern blots consisting of DNA digested with *Taq*I, *Pst*I or *Hind*III (Figure 4.6). For the *Taq*I enzyme, hybridisation of the 1.4 kb probe produced either only a 2.5 kb fragment or a 2.5 kb fragment plus a 6.4 kb fragment depending whether or not the individual DNA contained the previously described *Taq*I polymorphism (Chapter 3.3.3). A 15 kb fragment was detected upon hybridisation of the 1.4 kb probe on DNA digested with *Pst*I and a 7.4 kb fragment with *Hind*III. The

A

← 15 kb

B

← 7 kb

C

← 6.4 kb

← 2.5 kb
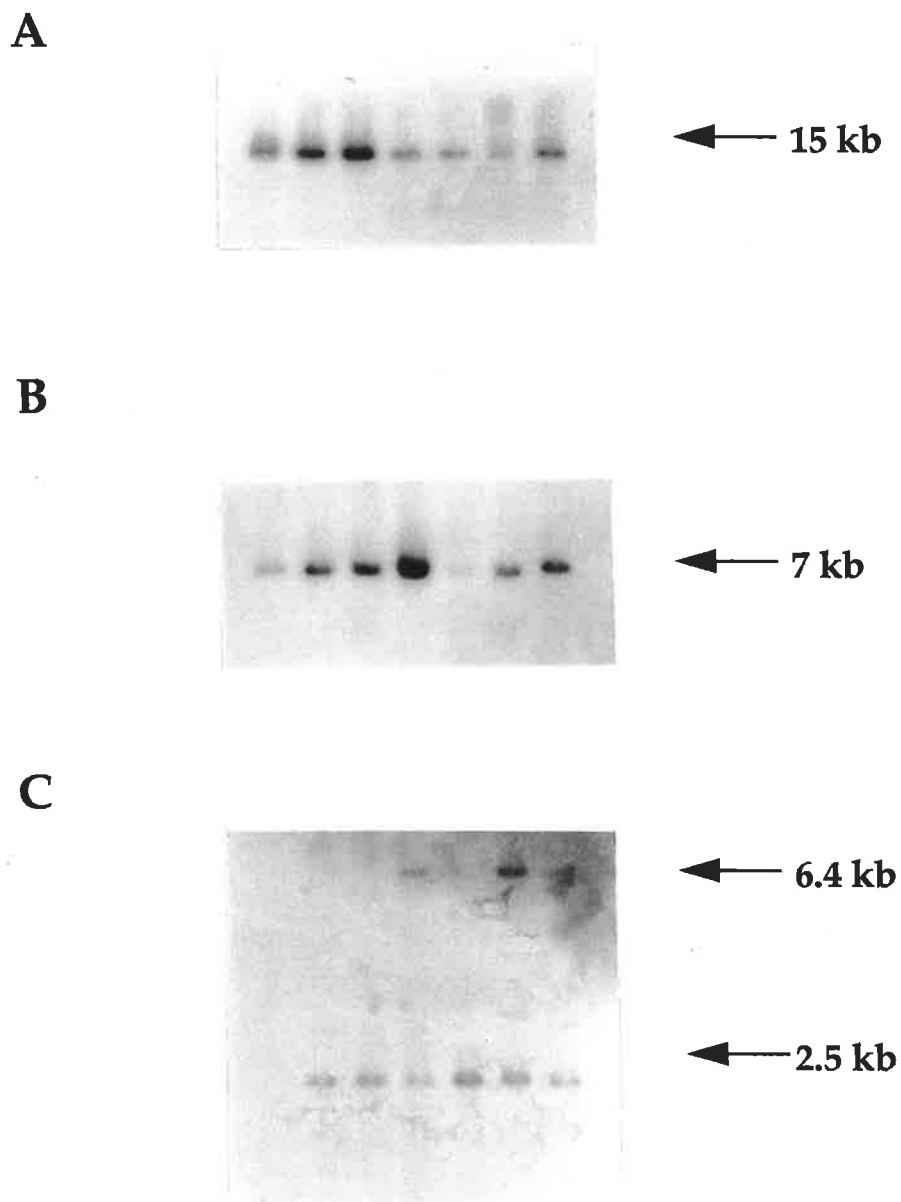
Figure 4.6
Each lane in the above Southerns blots consists of genomic DNA isolated from normal, unrelated individuals which was digested with either *Pst*I (A), *Hind*III (B) or *Taq*I (C). The Southerns were hybridised with the 1.4 kb human UPK1B genomic probe. For the *Pst*I enzyme, the 1.4 kb probe detected a 15 kb band and for the *Hind*III-digested DNA, a 7.4 kb band. One or two bands were detected for the *Taq*I-digested DNA depending on whether or not the individual DNA contained the *Taq*I polymorphism.

possibility of UPK1B nonprocessed pseudogenes present in tandem with the active gene (Webb et al., 1990a) at 3q13.3-21 is unlikely because only a single band is detected by Southern analysis, using either the 778 bp or the 1.4 kb UPK1B genomic probes. These results indicate that only one copy of the UPK1B gene exists in the human genome in the nonprocessed form, that is, containing both exonic and intronic sequences.

4.3.4 Detection of processed UPK1B pseudogenes

Processed pseudogenes are inactive genomic sequences that resemble the RNA transcript. These pseudogenes lack introns, in contrast with the interrupted structure of the active gene and would be expected to be found at different genomic loci as they probably integrate at random into the genome. As both the 778 bp and 1.4 kb UPK1B probes contain predominantly intronic sequence (Chapter 3), it is possible that processed pseudogenes of UPK1B, undetected by the mapping of the UPK1B gene, do exist. This possibility has not been excluded because the 119 bp of total exonic DNA in the 778 bp probe would probably be too short to hybridise successfully to any version of UPK1B (Webb et al., 1994). Also, the 1.4 kb genomic probe consists almost entirely of intronic sequence, except for 56 bp (4%) of exonic sequence which includes the RC-TM3 primer originally used to amplify the 1.7 kb PCR product by inverse PCR (Chapter 3). Processed pseudogenes may therefore not be detected because of a lack of substantial contiguous homology to the two probes used. The failure to detect subpeaks during scoring of the 778 bp and 1.4 kb genomic UPK1B probes, which would indicate the presence of processed pseudogenes, may be because of the small proportion of exonic sequences present in the genomic probes. Hybridisation of

the total UPK1B cDNA on a Southern consisting of normal genomic DNA digested with *Taq*I, produced five different-sized bands (Chapter 3- Fig. 3.15). However, as the total genomic structure of the human UPK1B gene is yet to be determined, these fragments may simply represent the number of *Taq*I restriction sites to be found within the UPK1B gene itself and not any processed pseudogenes.

A mapping experiment, using the total human UPK1B cDNA as a probe, would determine if a processed pseudogene of UPK1B did exist. A peak detected on any chromosomal location besides 3q13.3-21 would be indicative of a pseudogene. The human UPK1B cDNA coding for the putative open reading frame of the UPK1B protein has been cloned and characterised (Chapter 3.3.1). Digestion of the UPK1B/pGEM construct with *Sac*II and *Sac*I released the UPK1B cDNA insert. The 832 bp UPK1B cDNA probe was labelled to a specific activity of 4.40×10^7 CPM/µg and hybridised to a number of slides which were exposed for 27-55 days. The major peak was detected at chromosome 3q13.3-21, consisting of 10% of all grains scored with the two tallest columns of 42 and 27 grains (Figure 4.7). However, sub-peaks of at least 7 grains were also observed on chromosomes 3q26-27, 6p, 9q and 11q, as well as a number of minor sub-peaks of approximately 5 grains on a number of chromosomes.

4.3.5 Cloning of a mouse Upk1b genomic fragment

A mouse Upk1b genomic fragment was cloned by PCR techniques from mouse genomic DNA using the TM3 and ECD primers (Figure 4.8). These primers were originally designed from the highly homologous mink TI1 and bovine UPKIb
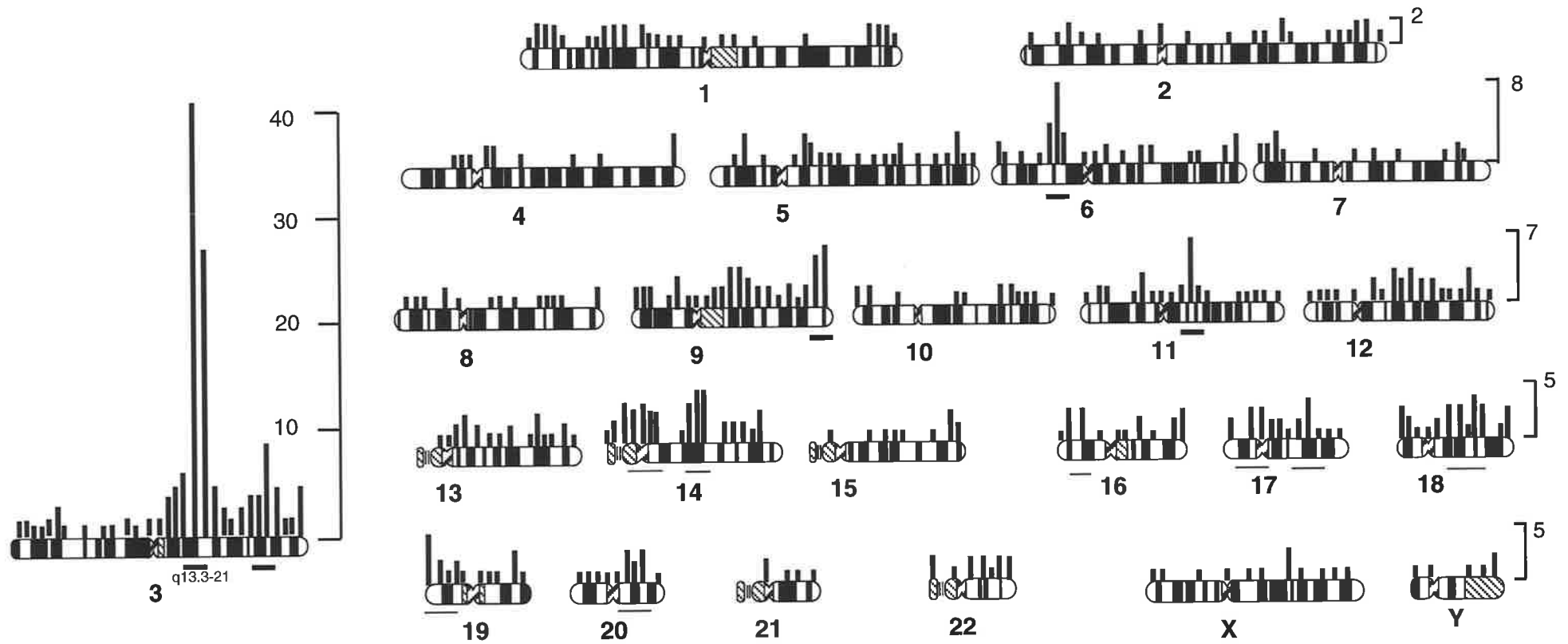
Figure 4.7 Distribution of grains over the whole human karyotype using the human UPK1B cDNA probe. The major peak of grains is localised over 3q13.3-21, however there are a number of sub-peaks over 3q, 6p, 9q and 11q, indicated by a thick underline, as well as a number of minor sub-peaks over chromosomes14, 16, 17, 18, 19 and 20, indicated by a thin underline.

A

TM3

77 bp

42 bp

574 bp

ECD

693 bp mouse genomic Upk1b

B

693 bp ⟶

120 bp ⟶
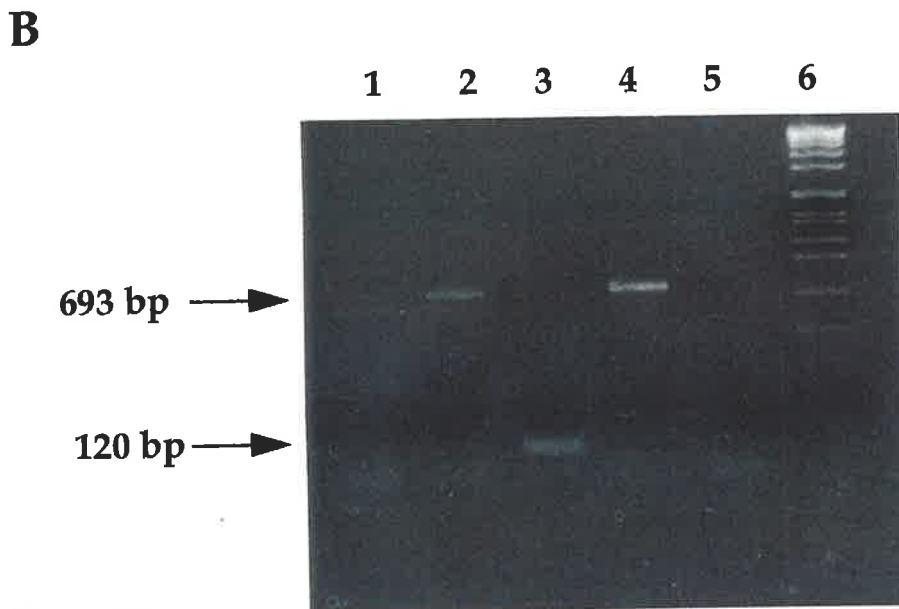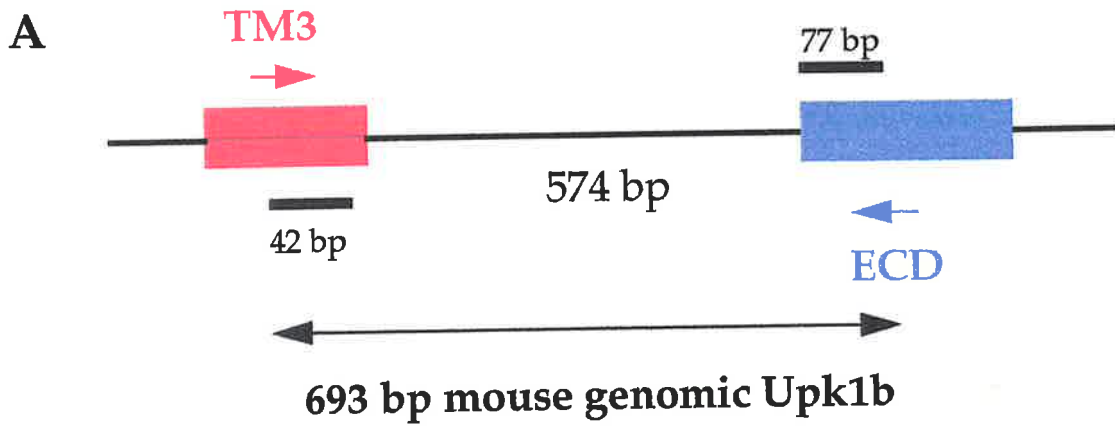
Figure 4.8

A.
Positioning of the TM3 and ECD primers used to amplify a 693 bp mouse Upk1b genomic fragment. The red box represents the putative TM3 exon and the blue box, the ECD exon. The black lines represents intronic sequence.

B.
Amplification of the 693 bp mouse Upk1b PCR product using TM3 and ECD primers.
Lane 1: mouse genomic DNA
Lane 2: human genomic DNA
Lane 3: positive control (TI1/pBluescript)
Lane 4: positive control (778 bp UPK1B genomic/pGEM)
Lane 5: negative control (no DNA)
Lane 6: SPP1/*Eco*RI marker

cDNA sequences and were used to amplify the 784 bp human UPK1B genomic fragment (Chapter 3.3.2). Using an annealing temperature of 60°C, the TM3 and ECD primers directed the amplification of a 693 bp mouse Upk1b genomic product, which was cloned into the pGEM-Easy vector and sequenced (Appendix VI). GenBank Accession Number: AF073956. Sequence analysis revealed that the 693 bp product consisted of two putative exonic sequences of lengths 42 and 77 bp interspersed with a 574 bp intron (Figure 4.8). The total 119 bp of mouse Upk1b exonic sequence has 94% and 95% homology with the corresponding sequences of human UPK1B and bovine UPKIb cDNA sequences (Chapter 3.3.2 and Yu et al., 1994). To isolate a probe for mapping, the mouse Upk1b/pGEM plasmid was digested with the NotI enzyme to release a 727 bp genomic fragment of Upk1b. Hybridisation of the 727 bp mouse Upk1b probe onto Southern blots of mouse genomic DNA digested with PstI or PvuII enzymes generated a single band suggesting the probe would not cross-hybridise to any other gene (Figure 4.9).

4.3.6 Localisation of Upk1b to mouse Chromosome 16

The 727 bp mouse Upk1b probe was tritium-labelled to a specific activity of 8.1 x 10^7 CPM/µg and hybridised *in situ* to chromosomes of C57BL and BALB/C mouse strains. The resultant slides were exposed for 33 or 55 days and initial scoring showed only one major peak of silver grains to chromosome 16, representing 28.2% of all grains (Figure 4.10). The background signal of grains was distributed over all the other mouse chromosomes with the largest cluster being of only 3 grains. The grains over chromosome 16 clustered to two tall columns, of 25 and 14 grains, over bands 16B5 and 16C1 respectively. An independent, higher
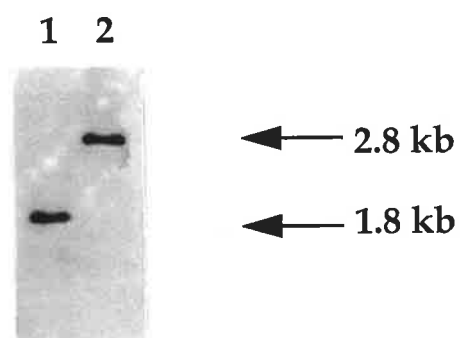
Figure 4.9
Genomic DNA was isolated from C57/Bl mice and digested with either *Pst*I (Lane 1) or *Pvu*II (Lane 2). The DNA was run on a 1% gel overnight and transferred to nylon membrane. The Southern blot was hybridised with the 727 bp mouse Upk1b genomic probe. Only one band was detected for both enzymes, 1.8 kb for the *Pst*I enzyme and 2.8 kb for *Pvu*II, suggesting the mouse Upk1b probe would only detect one peak for *in situ* hybridisation and would not cross-hybridise with other genes.
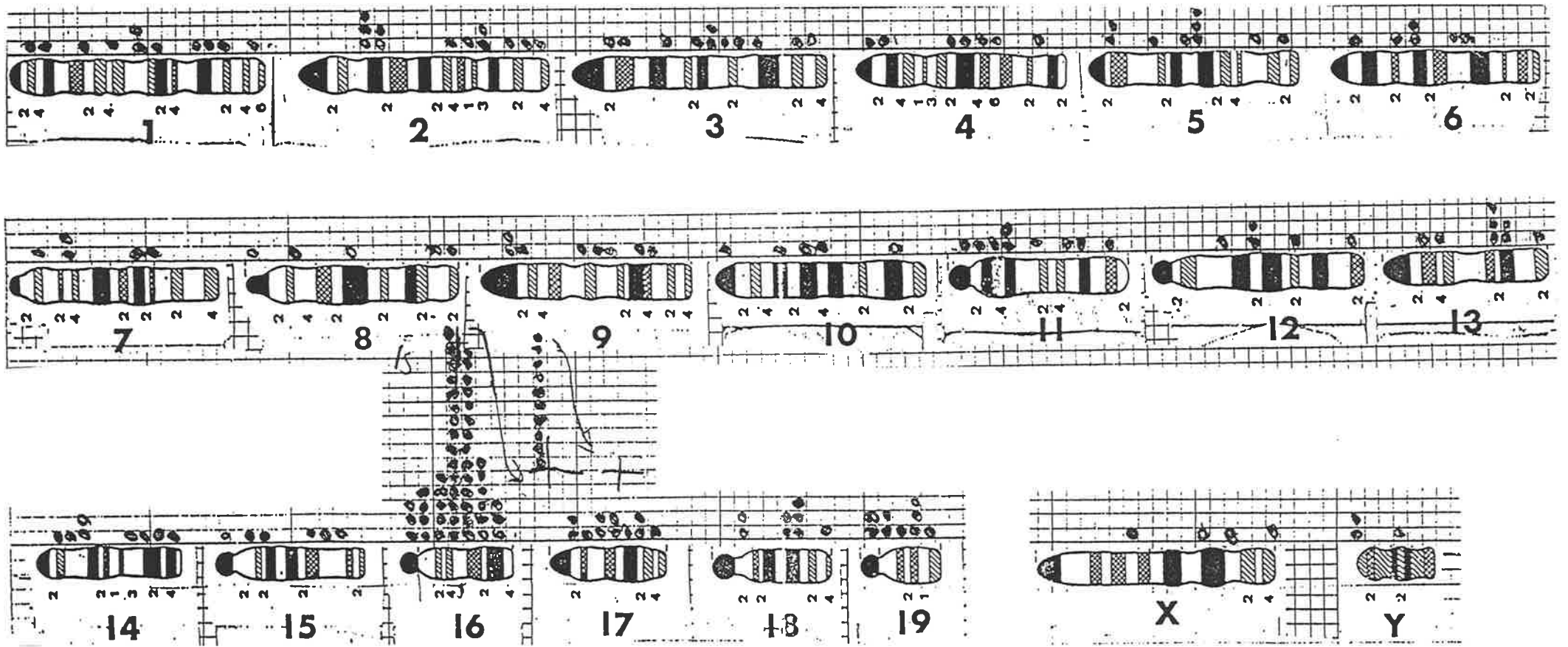
Figure 4.10
Initial scoring of the distribution of grains over the mouse karyotype from *in situ* hybridisation of the mouse Upk1b genomic probe. The major peak of grains is localised over chromosome 16, representing 28.2% of all grains. The two tallest columns of this peak contain 25 and 14 grains, over 16B5 and C1.

resolution score of only chromosome 16 in both C57BL and BALB/C mouse strains confirmed the initial mapping and further defined the localisation to bands 16B5-C2 (Figure 4.11).

4.4 DISCUSSION

The human uroplakin 1B gene has been mapped by *in situ* hybridisation to chromosome 3q13.3-21. Two contiguous human UPK1B genomic probes of lengths 778 bp and 1.4 kb were used to map *UPK1B* and both probes gave the same chromosomal location. This location of human UPK1B on chromosome 3q is in agreement with the synteny observed between loci on bovine chromosome 1 and human chromosome 3. The previous mapping of the bovine uroplakin Ib gene (Ryan et al., 1993) gave an indication of the possible location of the gene in the human genome, but this information was not divulged to the scorer (G.C.W) so as to remove any bias from the scoring of the grains.

Other genes that map to both human chromosome 3 and bovine chromosome 1, include pituitary transcription factor (PIT1), γs-crystallin (CRYGS), uridine monophosphate synthase (UMP) and kininogen (KNG) (Harlizius et al., 1995, van Rens et al., 1989, Qumsiyeh et al., 1989 and Aleyasin et al., 1997). Bovine chromosome 1 (BTA1) has loci found on two different human chromosomes, 3 (HSA3) and 21 (HSA21). The loci found on these two chromosomes are not conserved in tandem because the synteny between BTA1 and HSA21 is interspersed with a cluster of genes found on HSA3 (Figure 4.12). There appears to be an evolutionary breakpoint between the superoxide dismutase 1 (SOD1)

118
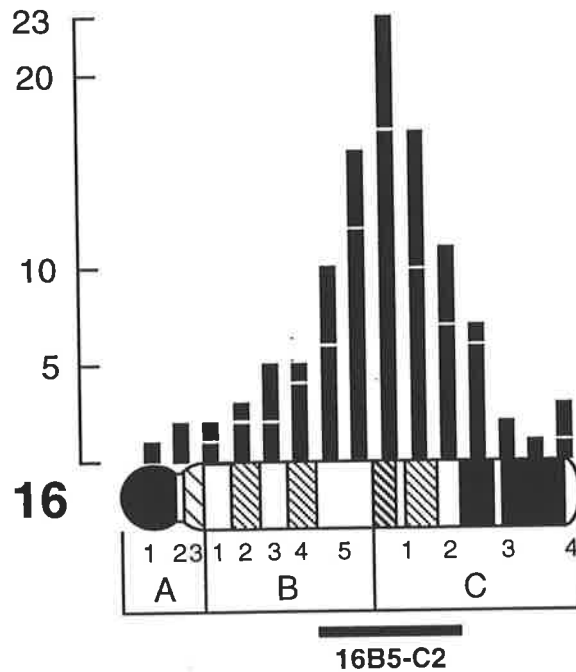
Figure 4.11

High resolution scoring of grains to mouse Chromosome 16 generated by *in situ* hybridisation of the 727 bp mouse Upk1b genomic probe. The five tallest columns are over bands 16B5-C2, the probable location of mouse Upk1b (as indicated by a bar). These five columns represent 71% of the grains. Scores below the gaps are from C57BL, those above the gaps, are from BALB/C mice.

(This figure was published in Webb, Finch and Cowled (1998))
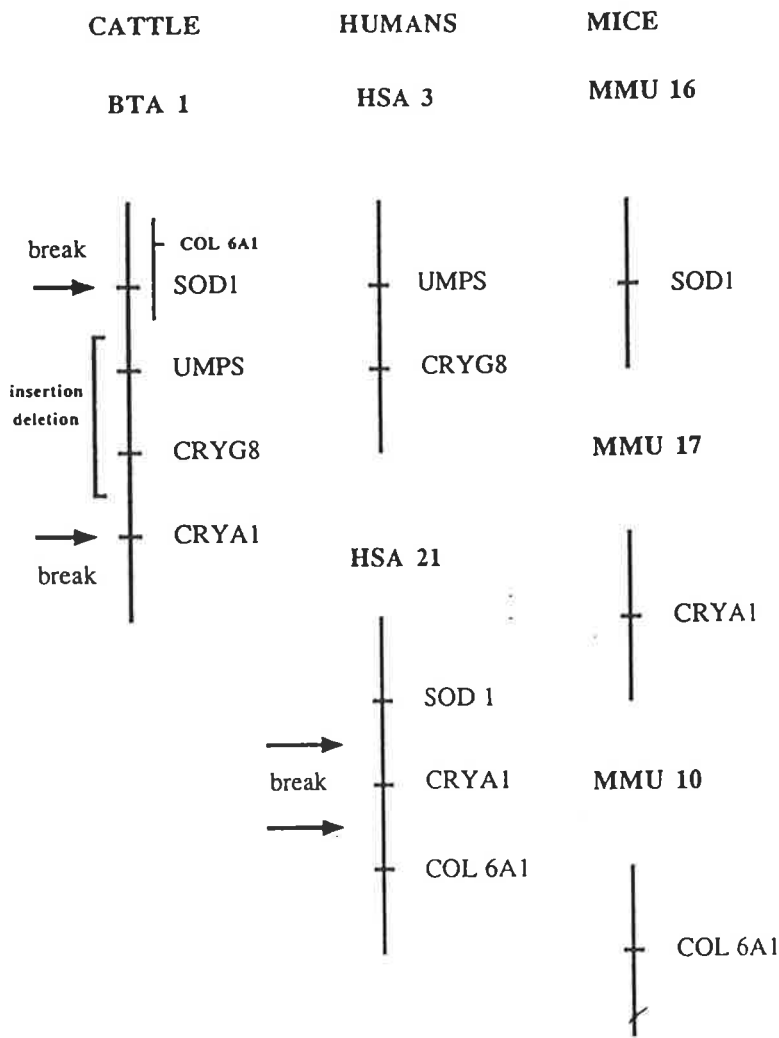
Figure 4.12
A diagram of the conservation of loci between bovine chromosome 1 and human and mouse chromosomes. The synteny between bovine chromosome 1 and human chromsome 21 is interspersed with genes from human chromosome 3. The uroplakin 1b gene is located on bovine chromosme 1, human chromosome 3 and mouse chromosome 16.

(This figure was taken from Barendse et al., 1993).

gene, which maps to human chromosome 21q22.1 and the UMP gene, which maps to human chromosome 3q13 as both of these genes map to bovine chromosome 1 (Barendse et al., 1993). Further characterisation of this breakpoint was defined by comparative mapping of the PIT1 gene which maps to human chromosome 3p11 and was found to map between the SOD1 and UMP genes on bovine chromosome 1 (Harlizius et al., 1995). Therefore, a disruption of synteny occurs between bovine chromosome 1 and human chromosome 3. Unfortunately, there are no details of the exact positioning of UPKIb in relation to other genes on bovine chromosome 1 as the localisation of the bovine uroplakin Ib gene was determined by bovine x rodent somatic cell hybrids (Ryan et al., 1993).

The cloning of a mouse Upk1b genomic fragment revealed high sequence conservation of putative exonic sequence with previously cloned human and bovine uroplakin 1B cDNA sequences (Chapter 3.3.2 and Yu et al., 1994). The mapping of mouse Upk1b to mouse Chromosome 16B5-C2 is consistent with conserved synteny in this chromosome region and human chromosome 3q. By comparison with human chromosome region 3q13.3-21 to homeologous loci on mouse chromosomes, the predicted location of the mouse homeologue of human UPK1B was to mouse Chromosome 16 (Naylor et al., 1996). There are a number of genes which map both to mouse Chromosome 16 and human chromosome 3q including CD80 which has been physically localised to mouse 16B5 and human 3q13.3-q21 (Reeves et al., 1997).

The chromosomal location of a gene may give an indication as to the nature of its particular function. In this case, the uroplakin 1B gene maps to a region of the genome which is not yet particularly well characterised in human disease in relation to either cytogenetic deletions or allelic loss by microsatellite analysis. The main hypothesis of this thesis is that the normal function of the human uroplakin 1B gene is disrupted in cancer. The cancer in which the human UPK1B gene product is most likely to be implicated is bladder cancer, as the mammalian uroplakins are specifically expressed in the bladder (Yu et al., 1994). Three studies have looked at chromosome 3q abnormalities in bladder cancer. The first study, a cytogenetic investigation of untreated transitional cell carcinomas of the bladder revealed structural abnormalities or loss of 3q chromosomes in three out of ten tumours analysed. Two of the three tumours with chromosome 3q abnormalities had lost both copies of the chromosome 3q arm (Atkin et al., 1985). Secondly, an allelotype study to determine the regions of chromosomes frequently deleted in bladder cancer used a *Hinf*I restriction fragment length polymorphism to look for loss of heterozygosity at 3q21-qter using the pEFD64.1 probe, which is located at the D3S42 locus (Knowles et al., 1994). Loss of heterozygosity at this locus was found to occur at a rate of only 5.2%, but was found to be significantly associated with high grade tumours. The third study primarily investigated the abnormalities of the short arm of chromosome 3 in bladder cancer, but did examine two markers on the long arm of chromosome 3; 3q13.2-13.3 (D3S1267) and 3q26.2-27 (GLUT2). The frequency of loss of heterozygosity at D3S1267 was 5.9% and at the GLUT2 locus was 3.4% (Li et al., 1996). These studies are not exhaustive of the total potential loss of heterozygosity of the long arm of chromosome 3, as they reflect

mainly centromeric and telomeric regions of 3q. However, the results suggest that loss of heterozygosity of chromosome 3q is infrequent in bladder cancer.

Chromosomal mapping of the human UPK1B gene using partial genomic probes did not rule out the possibility of the existence of UPK1B pseudogenes. Nonprocessed pseudogenes retain the same genomic structure as the active gene, including introns and are most likely to be found in tandem with the active gene. The nonprocessed pseudogene is believed to originate due to a DNA duplication event which results in the production of two gene copies. Following this DNA duplication, one of the gene pair is inactivated by mutation and can usually be separated by differing migration in a gel (Sharp, 1983). A nonprocessed pseudogene would not be detected as a separate entity by *in situ* hybridisation if it was located in tandem with the active gene. The probe would only produce one peak, as the genes would be mapped to a common chromosomal location. It is unlikely that a nonprocessed pseudogene of *UPK1B* exists because Southern analysis using the two UPK1B genomic probes results in only one labelled band in most hybridisation experiments, suggesting that only copy of the gene exists in its nonprocessed form.

Processed pseudogenes are inactive genomic sequences that resemble the RNA transcript, therefore they lack introns and can be found at different genomic loci as they can integrate at random into the genome (Sharp, 1983). The probes used for the chromosomal localisation of the UPK1B gene were genomic probes consisting of mainly intronic sequence with only very short exonic sequences. Therefore, a processed pseudogene of *UPK1B* could have escaped detection by the chosen

probes. Genomic probes were chosen for *in situ* hybridisation as the complete genomic structure of the UPK1B gene remains unknown. Therefore, the size of the introns may have been too large to obtain a precise mapping using a cDNA probe. The use of genomic probes also avoided the possibility of the probe annealing to other tetraspan genes because the greatest homology between the tetraspan genes is found in the coding regions. Hybridisation of the total UPK1B cDNA probe onto a number of Southern blots of normal genomic DNA resulted in the detection of five different-sized fragments, implying that processed pseudogenes may exist. One of these bands, a 2.5 kb fragment was also detected when the Southern was hybridised with the 778 bp or 1.4 kb human UPK1B genomic probes. However, with the current limited knowledge of UPK1B genomic structure, it was not possible to distinguish between potential processed pseudogenes, other tetraspan genes and the number of restriction sites within UPK1B genomic DNA.

Hybridisation of the human UPK1B cDNA onto human chromosomes revealed a major peak on chromosome 3q13.3-21 as expected, once again confirming the original localisation of the human UPK1B gene. However, four sub-peaks of seven or more grains were also present on chromosomes, 3q26-27, 6p, 9q and 11q, as well as a number of minor sub-peaks. A search of the literature on tetraspan proteins revealed that none of these sub-peak locations were the site of any known tetraspan genes. The major similarities between the tetraspan family members is not the amino acid conservation but in the structure of the tetraspan proteins, consisting of four hydrophobic domains. Therefore it is unlikely that cross-hybridisation between tetraspan genes would occur. The tetraspan gene most

likely to have a high degree of homology to UPK1B is the putative human uroplakin Ia gene (39% amino acid homology between bovine UPKIb and UPKIa) which has not been cloned and therefore no human chromosomal location identified (Yu et al., 1994). The uroplakin Ia gene has been mapped to bovine chromosome 18 and mouse chromosome 7 and would be predicted to be located on human chromosome 19 (Ryan et al., 1993). Only a minor sub-peak is located on chromosome 19, therefore the peak is most likely to be due to background noise rather than cross-hybridisation of UPK1B cDNA to human uroplakin Ia. A recently isolated tetraspan gene, TM4SF5, (Muller-Pillasch et al., 1998) is located on chromosome 17p13.3. Again, the minor sub-peaks located in this vicinity from the human uroplakin 1B cDNA *in situ* hybridisation experiment are most likely to be due to background grains and not a specific localisation.

The likelihood of the sub-peaks representing UPK1B-like genes is minimal if it is assumed that related genes would be expressed, unlike pseudogenes. Hybridisation of UPK1B cDNA onto Northern blots of RNA isolated from normal bladder tissue detects two transcripts as described in Chapter 5. One of the sub-peaks, therefore, could take account for one of the UPK1B mRNA transcripts, except that it would be expected to have an approximate equal number of grains as those found on the major peak on chromosome 3q13.3-21, as both transcripts are detected with equal intensity. The most likely explanation for the two transcripts of UPK1B is that there is alternate splicing of the RNA (Chapter 5). The transcripts detected by Northern analysis however, only take into account genes expressed in the bladder, leaving the possibility of expression of UPK1B-like genes in other tissues. The finding of four major sub-peaks does not contradict the detection of

five bands upon hybridisation of the UPK1B cDNA onto the Southern blot containing *Taq*I-digested DNA. It appears most likely therefore, that the sub-peaks may represent processed pseudogenes of UPK1B. An argument against the presence of UPK1B processed pseudogenes is that such pseudogenes usually show more prominent peaks with tritium localisation (Webb et al., 1990b, Weil et al., 1997) than those found with UPK1B cDNA.

Many of the human tetraspan genes have been assigned to their particular chromosomes. It is of interest that one of the tetraspan genes, L6, also maps to the long arm of chromosome 3. The human L6 gene has been mapped to chromosome 3q21-25, close to a UPK1B cDNA subpeak at 3q25-26, by Southern analysis of a panel of human x rodent somatic cell hybrids (Virtaneva et al., 1994). It has been previously suggested that the localisation of the mouse CD81 and CD37 tetraspan family genes to mouse Chromosome 7 provides evidence for the theory that the tetraspan genes arose divergently from the same ancestral gene as a result of an ancient chromosomal duplication (Wright et al., 1993). Other evidence which suggests that the tetraspan genes arose divergently from an ancestral gene is the striking similarity of genomic as well as protein structure amongst the tetraspan family members (Wright et al., 1993, Wright et al., 1994). Mapping of some of the human tetraspan genes has also revealed similar chromosomal locations, for example, the CD63 gene maps to chromosome 12q12-q14 (Hotta et al., 1988) while CD9 also maps to chromosome 12, but to the short arm p13 (Benoit et al., 1991). Both the TAPA-1 and SFA-1 genes map to chromosome 11p15.5 (Virtaneva et al., 1994, Hasegawa et al., 1997a). The KAI1 gene is also located on chromosome 11, but has been mapped to 11p11.2 (Kawana

et al., 1997). The mapping of the human uroplakin 1B gene to the same chromosome as the L6 gene and in particular to the same chromosome 3 arm, 3q, also adds to the argument that the tetraspan genes arose from ancestral genes that diverged through chromosomal duplication. Other tetraspan genes which have been mapped include the human CD53 gene, on chromosome 1p21-p13.3 (Gonzalez et al., 1993), human CD37 on chromosome 19p13-q13.4 (Virtaneva et al., 1993) and A15 which is located on chromosome Xq11 (Virtaneva et al., 1994).

Summary

This chapter has described the mapping of the human uroplakin 1B gene to chromosome 3q13.3-21, using two independent, but contiguous human uroplakin 1B genomic probes. The use of a human uroplakin 1B cDNA probe confirmed the location and also revealed four major sub-peaks of grains and a number of minor sub-peaks. The detection of these sub-peaks is unlikely to be due to cross-hybridisation with other tetraspan sequences or UPK1B-like genes but might be due to UPK1B processed pseudogenes, however the peaks are not as strong as found for other processed pseudogenes. The chromosomal location of the human uroplakin 1B gene is not a region known to be frequently deleted in bladder cancer, therefore not providing evidence for uroplakin 1B as a tumour suppressor. Human chromosome 3q13.3-21 is a region which has homeology with bovine chromosome 1. The cloning of a mouse Upk1b genomic fragment allowed the mapping of mouse Upk1b to Chromosome 16B5-C2, a region which maintains synteny with both human chromosome 3q and bovine chromosome 1.

CHAPTER 5

ANALYSIS OF EXPRESSION OF HUMAN UROPLAKIN 1B MRNA

5.1 INTRODUCTION

Expression of bovine uroplakin Ib mRNA has been detected in bovine bladder and cultured urothelial cells by Northern analysis. The bovine liver, kidney (excluding renal pelvis), lung, brain and epithelium from the esophagus, snout and intestines do not express UPKIb, suggesting bladder-specific patterns of expression of the bovine UPKIb gene (Yu et al., 1994). However, the mink TI1 cDNA was isolated from the mink lung epithelial cell line, CCL64, and abundant mink TI1 mRNA expression was detected in this cell line by Northern analysis (Kallin et al., 1991). In this chapter, the human UPK1B gene will be investigated for its expression in normal urothelial tissue. Due to the difficulty in obtaining fresh normal human tissue to isolate RNA, only the human bladder was examined. However, a number of murine tissues, including bladder and lung, were analysed to assess the conservation of bladder-specific UPK1B expression across mammalian species.

Studies of many of the tetraspan family genes, for example, CD63, KAI1 and CD9, have revealed altered patterns of expression in a variety of malignancies in comparison to normal tissue. The CD63 gene, not expressed in normal melanocytes, is highly expressed in early melanomas, but expression is lost in advanced melanomas (Hotta et al., 1988). Down-regulation of KAI1 mRNA expression has been detected in prostate and pancreatic cancer (Dong et al., 1996; Guo et al., 1996). Reduced expression of CD9 is an indicator of poor prognosis in breast and non-small cell lung cancer (Higashiyama et al., 1995; Miyake et al., 1996). One of the aims of this study was to compare the levels of mRNA expression of human UPK1B between normal urothelial tissue, bladder tumours,

and bladder carcinoma cell lines using Northern analysis. UPK1B mRNA expression was also investigated by the more sensitive RT-PCR method.

The possibility of a relationship between expression of the human UPK1B and transforming growth factor beta (TGFβ) genes in bladder cancer was explored because the original TI1 studies suggested that the expression of mink TI1 was regulated by this cytokine (Kallin et al., 1991). Previous studies have revealed conflicting results for the expression pattern of TGFβ1 in bladder cancer. Using Northern analysis, Coombs et al., (1993) showed a decrease in the levels of expression of TGFβ1 which correlated with the progression of bladder cancer. However, Miyamoto et al., (1995) used a quantitative RT-PCR method to show that expression of TGFβ1 was up-regulated during early stage bladder cancer, compared to normal urothelium and was decreased in more advanced and invasive tumours. In the current study, bladder tumours and bladder cancer cell lines were analysed for the levels of expression of both UPK1B and TGFβ mRNA, to look for a correlation between their expression patterns in cancer.

To address the above aims, a partial human UPK1B cDNA probe to be used for Northern analysis, was cloned by PCR-based strategies. Expression of the human UPK1B gene had not previously been analysed, nor had human UPK1B cDNA probes been cloned. A partial human UPK1B cDNA probe was isolated using PCR primers designed from the highly homologous mink TI1 and bovine UPKIb cDNA sequences. The total UPK1B cDNA open reading frame was later cloned and sequenced as described in Chapter 3, so the identity of the partial UPK1B

cDNA probe isolated for Northern analysis could be verified by sequence comparison.

5.2 METHODS

5.2.1 Isolation of a partial human UPK1B cDNA probe

RNA was isolated from normal urothelium (2.2.2), and cDNA synthesised by reverse-transcription with an oligo (dT) primer (2.2.8). The TM1 and ECD2 primers (Table 2.1) were used to amplify a 415 bp human uroplakin 1B cDNA product at an annealing temperature of $60^{\circ}C$ (2.2.7). The PCR product was cloned into the pGEM-T vector (2.2.10), transformed into competent cells (2.2.14) and a positive colony propagated (2.2.15). The TM1-ECD2/pGEM plasmid was isolated using the Qiagen midi plasmid kit (2.2.16), sequenced using the pGEM primers (Table 2.1) and aligned to mink TI1 and bovine UPKIb cDNA sequences using GeneJockey (2.2.17). The GeneJockey program was also later used to compare the partial 415 bp cDNA sequence with the human UPK1B cDNA ORF.

5.2.2 Isolation of a human TGFβ1 cDNA probe

Total RNA was extracted from normal urothelium (2.2.2) and reverse-transcribed using an oligo (dT) primer (2.2.8). The TGFβ-5′ and TGFβ-3′ primers (Table 2.1) were used at an annealing temperature of $55^{\circ}C$ to amplify a 663 bp human TGFβ PCR product (2.2.7).

5.2.3 Bladder cancer cell lines and tissues

The TCC-SUP, ScaBER, J82 and T24 bladder carcinoma cell lines were obtained from the American Type Culture Collection (Rockville, MD). The 5637 bladder carcinoma cell line was kindly provided by Dr. D. Leavesley. All cell lines were maintained in Dulbecco's Modified Eagle's medium, pH 7.4, supplemented with 10% fetal calf serum at 37°C in an atmosphere of 5% CO_2 (2.2.18). Bladder cancer tissues were collected with informed consent from either transurethral resection or radical cystectomy, snap frozen in liquid nitrogen and stored at -80°C. Any underlying muscle was removed before the sample was analysed. Samples of normal human bladder, ureter and renal pelvis tissues were also collected and snap frozen. The patients who supplied the normal samples of urothelium did not suffer from any malignant urological diseases.

5.2.4 Detection of expression of UPK1B mRNA by Northern analysis

Total RNA was isolated from normal ureter, renal pelvis, bladder cancer tissue and bladder cancer cell lines as previously described (2.2.2). Ten μg of RNA was electrophoresed on a 1% agarose/formaldehyde gel overnight and then transferred to nylon membrane (2.2.5). The Northern blot was placed in a hybridisation bottle at 42°C with the pre-hybridisation solution for 3 hours (2.2.6). The TM1-ECD2/pGEM probe was labelled with α^{32}P-dATP using the Gigaprime kit (2.2.6) and the denatured probe added to the Northern blot and allowed to hybridise overnight. The Northern blot membrane was washed to a medium stringency of 1 x SSC/0.1% SDS at 60°C, then exposed to autoradiography film for 1-5 days and developed (2.2.6).

5.3 RESULTS

5.3.1 Isolation of a human UPK1B cDNA probe

A partial human UPK1B cDNA clone was isolated by RT-PCR, using RNA isolated from normal human ureter as a template. The ureter, like the bladder and renal pelvis, contains urothelial cells lining the luminal surface. PCR primers were designed from mink TI1 and bovine UPKIb cDNA sequences which code for the first transmembrane domain and the putative second exon of the large extracellular domain. These primers, TM1 and ECD2, amplified a 415 bp human UPK1B product which was cloned into the pGEM-T vector (Figure 5.1) and sequenced (Appendix VII). The cDNA sequence of the 415 bp TM1-ECD2 product was compared to the mink TI1 and bovine UPKIb cDNA sequences. The homology between human and the mink TI1 cDNA over this region was 94% and was 92% between human and bovine UPKIb cDNA. The TM1-ECD2 sequence was also later confirmed as human UPK1B by comparison with the 783 bp human UPK1B cDNA coding for the ORF cloned in Chapter 3.

5.3.2 Human uroplakin 1B mRNA is expressed in normal urothelium

Northern blots were prepared from RNA isolated from either normal ureter or renal pelvis. Expression of human UPK1B mRNA was detected by hybridisation of the 415 bp human TM1-ECD2 cDNA probe. Abundant levels of expression of the human UPK1B gene were detected in the form of two mRNA transcripts of approximate sizes 1.8 kb and 1 kb (Figure 5.2). In all RNA samples, the level of intensity of the two RNA species was almost identical. The 1.8 kb mRNA transcript is the predicted size of the human uroplakin 1B mRNA, by analogy

A

TM1

11   101   793

| ATG | human UPK1B cDNA ORF | TAA |

516

ECD2

415 bp human UPK1B cDNA

B

1   2   3   4
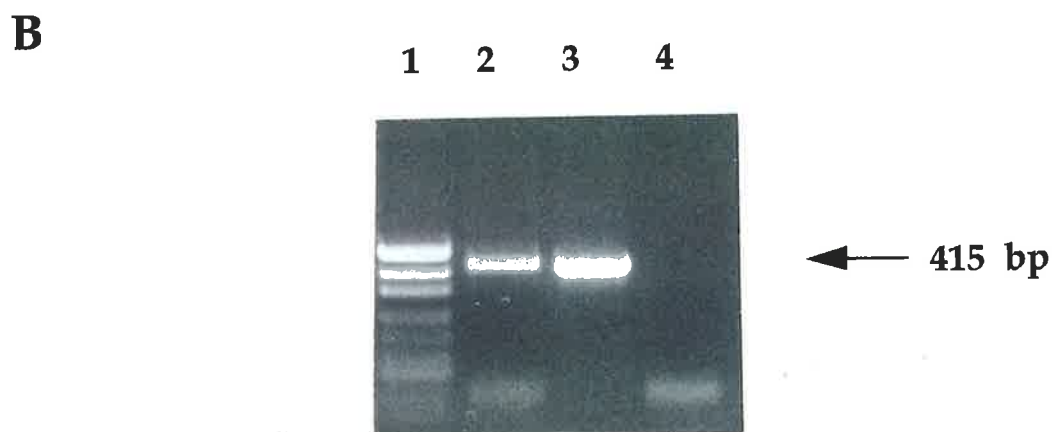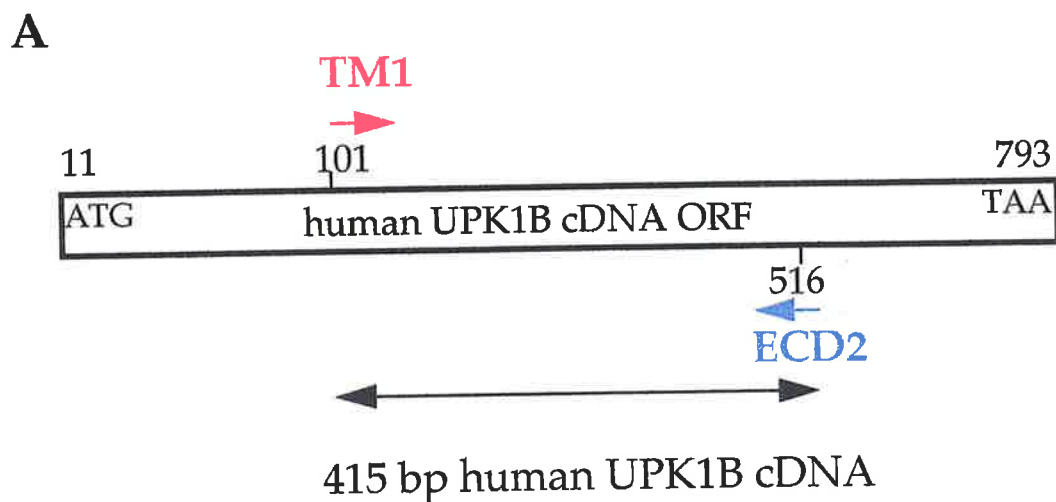
←—— 415 bp

Figure 5.1

A. Diagrammatic representation of the positioning of the TM1 and ECD2 primers used to amplify the 415 bp human UPK1B cDNA probe.

B. RNA was isolated from normal urothelium and reverse-transcribed using oligo(dT) primers. The TM1 and ECD2 primers directed the PCR amplification of the 415 bp human UPK1B cDNA product.

Lane 1: pUC19/*Hpa*II marker
Lane 2: normal human urothelium cDNA
Lane 3: positive control -TI1/pBluescript plasmid
Lane 4: negative control (no DNA)
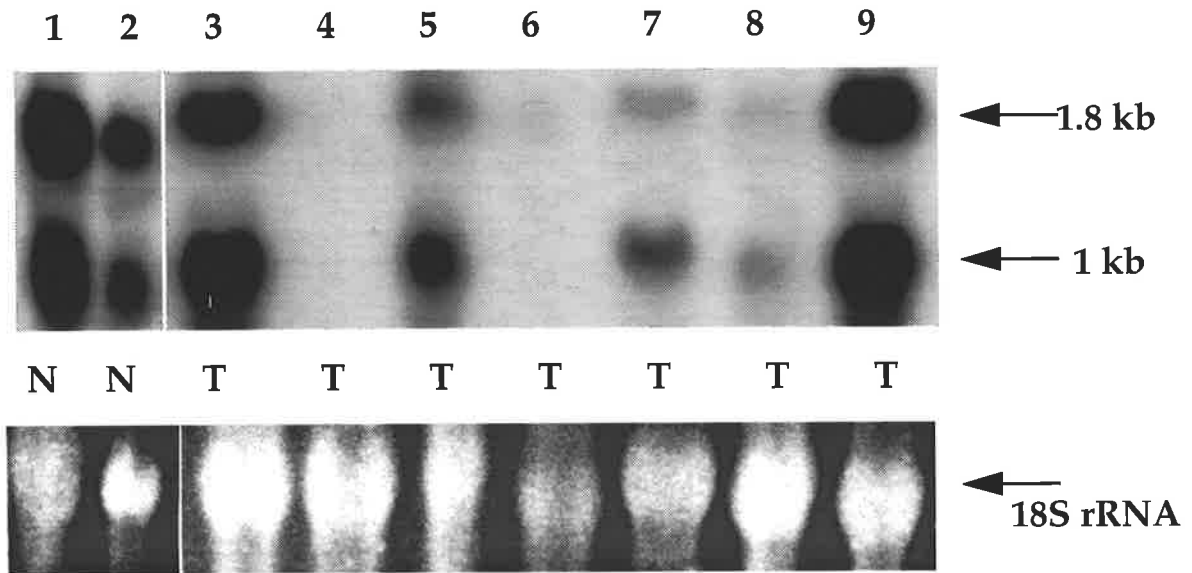
Figure 5.2
RNA was isolated from normal (N) urothelium and bladder tumour (T) tissue.
Northern analysis of these RNAs using the 415 bp human TM1-ECD2 (UPK1B)
probe detected two transcripts of sizes 1.8 kb and 1 kb, in the upper panel.
Expression of UPK1B mRNA was detected in both normal RNA samples (lanes
1 and 2), however, only in lanes 3, 5 and 9 did tumours show expression levels
comparable to normal urothelial expression. In lane 7, expression is reduced
and in lanes 4, 6 and 8 there is no detectable expression of UPK1B in tumours.
The level of expression was assessed by visual determination, using the
ethidium bromide-stained 18S rRNA in the lower panel as a control for loading.

with the 1.8 kb mink and 1.9 kb bovine mRNA transcripts. The smaller transcript, of approximately 1 kb was not detected in either mink TI1 or bovine uroplakin Ib Northern blots (Kallin et al., 1991 and Yu et al., 1994).

5.3.3 Expression of UPK1B mRNA is frequently lost in bladder cancer

Northern analysis of RNA isolated from sixteen bladder cancer tissues of varying stage and grade (Table 5.1) revealed that seven of the bladder tumours had no detectable expression of UPK1B mRNA, four had reduced expression and five had levels of UPK1B expression which were comparable to the levels of expression found in normal urothelial tissue (Figure 5.2). Therefore, in 68% of bladder tumours, mRNA expression of human UPK1B was either lost or markedly reduced. Reduction of expression of UPK1B was determined by visual examination of the Northern blots, comparing the intensity of the hybridisation signal between tumour RNA and normal urothelium RNA. Loading was controlled for by ethidium-bromide staining of the RNA. Similarly, no UPK1B expression was detected by Northern analysis from RNA extracted from five human bladder cancer cell lines using the human TM1-ECD2 cDNA probe (Figure 5.3). The cell lines analysed were J82, T24, TCC-SUP, 5637 and ScaBER.

Table 5.1 Relationship between level of expression of UPK1B mRNA, bladder tumour grade and depth of tumour invasion

Patient	UPK1B expression	Bladder tumour grade	Depth of invasion
SC	reduced	II	non-invasive
LH	normal	II-III	non-invasive
EG	reduced	II	non-invasive
BH	lost	II	non-invasive
DAR	normal	I-II	non-invasive
LS	normal	II	non-invasive
DC	lost	II-III	muscle invasion
JM	lost	high grade	muscle invasion
HD	lost	III	muscle invasion
BC	normal	III	muscle invasion
T W	lost	III	muscle invasion
DR	normal	III	muscle invasion
KR	lost	na	vascular invasion
CD	reduced	II-III	lamina propria
LM	lost	III	na
MK	reduced	na	na

(na = data not available)
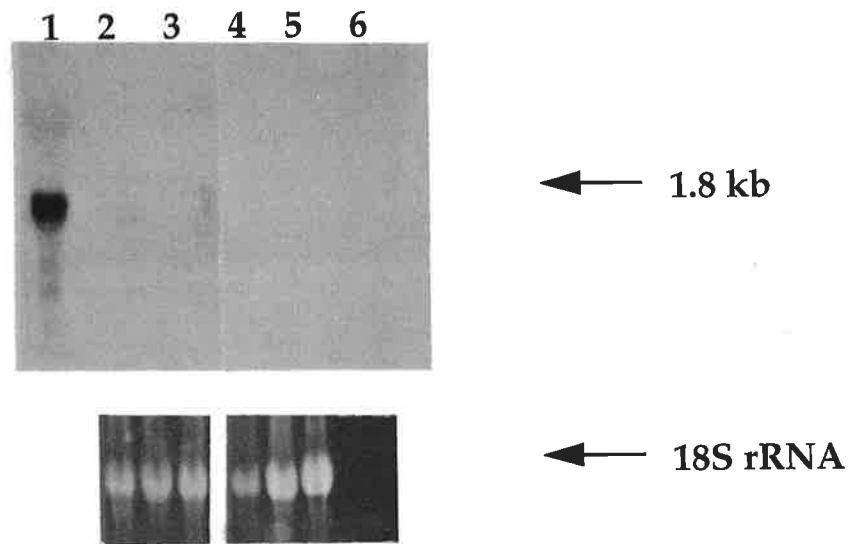
Figure 5.3
The upper panel shows the Northern analysis of RNA isolated from bladder cancer cell lines hybridised with the 415 bp human TM1-ECD2 probe. None of the five bladder cancer cell lines have detectable levels of expression of UPK1B. In lane 1 is RNA isolated from the mink lung epithelial cell line, CCL64 which does express TI1/UPK1B. In the lower panel is the ethidium bromide-staining of 18S rRNA to control for loading.

Lane 1: mink (CCL64)
Lane 2: ScaBER
Lane 3: J82
Lane 4: TCC-SUP
Lane 5: T24
Lane 6: 5637

5.3.4 Analysis of expression of human UPK1B mRNA by RT-PCR

None of the five bladder cancer cell lines had detectable expression of UPK1B mRNA by Northern analysis. RNA isolated from the bladder cancer cell lines was also analysed by the more sensitive RT-PCR method. The bladder cancer cell lines, J82, TCC-SUP, T24, ScaBER and 5637 all produced the expected 202 bp RT-PCR fragment with the TM3 and ECD2 primers. However, none of the bladder cancer cell lines amplified the expected 482 bp RT-PCR product using the TM3 and 3'ORF primers (Figure 5.4).

Many of the bladder tumours had no detectable expression of UPK1B mRNA by Northern analysis. The RNAs isolated from the tumours were also analysed for expression of UPK1B by the more sensitive RT-PCR method. RNA from eight bladder tumours was analysed by RT-PCR for expression of UPK1B mRNA. All eight samples yielded the predicted RT-PCR products of sizes 198 bp and 106 bp respectively when using the primer pairs of TM2-ECD and R-ECD-ECD2 (Figure 5.5a). However, only five of the bladder tumours produced RT-PCR products when either the TM2-3'ORF or TM3-3'ORF primers pairs were used (Figure 5.5b). Using TM2-3'ORF or TM3-3'ORF primers, the PCR conditions were extended to include 40 cycles rather than the normal 35 cycles, but again three of the eight bladder cancer RNAs failed to yield RT-PCR products. The RT-PCR results from RNA isolated from the bladder tumours and bladder cancer cell lines were compared with the expression levels of UPK1B mRNA detected for the corresponding RNA samples by Northern analysis. These comparisons revealed that eight out of nine bladder tumours or bladder cancer cell lines with no

Figure 5.4

A.

Diagrammatic representation of the positioning of the two primer pairs used to amplify UPK1B RT-PCR products from RNA isolated from human bladder cancer cell lines. The predicted size product from the TM3 and ECD2 primers was 202 bp and from the TM3 and 3'ORF primers, 482 bp. The numbering represent the position of the primer in respect to the human UPK1B cDNA ORF.

B.

RT-PCR analysis of RNA isolated from human bladder cancer cell lines and reverse-transcribed using an oligo(dT) primer. The TM3 and ECD2 primers were used at an annealing temperature of 60°C to amplify a 202 bp product. The PCR products were run on a 2% agarose gel. The 202 bp TM3-ECD2 RT-PCR product was amplified from RNA from all five bladder cancer cell lines.

Lane 1: pUC19/*Hpa*II marker
Lane 2: J82
Lane 3: TCC-SUP
Lane 4: T24
Lane 5: ScaBER
Lane 6: 5637
Lane 7: Positive control (mouse bladder)
Lane 8: Positive control (TM3-3'ORF/pGEM plasmid)
Lane 9: Negative control (no DNA)

C.

RT-PCR analysis of RNA isolated from human bladder cancer cell lines and reverse-transcribed using an oligo(dT) primer. The TM3 and 3'ORF primers were used at an annealing temperature of 60°C to amplify a 482 bp UPK1B cDNA product. The PCR products were run on a 1.5% agarose gel. No RT-PCR product was amplifed from any of the RNAs isolated from the five bladder cancer cell lines using the TM3 and 3'ORF primers.

Lane 1: SPP1/*Eco*RI marker
Lane 2: J82
Lane 3: TCC-SUP
Lane 4: T24
Lane 5: ScaBER
Lane 6: 5637
Lane 7: Positive control (mouse bladder)
Lane 8: Positive control (TM3-3'ORF/pGEM plasmid)
Lane 9: Negative control (no DNA)

A

TM3 ECD2

202 bp (B)

11 314 516 793

ATG human UPK1B cDNA ORF TAA

796

482 bp (c)

TM3 3'ORF
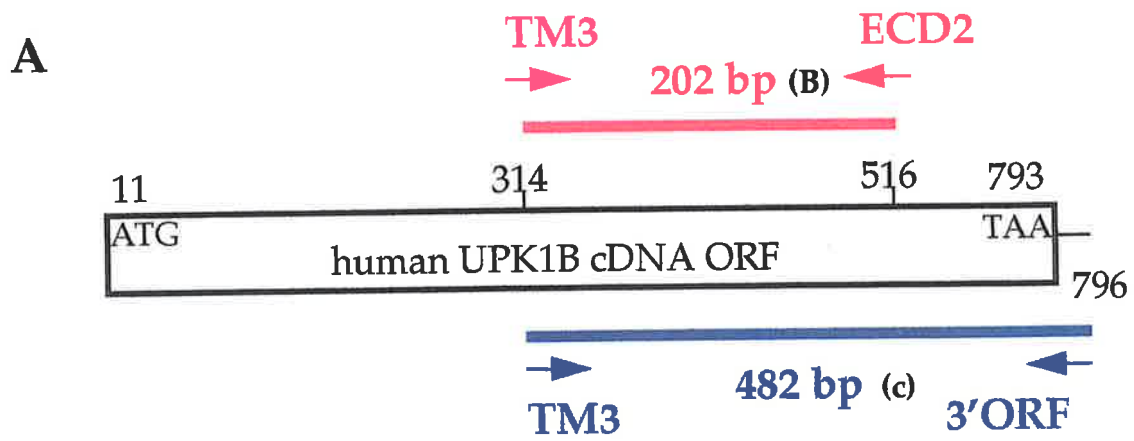
B

1 2 3 4 5 6 7 8 9

← 202 bp

C

1 2 3 4 5 6 7 8 9

← 482 bp

Figure 5.5a

A. Diagram of the positioning of the 2 sets of primer pairs used to amplify RT-PCR products from bladder tumour RNA. The expected size fragment from the TM2-ECD primers is 198 bp and 106 bp from the R-ECD and ECD2 primers. The R-ECD primer is the ECD primer in the sense orientation. Positive controls for both PCR amplifications was the TM1-ECD2/pGEM plasmid.

B. RNA was isolated from bladder tumours and reverse-transcribed using oligo(dT). PCR products were amplified using the TM2 and ECD primers. The PCR products were run on a 1.5% gel.

Lane 1: Positive control
Lane 2: pUC19/*Hpa*II marker
Lane 3: Normal urothelium
Lane 4: Tumour BC
Lane 5: Tumour CD
Lane 6: Tumour HD

Lane 7: Tumour EG
Lane 8: Tumour LM
Lane 9: Tumour LS
Lane 10: Tumour DC
Lane 11: Tumour JM
Lane 12: Negative control (no DNA)

C. RNA was isolated from bladder tumours and reverse-transcribed using oligo(dT). PCR products were amplified using R-ECD and ECD2 primers and were run on a 2% gel.

Lane 1: pUC19/*Hpa*II marker
Lane 2: Normal urothelium
Lane 3: Tumour BC
Lane 4: Tumour CD
Lane 5: Tumour HD
Lane 6: Tumour EG

Lane 7: Tumour LM
Lane 8: Tumour LS
Lane 9: Tumour DC
Lane 10: Tumour JM
Lane 11: Positive control
Lane 12: Negative control (no DNA)

A

TM2　　　　ECD
198 bp(B)

R-ECD　　　ECD2
106 bp(C)

11　　　236　　　410/434　516　793

ATG　　human UPK1B cDNA ORF　　TAA

B

1 2 3 4 5 6 7 8 9 10 11 12

← 198 bp

C

1 2 3 4 5 6 7 8 9 10 11 12

← 106 bp
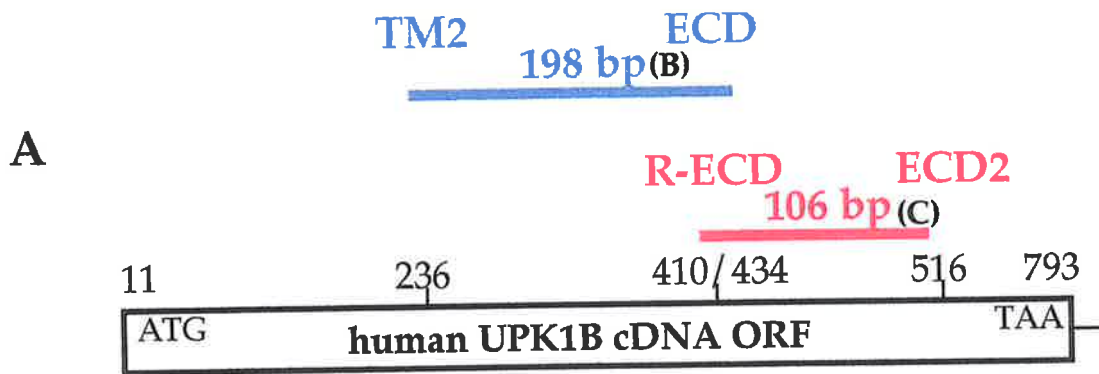
Figure 5.5 b

D. Diagram of the positioning of the 2 sets of primers used to amplify RT-PCR products from bladder tumour RNA. The predicted size of the TM3-3'ORF primer pairing was 482 bp and 560 bp is expected from the TM2-3'ORF primers. Positive controls for both PCRs were the TM3-3'ORF/pGEM plasmids.

E. RNA was isolated from bladder tumours and reverse-transcribed using an oligo(dT) primer. PCR products were amplified using the TM3 and 3'ORF primers. The PCR products were run on a 1.5% gel.

Lane 1: pUC19/*Hpa*II marker
Lane 2: Normal urothelium
Lane 3: Tumour BC
Lane 4: Tumour CD
Lane 5: Tumour HD
Lane 6: Tumour EG

Lane 7: Tumour LM
Lane 8: Tumour LS
Lane 9: Tumour DC
Lane 10: Tumour JM
Lane 11: Positive control
Lane 12: negative control (no DNA)

F. RNA was isolated from bladder tumours and reverse-transcribed using an oligo(dT) primer. PCR products were amplified using the TM2 and 3'ORF primers. The PCR products were run on a 1.5% gel.

Lane 1: pUC19/*Hpa*II marker
Lane 2: Normal urothelium
Lane 3: Tumour BC
Lane 4: Tumour CD
Lane 5: Tumour HD
Lane 6: Tumour EG

Lane 7: Tumour LM
Lane 8: Tumour LS
Lane 9: Tumour DC
Lane 10: Tumour JM
Lane 11: Positive control
Lane 12: negative control (no DNA)
Lane 13: SPP1/*Eco*RI marker

D

E

482 bp

F

560 bp
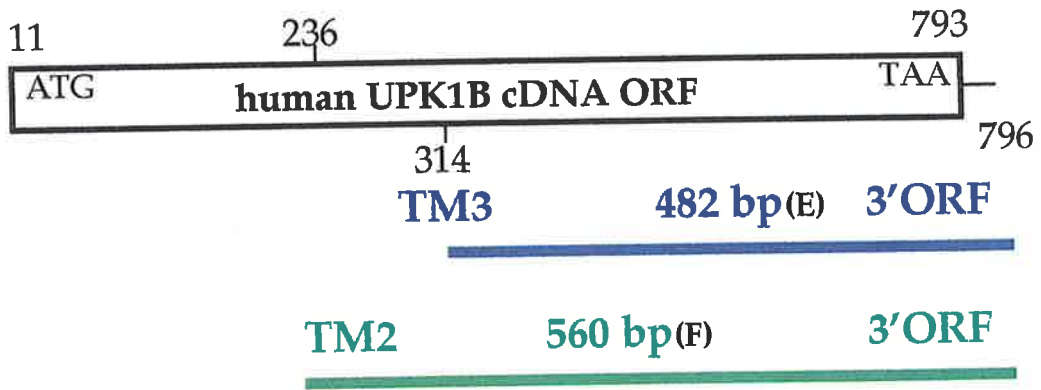
UPK1B mRNA expression by Northern analysis, also produced no RT-PCR product when the 3'ORF primer was used as the antisense PCR primer (Table 5.2).

Table 5.2 Comparison of the detection of expression of UPK1B mRNA by RT-PCR and Northern analysis

Cell line or bladder tumour patient	RT-PCR ECD or ECD2	RT-PCR 3'ORF	Northern analysis
J82	yes	no	lost
TCC-SUP	yes	no	lost
T24	yes	no	lost
ScaBER	yes	no	lost
5637	yes	no	lost
HD	yes	no	lost
LM	yes	no	lost
DC	yes	no	lost
JM	yes	yes	lost
CD	yes	yes	reduced
LS	yes	yes	normal
BC	yes	yes	normal
EG	yes	yes	reduced

5.3.5 UPK1B and TGFβ1 in bladder cancer

It is known that expression of the TI1/UPK1B gene is regulated by transforming growth factor β1 in mink lung epithelial cells (Kallin et al., 1991). A TGFβ1 cDNA clone was isolated by designing TGFβ-specific primers to amplify a PCR product from cDNA synthesised from normal urothelial RNA (Figure 5.6a). The TGFβ1 primers amplified a region of 663 bp between the nucleotides 579 and 1201 to produce a TGFβ1 probe which was used to hybridise to Northern blots consisting of RNA isolated from normal urothelium, bladder tumours or bladder cancer cell lines. Northern analysis detected expression of TGFβ1 mRNA in normal urothelium. Of twelve tumours analysed, only four expressed TGFβ1 (Figure 5.6b). There was no correlation between expression of TGFβ1 mRNA and expression of human UPK1B mRNA. For example, there were tumours which expressed TGFβ1 but not UPK1B and also tumours with no TGFβ1 expression but abundant levels of UPK1B (Table 5.3). Analysis of the bladder cancer cell lines for expression of TGFβ revealed a high level of expression in the T24 cells (as has been previously reported (Derynck et al., 1985)), some expression in the TCC-SUP and 5637 cells but no expression in the J82 cell line. From previous Northern blots (Chapter 5-Figure 5.3) it is known that none of these bladder cancer cell lines express any detectable levels of UPK1B mRNA. These results suggest that there is no correlation between expression of TGFβ and UPK1B mRNA in bladder cancer.

Figure 5.6a

A.
Diagram of the position of the TGFβ (5′ and 3′) primers used to amplify a 663 bp TGFβ cDNA PCR product between nucleotides 579 and 1201 of the 2745 bp TGFβ cDNA.

B.
RNA was isolated from normal urothelium and reverse-transcribed using an oligo(dT) primer. The TGFβ-5′ and 3′ primers amplified a 663 bp product run on a 1.5% agarose gel.

Lane 1: SPP1/EcoRI marker
Lane 2: Normal urothelium
Lane 3: Negative control (no DNA)
Lane 4: pUC19/HpaII marker

A

TGFβ-5′   TGFβ-3′

663 bp

1   2745

TGFβ1 cDNA

579   1201

B

1 2 3 4

← 663 bp
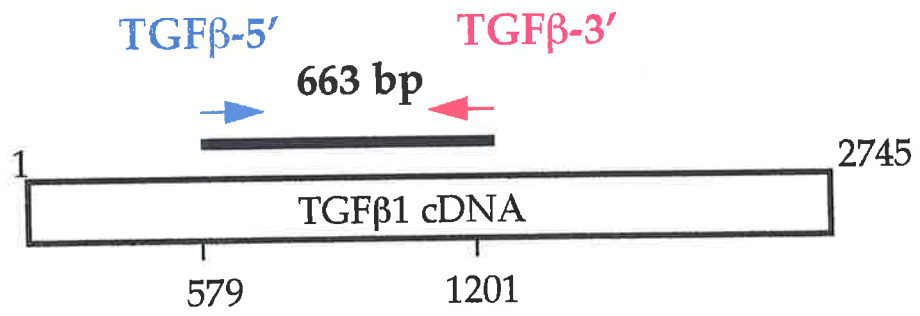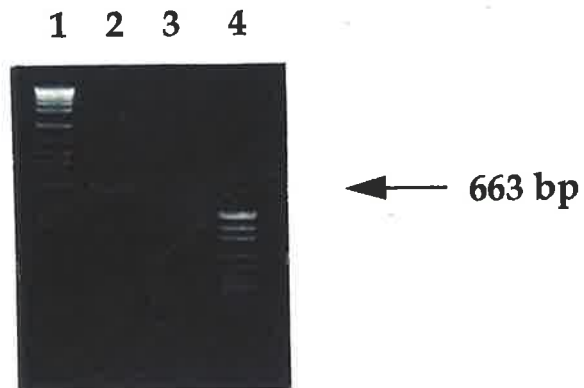
Figure 5.6b

C.

Total RNA was isolated from the J82, TCC-SUP, T24 and 5637 bladder cancer cell lines and electrophoresed on a 1% agarose/formaldehyde gel overnight and transferred to nylon membrane. In the upper panel is the Northern blot hybridised with the 663 bp TGFβ cDNA probe. In the lower panel is a parallel ethidium-bromide staining of 18S rRNA as a control for loading.

Lane 1: J82
Lane 2: TCC-SUP
Lane 3: T24
Lane 4: 5637

D.

Total RNA was isolated from bladder tumours and normal urothelium, electophoresed on a 1% agarose/formaldehyde gel and transferred to nylon membrane. In the upper panel is the hybridisation of the 663 bp TGFβ probe to the Northern blot. In the lower panel is a parallel ethidum-bromide staining of 18S rRNA as a control for loading.

Lane 1: Normal urothelium
Lane 2: Normal urothelium
Lane 3: Tumour DAR
Lane 4: Tumour MK
Lane 5: Tumour KR
Lane 6: Tumour BH
Lane 7: Tumour BC

Lane 8: Tumour DC
Lane 9: Tumour CD
Lane 10: Tumour HD
Lane 11: Tumour EG
Lane 12: Tumour LM
Lane 13: Tumour LS
Lane 14: Tumour JM

C

1 2 3 4

← TGFβ

← 18S rRNA

D

1 2 3 4 5 6 7 8 9 10 11 12 13 14

← TGFβ

← 18S rRNA
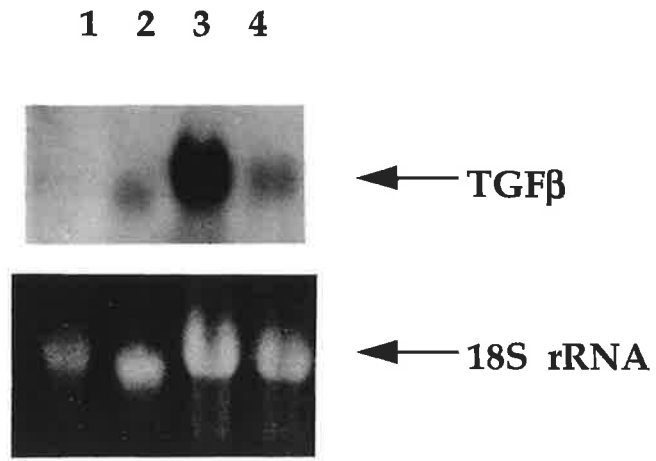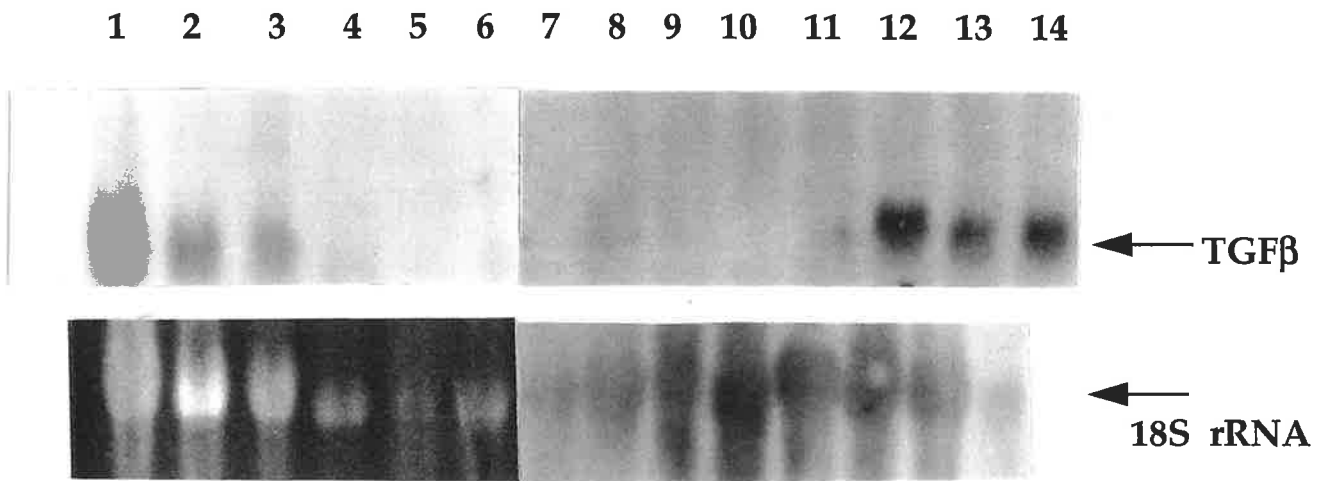
Table 5.3 Relationship between expression of UPK1B and TGFβ1 mRNAs

Bladder tumour patient	Expression of UPK1B	Expression of TGFβ
HD	lost	no
DC	lost	no
LM	lost	yes
JM	lost	yes
KR	lost	no
BH	lost	no
MK	reduced	no
EG	reduced	no
BC	normal	no
DAR	normal	yes
LS	normal	yes
CD	reduced	no

5.3.6 UPK1B is expressed in mouse bladder

As fresh normal human tissue was difficult to obtain, a range of mouse tissues were used to look for expression of UPK1B in other tissues besides bladder. RNA was isolated from mouse bladder, liver, heart, lung and spleen. There is high cDNA sequence homology between mink TI1 and human UPK1B, therefore it was assumed this high sequence conservation would also extend to mouse

Upk1b and allow detection of mouse Upk1b mRNA, using mink TI1 or human UPK1B probes. A mouse genomic sequence cloned in Chapter 4, showed 94% and 95% homology to human UPK1B and mink TI1 respectively, over 119 bp of exonic sequence. Northern analysis of mouse bladder, liver and lung using either a mink TI1 cDNA probe or the human UPK1B TM1-ECD2 cDNA probe produced the same result. The only tissue which expressed uroplakin 1B mRNA to levels detectable by Northern analysis was the bladder. The TM3 and ECD primers were used to amplify a mouse Upk1b RT-PCR product. The more sensitive RT-PCR approach showed expression in mouse bladder and also the liver (Figure 5.7). A larger PCR product, approximately 800 bp, was amplified from genomic DNA, confirming that the product from bladder and liver was derived from an RNA template. These results suggest that the uroplakin 1B gene is not exclusively expressed in the bladder in the mouse.

5.4 DISCUSSION

In this chapter, a partial human UPK1B cDNA probe was cloned to allow detection of expression of UPK1B mRNA. This probe was verified by comparison of its sequence with the human UPK1B cDNA open reading frame sequence as described in Chapter 3. Northern analysis using the 415 bp UPK1B cDNA probe revealed that the human UPK1B gene was expressed in normal urothelial tissue. The expression of the human UPK1B gene in the bladder is in agreement with the study of other uroplakin genes in different mammalian species which also have demonstrated urothelial expression (Wu et al., 1994). Unexpectedly, two human UPK1B mRNA transcripts were detected by Northern analysis. The

A

1　2　3

← ~ 1.8 kb

← 18S rRNA

B

1 2 3 4 5 6 7 8 9 10

← 693 bp

← 120 bp
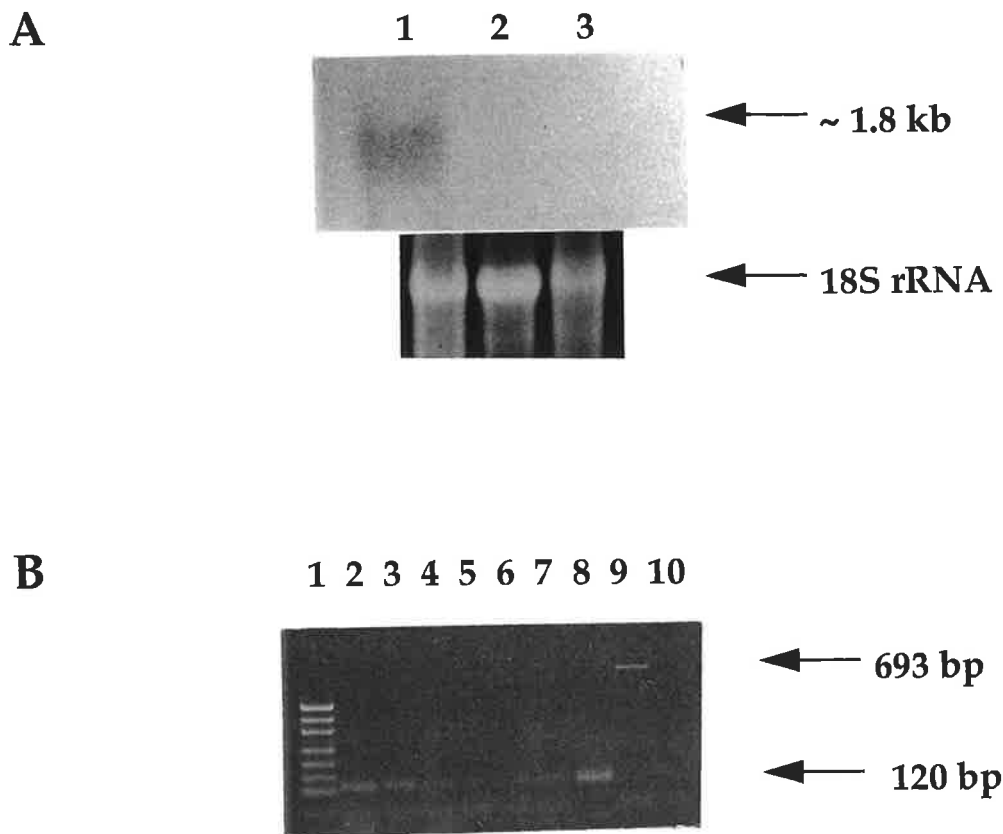
Figure 5.7

A. In the upper panel is the Northern analysis of RNA isolated from mouse bladder, liver and lung, hybridised with the mink TI1 cDNA probe. In the lower panel is the parallel ethidium-bromide staining of 18S rRNA.
Lane 1: bladder, lane 2: liver, lane 3: lung.

B. RT-PCR analysis of RNA isolated from mouse tissues using the TM3 and ECD primers. The approximately-sized 120 bp PCR product was run on a 2% gel. A larger 693 bp PCR product was amplifed from mouse genomic DNA.

Lane 1: pUC19/*Hpa*II
Lanes 2 & 3: bladder
Lane 4: liver
Lane 5: lung
Lane 6: heart

Lane 7: spleen
Lane 8: positive control (TI1/pBs)
Lane 9: mouse genomic DNA (no RT)
Lane 10: negative control

larger 1.8 kb transcript was consistent with the lengths of the mink TI1 and bovine UPKIb transcripts. There was no mention of a second mRNA transcript for either mink TI1 or bovine uroplakin Ib mRNA (Kallin et al., 1991, Yu et al., 1994). The origin of the 1 kb band remains unknown. The probe used, TM1-ECD2, was designed to consist primarily of cDNA which would code for the extracellular domain of the protein, which is the region most diverse between the tetraspan genes, to prevent cross-hybridisation to other tetraspan genes. This region of DNA has very little homology with the gene closest in sequence homology to UPK1B, the bovine uroplakin Ia gene, (FASTA analysis, (Pearson et al., 1985)), therefore it is unlikely to represent this mRNA species. Evidence supporting the theory that the 1 kb transcript detected is not human UPKIa, is that in the cow, the uroplakin Ia gene expresses a larger 1.3 kb transcript.

A possible explanation for the presence of the 1 kb transcript is that it arises from alternately splicing or may occur as a result of a second transcription start site of the UPK1B gene. The intensity of the lower 1 kb transcript was always identical to the higher 1.8 kb transcript regardless of the stringency used to wash the Northern blots after hybridisation. There have been reports of alternate transcripts in three other tetraspan genes. The SAS gene encodes two major transcripts of sizes 1.7 and 0.9 kb. However, unlike the UPK1B transcripts, which are of equal intensity, the longer SAS 1.7 kb transcript is consistently more abundant. In both the astrocytoma cell line, SF268, and the leukemia cell line, HL60, there is no detectable 0.9 kb transcript but a highly expressed 1.7 kb transcript. A faint band at 4.0 kb is also detected in some cell lines but is thought to be due to incomplete splicing rather than an alternately spliced product

(Jankowski et al., 1994). The L6 gene encodes two transcripts of sizes 1.8 and 1.2 kb. The smaller 1.2 kb transcript is presumed to correspond to the cDNA isolated in the original cloning strategy and the larger transcript may be either an alternately spliced transcript or a processing intermediate. However, the larger transcript appears to be more abundantly expressed (Marken et al., 1992). The NAG-2 tetraspan gene also transcribes two different size mRNA species. The 1.5 kb mRNA transcript of NAG-2 is present in all human tissues except the brain. A larger 6.5 kb mRNA transcript of NAG-2 is detected only in heart and skeletal muscle, along with the 1.5 kb mRNA (Tachibana et al., 1997).

Analysis of mRNA of human uroplakin 1B in bladder tumours and bladder carcinoma cell lines revealed a high incidence of reduced or lost expression in comparison to normal urothelium. These results suggest that the normal function of uroplakin 1B is frequently disrupted in bladder cancer and suggests a role for the human UPK1B gene in the pathogenesis of bladder cancer. The small sample size of bladder tumours available to be analysed precluded correlations between levels of uroplakin expression and degree of invasion and cancer stage. However preliminary results suggest that those tumours without uroplakin 1B expression are also invasive in nature. Antibodies directed against UPK1B were not available for this study, so immunohistochemical localisation of UPK1B protein was not possible, nor was correlation between the dysregulation of mRNA expression and protein expression.

In agreement with the current study, loss of expression of other uroplakins has been reported to be a common event in transitional cell carcinoma of the bladder.

Immunohistochemistry, using rabbit antibodies against uroplakin III, of paraffin sections of transitional cell carcinoma (TCC), detected positive reactions in all stages of bladder cancer. Although 88% of papillary noninvasive TCC were positive, only 53% of invasive TCCs and 66% of metastatic TCCs were positive for uroplakin III protein expression (Moll et al., 1995). Similarly, expression of uroplakin II was only found in 40% of transitional cell carcinomas (Wu et al., 1998). Ogawa et al., (1996) studied the patterns of expression of uroplakins in various degrees of chemically-induced hyperplasia and carcinoma in the rat bladder. In chemically-induced simple hyperplasia, expression of uroplakins was observed in the intermediate cells as well as in the normal location on the luminal surface but the staining patterns remained orderly. In bladder carcinomas, expression was absent from the luminal surface and only a small amount of focal staining was observed in the intermediate cells. These studies all suggest that disruption of expression of uroplakins is involved in the progression of transitional cell carcinomas and are in good agreement with the data presented above.

RT-PCR analysis of both the bladder carcinoma cell lines and the bladder tumours has revealed an interesting but unexplained correlation between lack of expression of UPK1B by Northern analysis and the inability to amplify the total UPK1B cDNA coding for the open reading frame by RT-PCR. These results indicate that the cell lines and tumours with no detectable Northern expression of UPK1B may have the 3'ORF primer sequence missing at either the DNA level or the RNA level. More information is required regarding the genomic structure of UPK1B to allow amplification of this region to determine if the

sequence of the 3'ORF primer is still present at the DNA level in those tumours which failed to amplify RT-PCR products using the 3'ORF primer. The RNA used as a template for the RT-PCR was primed using an oligo (dT) primer, therefore, the polyA tail of the UPK1B mRNA must still be intact in these tumour and carcinoma cell line RNAs.

There appears to be no correlation between the expression of UPK1B and TGFβ mRNA. This is surprising as, at least in the CCL64 cell line, expression of mink TI1 appears to be regulated by TGFβ. However, the expression of mink TI1 may be regulated by the growth arrest phase of the cycle rather than specifically TGFβ. This hypothesis is consistent with the results of experiments conducted by Kallin et al., (1991) showing that inducing growth arrest by growing cells in 0.5% serum also results in a substantial rapid increase in mink TI1 expression in CCL64 cells. The up-regulation of TGFβ expression starts a cascade of events which result in growth arrest. The signalling pathway of TGFβ is very complex and it may be simplistic to assume that TGFβ directly regulates the expression of TI1. A more reasonable hypothesis may be that TGFβ-induced growth arrest, rather than growth arrest induced by other genes or factors, favours regulation of TI1 expression. However, some of the bladder tumours do express UPK1B mRNA, but do not express TGFβ mRNA and this would suggest that UPK1B is not a direct downstream target of TGFβ signalling.

The expression of a novel gene in a particular tissue may offer an insight into the function of the gene. In this case, bovine uroplakin Ib mRNA expression is detected solely in bovine bladder tissue (Yu et al., 1994), which would suggest that the uroplakin Ib gene product has a bladder-specific function. However, the conflicting results of the detection of mink TI1 mRNA expression in a lung cell line, brings this assumption into question. Due to the unavailability of a range of normal human tissues, it was considered worthy to study murine tissues to assess conservation of bladder-specific UPK1B expression in the mouse. Expression of UPK1B mRNA was detected only in mouse bladder tissue by Northern analysis. However, the more sensitive RT-PCR method revealed some expression in the mouse liver. Yu et al., (1994) investigated the expression of bovine uroplakin Ib by Northern analysis and not by RT-PCR and claimed urothelial-specific expression. The inconsistency between expression of TI1 in the mink lung and the apparent bladder-specific bovine UPKIb expression is referred to by Yu et al., (1994). The authors suggested that the expression of TI1 in mink lung may occur because an original subpopulation of the mink lung epithelial, CCL64 cells were UPKIb/TI1-positive. Transcription of various tissue-specific genes have been detected by RT-PCR and subsequent Southern analysis in non-specific cells (Chelly et al., 1989). For example, transcripts of the erythroid-specifc β-globin gene and the muscle-specific aldolase A gene were detected in human fibroblasts, lymphoblastoid cell lines and hepatoma cells. This phenomenon of low levels of transcription of a tissue-specific gene in non-specific cells is termed "illegitimate transcription".

Studies of other uroplakin proteins have also revealed bladder-specific expression. The uroplakin III protein was detected in human bladder and not lung tissue by immunohistochemistry (Moll et al., 1995). To look at the molecular regulation of uroplakin II, a 3.6 kb 5′ flanking sequence of the mouse uroplakin II gene was used, in transgenic mouse experiments, to drive the expression of a bacterial *lacZ* reporter gene in urothelial tissue. The promoter region of the mouse uroplakin II gene was found to be capable of driving expression of a foreign gene in not only the bladder but also the hypothalamus region of the brain. However, expression of uroplakin II was found only in the bladder of a normal mouse by RT-PCR and not in any other tissue analysed (Lin et al., 1995).

In this chapter, the expression of human UPK1B mRNA has been detected in normal urothelium, however 68% of bladder tumours analysed have either a reduction or complete loss of UPK1B expression. These results suggest that the normal function of the human uroplakin 1B gene is frequently disrupted in bladder cancer. Therefore, loss of expression of UPK1B may have a role in the pathogenesis of bladder cancer. It is possible that UPK1B is acting as a metastasis suppressor or a tumour suppressor, in analogy with the tetraspan genes, CD63, CD9 and KAI1. However, the loss of expression of UPK1B may not be an initiating event but simply a consequence of the loss of differentiation that a cancerous tissue undergoes as the tumour progresses and infiltrates the normal tissue and if this is the case, the UPK1B gene may be useful as a marker of progression of bladder cancer.

CHAPTER 6

MOLECULAR MECHANISMS OF THE FREQUENT LOSS OF UROPLAKIN 1B EXPRESSION IN BLADDER CANCER

6.1 INTRODUCTION

In the previous chapter, the expression of human UPK1B mRNA was shown to be frequently lost or reduced in bladder tumours. The aim of this chapter was to investigate the possible molecular mechanisms involved in the down-regulation of expression of UPK1B mRNA detected in bladder cancer.

A number of other tetraspan genes also have altered patterns of expression in cancer and five independent studies have explored the possibility of DNA alterations being involved in the observed aberrant expression. The first two studies investigated KAI1, which decreases in protein expression as prostate cancer progresses, for a possible molecular mechanism to account for the loss of KAI1 expression in prostate cancer. A study analysing DNA isolated from American patients, failed to detect allelic loss in prostate cancer, using the D11S1344 microsatellite marker located in the same chromosome region as the KAI1 gene, chromosome 11p11.2 (Dong et al., 1996). However, an earlier Japanese study, using the same D11S1344 marker, found allelic loss or imbalance in 70% of informative patients with metastases of prostate carcinoma (Kawana et al., 1997). The possibility of mutations in the KAI1 gene was explored by the amplification by PCR of each of the eleven KAI1 exons. PCR products obtained from DNA isolated from prostatic metastases and matched normal prostatic tissue were compared by single-strand conformation polymorphism (SSCP) for mutations. There was no band shift observed for any of the ten metastases, indicating that none had a mutation of the KAI1 gene (Dong et al., 1996).

The L6 protein, another member of the tetraspan family, is over-expressed in a number of malignancies, including lung cancer. Southern analysis of the L6 gene revealed identical banding patterns between the lung carcinoma cell line A549, which overexpresses L6 and the melanoma cell line H3606, which has no detectable levels of expression of L6, suggesting that gross gene rearrangements or amplifications are not involved in the overexpression of the L6 gene (Marken et al., 1992). The CD63 gene has varied levels of expression in different melanoma cell lines. However Southern analysis of these cell lines did not reveal any amplification or rearrangement of the CD63 gene to account for the differing degrees of expression (Hotta et al., 1988). The TALLA-1 tetraspan gene is expressed as a surface marker in T-ALL (T-cell acute lymphoblastic leukemia) cell lines but not on normal peripheral blood mononuclear cells or immortalised T cell lines. Southern blot analysis of T-ALL cell lines failed to detect any gross genomic alteration of the TALLA-1 gene (Takagi et al., 1995). In summary, none of the studies thus far have found a molecular mechanism which could explain the observed altered patterns of expression of the tetraspan genes in cancer.

This chapter describes the search for genetic alterations of the human UPK1B gene in bladder cancer. Molecular analyses were performed on DNA isolated from bladder tumours and bladder cancer cell lines to determine if the observed down-regulation of expression of the UPK1B gene was caused by gross gene rearrangements or allelic loss. Southern analysis was used to determine if there were different banding patterns between normal DNA and bladder tumour DNA, which would indicate the presence of large gene deletions, amplifications or rearrangements. Microsatellite markers were used to look for allelic loss of the

chromosome region 3q13.3-q21 in bladder cancer, where the human UPK1B gene is located (Chapter 4).

6.2 METHODS

6.2.1 Southern analysis of the human UPK1B gene

Genomic DNA was isolated from peripheral blood leukocytes, cell lines and bladder tumour tissue (2.2.1). The DNA was digested with either *Taq*I, *Kpn*I, *Xba*I or *Pst*I restriction enzymes overnight and then electrophoresed in 1% agarose gels for 16 hours and transferred to nylon membranes as described in 2.2.4. The probes used for hybridisation were the 778 bp human genomic probe (3.3.2), the TM3-3'ORF cDNA (3.3.1) and the UPK1B cDNA ORF (3.3.1). All probes were labelled with α^{32}P-dCTP using the Gigaprime kit (2.2.6) and hybridised as described in 2.2.6.

6.2.2 Microsatellite analysis using polymorphic markers

PCR primers (Table 2.1) were synthesised to amplify the microsatellite repeats for both D3S1278 and D3S1292 (Gyapay et al., 1994). Each PCR, using either of the two sets of primers, was carried out at an annealing temperature of 55°C (2.2.7) with the addition of 1μCi α^{32}P-dATP per reaction. The PCR products were denatured at 95°C for 5 min and separated by gel electrophoresis on 6% (19:1) polyacrylamide urea gels for 3 hours at 40 W in 1 x TBE (2.2.3). The gels were vacuum-dried, exposed to autoradiography film and developed after 1-2 days.

6.3 RESULTS

6.3.1 Rearrangements of the human uroplakin 1B gene

The frequent loss or reduction of UPK1B mRNA expression in bladder cancer could be due to gross rearrangements of the UPK1B gene in these tissues. To test this hypothesis, DNA was isolated from a series of peripheral blood leukocytes (PBL) from donors (as normal controls) and bladder tumours and digested with the *Taq*I restriction enzyme. The resultant Southern blot was hybridised with a 778 bp human genomic UPK1B probe. In both normal DNA and fifteen tumour DNA samples, a single 2.5 kb band was detected (Figure 6.1). After the blot was stripped (2.2.6), hybridisation of the total human UPK1B cDNA ORF on the same Southern blot produced five bands in both normal and tumour samples of sizes, 5.0, 4.0, 3.5, 2.7 and 2.5 kb (Figure 6.1).

Analysis of Southern blots containing DNA from four bladder cancer cell lines hybridised with the 778 bp UPK1B genomic probe revealed one band of 15 kb when the DNA was digested with *Pst*I and 7.2 kb on digestion with *Xba*I (Figure 6.2). Digestion of DNA from five bladder cancer cell lines with *Kpn*I resulted in a single 8 kb band when probed with the TM3-3'ORF UPK1B cDNA probe (Figure 6.2). The intensity of bands in all of the above Southerns did not differ sufficiently from any of the bladder tumour DNAs in comparison to normal DNAs, to suggest that the UPK1B gene was amplified. For all of the above Southerns, identical banding patterns were observed for both normal, bladder tumour and cell line DNA. Ethidium bromide staining of the Southern gels showed equivalent loading of DNA. These results taken together indicate that large gene rearrangements, deletions or amplifications were not detected and
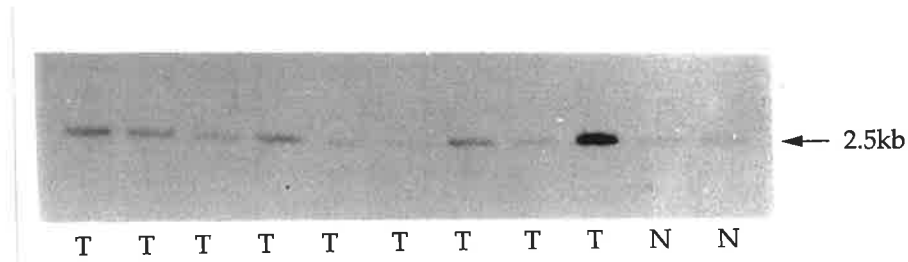
Figure 6.1

A.
Genomic DNA isolated from normal (N) PBL and bladder tumour (T) tissue was digested with *Taq*I. The Southern blot was hybridised with the 778 bp human UPK1B genomic probe. All samples produced an identical 2.5 kb band, indicating there is no rearrangement of the UPK1B gene.

B.
The Southern blot in (A.) was stripped and hybridised with the total human UPK1B cDNA ORF. Five bands were produced in normal and tumour samples of sizes 5, 4, 3.5, 2.7 and 2.5 kb.
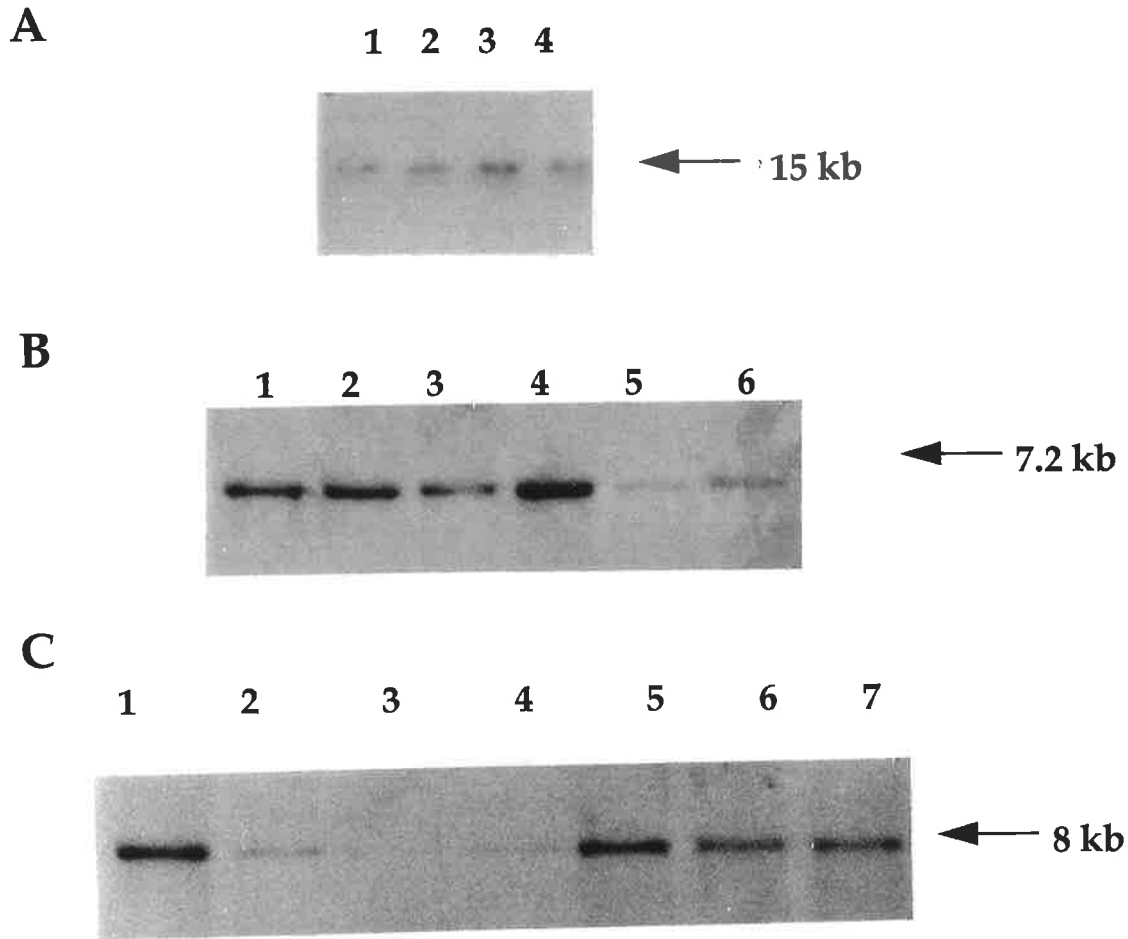
Figure 6.2

A. Southern analysis of DNA from four bladder cancer cell lines digested with *Pst*I and hybridised with the 778 bp human UPK1B genomic probe. One band of 15 kb was detected in all lanes, therefore no rearrangment of the UPK1B gene was observed. Lane 1: J82, lane 2: ScaBER, lane 3: TCC-SUP and lane 4: T24.

B.Southern analysis of DNA from four bladder cancer cell lines digested with *Xba*I and hybridised with the 778 bp human UPK1B genomic probe. One band of 7.2 kb was detected in all lanes, therefore no rearrangement of the UPK1B gene was detected. Lane 1: J82, lane 2: ScaBER, lane 3: TCC-SUP, lanes 4&5: normal genomic DNA and lane 6: T24.

C.Southern analysis of DNA from five bladder cancer cell lines digested with *Kpn*I and hybridised with the TM3-3'ORF UPK1B cDNA probe. One band of 8 kb was detected in all lanes, therefore no rearrangement of UPK1B was observed. Considerably less DNA was loaded on the Southern gel in lanes 2-4, accounting for the low intensity of hybridisation.
Lane 1: J82, lane 2: ScaBER, lane 3: TCC-SUP, lane 4: T24, lane 5: 5637and lanes 6 & 7: normal genomic DNA

therefore, are not the reason for the loss of mRNA expression of UPK1B in bladder cancer.

6.3.2 Allelic loss using polymorphic microsatellite markers

To investigate the possibility that allelic loss may result in loss of expression of the UPK1B gene in bladder cancer, a method to differentiate one allele from the other was required. Polymorphic microsatellite markers were chosen that mapped either side of the human uroplakin 1B gene, which is located on chromosome 3q13.3-21 (Chapter 4). The D3S1278 and D3S1292 markers are localised proximal, (3q13) and distal (3q21.3) respectively to chromosome 3q13.3-21 (Gyapay et al., 1994). These dinucleotide CA repeat markers are highly polymorphic between individuals allowing the two copies of chromosome 3 to be differentiated. The premise of this experiment was that if both markers flanking the UPK1B gene were lost in the tumour DNA, then it is highly likely that the gene located between the two markers had also been lost. DNA from fifteen matched normal PBL and bladder tumour samples was used as the template for separate PCR amplification of each marker (Figure 6.3). Comparison of the banding patterns between PCR products of each matched normal and tumour pair failed to reveal any difference, indicating that both alleles were retained in the bladder tumours (Figure 6.4). Therefore, allelic loss was not detected for either the D3S1292 or D3S1278 markers in any of the fifteen bladder tumours analysed.
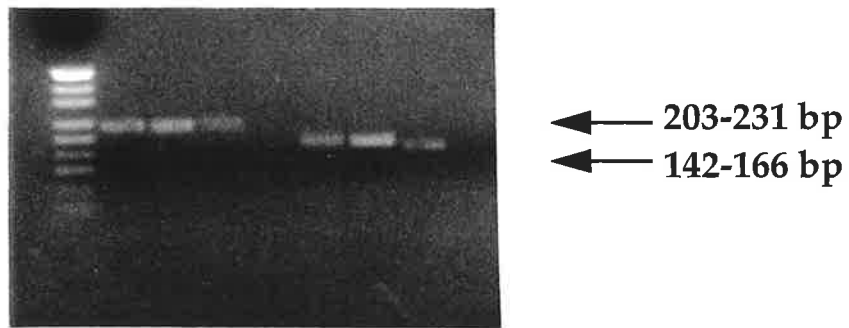
Figure 6.3
A representative agarose gel showing PCR amplification using either the D3S1278 or D3S1292 sets of primers from human genomic DNA. The PCR products were run on a 2% gel. The sizes of the D3S1278 PCR products range from 203-231 bp depending on the number of CA repeats. The sizes of the D3S1292 PCR products range from 142-166 bp depending on the number of CA repeats.

Lane 1: pUC19/HpaII marker

Lane 2: genomic DNA (D3S1278)

Lane 3: genomic DNA (D3S1278)

Lane 4: genomic DNA (D3S1278)

Lane 5: negative control

Lane 6: genomic DNA (D3S1292)

Lane 7: genomic DNA (D3S1292)

Lane 8: genomic DNA (D3S1292)

Lane 9: negative control

A

N-T N-T N-T N-T N-T N-T N-T N-T N-T N-T

B

N-T N-T N-T N-T N-T N-T N-T
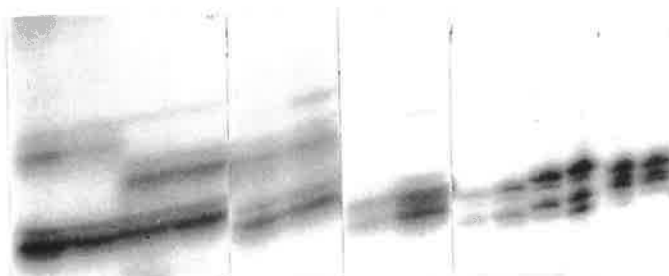
Figure 6.4

A.
Microsatellite analysis of the polymorphic marker D3S1292 in selected, matched normal (N) and tumour (T) DNA samples. PCR products were run on a 6% polyacrylamide gel. PCR product sizes ranged from 142-166 bp.

B.
Microsatellite analysis of the polymorphic marker D3S1278 in selected, matched normal (N) and tumour (T) DNA samples. PCR products were run on a 6% polyacrylamide gel. PCR product sizes ranged from 203-231 bp.

6.4 DISCUSSION

Expression of the human UPK1B gene is frequently lost or reduced in bladder cancer. The down-regulation of mRNA expression may be due to a number of different genetic aberrations. In this chapter, gross gene rearrangements and allelic loss were investigated as possible molecular mechanisms which may affect the levels of UPK1B mRNA. The possibility of gross gene rearrangements affecting UPK1B mRNA levels was considered because a large deletion or rearrangement of the UPK1B gene would obviously disrupt or prevent transcription. Allelic loss is often associated with tumour suppressor genes which frequently also have point mutations on the other intact allele in tumours. There is a possibility that the UPK1B gene product has tumour suppressive activity, as do other tetraspan proteins and allelic loss of UPK1B could account for the reduction of expression of some of the bladder tumours.

To establish if gross gene rearrangements were responsible for the down-regulation of UPK1B mRNA expression in bladder cancer, Southern analysis was performed on a number of tumours and cancer cell lines, using a variety of restriction enzymes and UPK1B probes. Particular enzymes and probes were chosen to give good coverage over the UPK1B coding region. There was no difference in banding pattern between normal and tumour DNA indicating that there was no gross rearrangement of the UPK1B gene. Therefore, it is not likely that large gene rearrangements or deletions are the reason for the frequent loss of UPK1B mRNA expression in bladder cancer. Amplification of the UPK1B gene in the bladder tumours was not expected as this would most likely result in an overexpression of UPK1B mRNA, but was also eliminated as a possible genetic

151

alteration in the tumours. Amplification of the tetraspan gene, SAS occurs in sarcoma and neuroblastoma cell lines which also overexpress SAS mRNA (Jankowski et al., 1994).

To search for allelic loss of the UPK1B gene, a restriction fragment length polymorphism within the gene could be used to allow differentiation of one allele from the other. There is a *Taq*I restriction fragment length polymorphism located within the UPK1B gene (Chapter 3.3.3) however with a rate of heterozygosity of 29% and a sample size of only fifteen tumours it was considered to be not sufficiently polymorphic to make this study viable. Microsatellite markers are often used for allelic loss studies, as these tandem DNA repeat sequences are abundant, highly polymorphic, are widespread across the genome and can be detected by PCR (Gonzalez-Zulueta et al., 1993). The two microsatellite markers, D3S1278 and D3S1292, were chosen for their close proximity to the UPK1B gene on chromosome 3q13.3-q21. The result of no allelic loss for either marker in the samples of bladder cancer is surprising because there is usually a background level of allelic loss for a chromosome arm in tumours. However, as previously discussed in Chapter 3, there have been no earlier studies that have looked precisely at chromosome 3q13.3-q21. The current sample size was quite small due to the limited availability of matched normal and tumour DNAs. However, this data would suggest that any deletion of the UPK1B gene is going to be a rare event in less than 10% of tumours. The tumour DNA samples examined for allelic loss included both tumours which expressed UPK1B mRNA as well as those which did not, as detected by Northern analysis.

These studies have not exhausted all the possible molecular mechanisms which could result in a loss or reduction of UPK1B expression. Potential mechanisms which have not yet been examined include point mutations, small deletions, promoter sequence abnormalities and hypermethylation of the UPK1B promoter or gene. Down-regulation of an upstream gene which regulates UPK1B expression may also result in lost or reduced UPK1B expression in bladder cancer. To investigate these possibilities, more information is required regarding the genomic structure of human UPK1B. The human UPK1B promoter would need to be cloned to enable the examination of potential mutations or methylation changes of the promoter between normal and tumour DNAs which may result in transcriptional silencing (Versteeg, 1997). To search for point mutations or small deletions, the single-strand conformation polymorphism (SSCP) technique could be used (Orita et al., 1989). The exon/intron boundaries of the human UPK1B gene would first need to be determined to allow amplification of the exon sequences to detect sequence anomalies between normal and tumour DNAs. It is also possible that an unknown upstream gene responsible for regulation of UPK1B expression is mutated in bladder cancer and that the loss of expression of UPK1B is just a downstream event of this initiating mutation. Analysis of the promoter sequence may reveal motifs for binding sites of particular proteins which may be involved in the regulation of UPK1B gene expression.

CHAPTER 7

ANTI-PROLIFERATIVE
ACTIVITY OF UROPLAKIN 1B

7.1 INTRODUCTION

To date, no studies have investigated the possible biological function of the human UPK1B gene. The initial insights into the function of UPK1B come from studies investigating its homologues, mink TI1 and bovine UPKIb. The mink TI1 gene was originally isolated from the mink lung epithelial cell line, CCL64. Expression levels of mRNA of mink TI1 were increased in CCL64 cells growth-arrested by either serum starvation or TGFβ1 treatment, however, CCL64 cells grown in normal growth conditions express only a basal level of TI1 mRNA (Kallin et al., 1991). The bovine uroplakin Ib gene codes for a protein product of the asymmetric unit membrane of the bladder and is expressed specifically in terminally differentiated urothelial cells (Yu et al., 1994). The preferential expression of TI1 during growth arrest and UPKIb in terminally differentiated cells implicates TI1/UPKIb as a potential negative regulator of the cell cycle and inducer of terminal differentiation.

The initial aim of this study was to monitor the effect of exogenous overexpression of TI1, under the control of a constitutive CMV promoter, on CCL64 cells. The hypothesis for this experiment was that constant high levels of expression of TI1 throughout the cell cycle, rather than just during the growth arrest stage, would be growth inhibitory to the cells. These experiments would determine if TI1 acts as a growth-suppressor.

It is also useful to draw analogies with other tetraspan proteins and their respective functions for an insight into the possible role of the UPK1B gene

product. As discussed in the literature review, while the precise functions of many of the tetraspans are unknown, some have roles in cell motility, activation, growth and adhesion. Recent studies have found that some of these tetraspan genes have reduced levels of expression in a range of human cancers, suggesting they may function as tumour suppressor genes. The CD63 gene, not expressed in normal melanocytes, is highly expressed in early melanomas, but expression is lost in advanced melanomas. Transfection of the CD63 gene into a CD63-negative melanoma cell line, resulted in suppression of tumorigenicity and reduced metastasis in nude mice (Radford et al., 1995). Down-regulation of KAI1 mRNA expression has been detected in prostate and pancreatic cancer (Dong et al., 1996, Guo et al., 1996). When transfected into metastatic prostate cancer cells, KAI1 suppressed lung metastases in nude mice (Dong et al., 1995). Reduced expression of CD9 is an indicator of poor prognosis in breast and non-small cell lung cancer (Miyake et al., 1996, Higashiyama et al., 1995). Transfection of CD9 into a metastatic mouse melanoma cell line resulted in suppression of the metastatic potential as demonstrated by a reduction in the formation of tumour foci in the lungs of nude mice (Ikeyama et al., 1993). These results all suggest that the CD63, KAI1 and CD9 gene products are capable of acting as metastasis suppressors.

The second section of this chapter examines the effect of exogenous expression of TI1 on human bladder cancer cell lines. Analysis by Northern blot of a number of bladder cancer cell lines failed to detect any UPK1B mRNA transcripts (Chapter 5.3.3). The aim of this section of the study was to determine if the restoration of expression of UPK1B in these cells would revert the transformed phenotype of

these cancerous cells. Two bladder cancer cell lines, 5637 and T24, were chosen for the transfection studies because of their differing properties. The 5637 cell line was derived from a well-differentiated transitional cell carcinoma and has high levels of expression of p53 protein, but contains a mutation resulting in an amino acid change from arginine to threonine (Cooper et al., 1994). 5637 cells are negative for retinoblastoma protein expression, but express both MDM2 and E-cadherin (Reiger et al., 1995). The T24 cell line was derived from an undifferentiated, invasive, grade III transitional cell carcinoma (Bubenik et al., 1973). The T24 cells express low levels of a novel mutant p53 protein with an inframe deletion of a tyrosine amino acid at codon 126 (Cooper et al., 1994) but are positive for retinoblastoma expression (Grimm et al., 1995). Neither 5637 or T24 cells express UPK1B mRNA transcripts to levels detectable by Northern analysis (Chapter 5.3.3).

The plasmid vector chosen to provide the exogenous expression of the TI1 gene was the pRc/CMV vector which contains a strong constitutive cytomegalovirus promoter and allows high levels of expression of cloned genes. The cDNA coding for the open reading frame of the mink TI1 protein was cloned into the multiple cloning site of the pRc/CMV vector in the 'sense' orientation with respect to the promoter. At the time these experiments were carried out, the total human UPK1B cDNA open reading frame had not yet been cloned. It was known that there was a high degree of homology between mink TI1 and bovine UPKIb cDNA sequences and it was assumed that this high degree of homology would also be conserved in the human UPK1B cDNA sequence. This assumption was later confirmed as the degree of homology of the cDNA coding

for the open reading frame of the protein between mink and human UPK1B was determined to be very high at 92% (Chapter 3.3.1), strongly suggesting that mink TI1 would be active in human cells.

This chapter reports the first efforts to gain an insight into the biological function of the UPK1B/TI1 gene. While it has been shown that the expression of UPK1B is down-regulated in bladder cancer, (Chapter 5.3.3), it is important to discover the normal function of the UPK1B gene in urothelial cells to determine its relevance in the pathogenesis of bladder cancer.

7.2   METHODS

7.2.1   Cloning of mink TI1 cDNA into pRc/CMV

The TI1/pBluescript plasmid (Figure 7.1) was linearised with the *Apa*I enzyme (2.2.11) at 25°C and purified through a Wizard Prep minicolumn (2.2.9). The *Apa*I-digested TI1/pBluescript plasmid was then digested with the *Hind*III enzyme at 37°C (2.2.11) to release an 848 bp *Apa*I/*Hind*III TI1 fragment which was purified from a low melting point agarose gel by Wizard Prep. (2.2.9). The pRc/CMV plasmid was also digested with the *Apa*I and *Hind*III enzymes (2.2.11), both of which have unique recognition sites in the polylinker. The 848 bp TI1 cDNA fragment was then ligated into the *Apa*I/*Hind*III pRc/CMV plasmid using T4 DNA ligase (2.2.12). The ligated plasmid was transformed into competent *E.coli* cells (2.2.14) and mini-preparations of the plasmid were isolated (2.2.15). Recombinant plasmids were detected by PCR analysis (2.2.7) using 5′CMV and 3′CMV primers (Table 2.1) and by restriction enzyme digestion using the *Kpn*I

and *Stu*I enzymes (2.2.11). A large-scale plasmid preparation of TI1/CMV was prepared using the Qiagen midi-plasmid kit (2.2.16).

7.2.2 Transfection of TI1/CMV

The CCL64, 5637 and T24 cell lines were seeded at 4×10^5 cells per $25cm^2$ flask and grown until approximately 50% confluent. The cells were transfected with 10 µg of plasmid, either TI1/CMV or CMV alone (2.2.19). All transfections were set up in duplicate and included transfection of an empty vector as a negative control. After approximately two weeks of selection with 400 µg/ml of the antibiotic, G418, the resultant colonies were counted. Individual colonies were manually picked and propagated in 2 ml of DMEM plus 10% FCS (2.2.18) in 24 well plates in the presence of continued G418 antibiotic selection. When the well became confluent, the cells were trypsinised and transferred to a small $25 \ cm^2$ flask. Cells from each clone were subsequently divided into three flasks and the cells were used to isolate DNA (2.2.1), RNA (2.2.2) or to make frozen stocks of the transfectants (2.2.18).

7.2.3 PCR analysis to detect the transfected TI1/CMV plasmid in CCL64, T24 and 5637 cell lines

DNA was isolated (2.2.1) from stable transfectant cell lines. PCR primers (5'CMV and 3'CMV) (Table 2.1) were designed from the pRc/CMV plasmid sequence to anneal to regions of DNA on either side of the multiple cloning site and, if used to amplify an empty pRc/CMV plasmid, yielded a product of 202 bp. The expected size of the PCR product amplified from the TI1/CMV plasmid, using

5′CMV and 3′CMV primers was 950 bp. The annealing temperature for this PCR was 63°C (2.2.7). Gene-specific primers, TM3 and ECD, (Table 2.1) were also used in conjunction with the CMV primers.

7.2.4 Detection of exogenous expression of TI1 mRNA by Northern analysis

Total RNA was isolated (2.2.2) from flasks containing cells transfected with either TI1/CMV or CMV plasmid alone, either two days after transfection or after approximately two weeks of G418 selection. Ten μg of RNA was electrophoresed on 1% formaldehyde gels and then transferred to nylon membrane as described in 2.2.5. The Northern blots were hybridised with either TI1/CMV or TI1/pBluescript plasmids, which were radiolabelled with α^{32}P-dCTP using the Gigaprime kit (2.2.6). After the blots were washed at high stringency, (0.1 x SSC, 0.1% SDS at 65°C), they were exposed to autoradiography film and developed (2.2.6).

7.2.5 Cloning of UPK1B ORF into the pTRE plasmid vector

The pTRE vector was linearised by digestion with the SacII enzyme (2.2.11) and then treated with calf intestinal alkaline phosphatase (CIP) to prevent self-ligation (2.2.12). The NH1B-3′ORF/pGEM plasmid containing the cDNA coding for the open reading frame of the human UPK1B protein (Chapter 3.3.1), was digested with the SacII and SacI enzymes (2.2.11) to excise the UPK1B cDNA ORF from the pGEM plasmid. The 802 bp SacI-UPK1B-SacII fragment was purified by

Wizard Prep (2.2.9) from a low-melting point agarose gel. Both the UPK1B fragment and the linearised pTRE vector were treated with 20 units of the Klenow enzyme (Bresatec) at 37°C for 30 mins, which adds on nucleotides to the overlapping restriction enzyme ends to create blunt ends. The UPK1B fragment was then ligated into the pTRE vector using T4 DNA ligase (2.2.12), transformed into competent *E.coli* cells (2.2.14) and mini-preparations of the plasmid were isolated (2.2.15). Recombinant plasmids were detected by PCR analysis (2.2.7) using 5'TRE and UPK1B-specific ECD2 primers (Table 2.1) and also by restriction enzyme analysis (2.2.11). A large-scale plasmid preparation of UPK1B/pTRE was isolated using the Qiagen midi-plasmid kit (2.2.16).

7.2.6 Transfection of the pTET-ON and UPK1B/pTRE plasmids

The 5637 cells were grown until approximately 50% confluent and then transfected with 10 µg of pTET-ON plasmid as described previously (2.2.19). Transfectant cells were propagated in 400 µg/ml G418 for two weeks and subsequent G418-resistant clones were manually picked and propagated in separate flasks. DNA was isolated from the transfected cells (2.2.1) and PCR analysis used to determine those clones which contained the pTET-ON plasmid. Primers, 5'TET-ON and 3'TET-ON (Table 2.1), were designed to amplify a 245 bp product from the pTET-ON plasmid, using an annealing temperature of 62°C (2.2.7). The second transfection (2.2.19) involved the co-transfection of two plasmids, UPK1B/pTRE and the pTK-HYG plasmid. Cells positive for the pTET-ON plasmid were transfected with the UPK1B/pTRE and pTK-HYG plasmids or as a control, pTRE and pTK-HYG. Transfectant cells containing these plasmids

were selected using hygromycin, as the pTK-HYG plasmid contains a hygromycin-resistance gene. G418 was also added to the culture to maintain selection for the pTET-ON plasmid.

7.3 RESULTS

7.3.1 Cloning of mink TI1 cDNA into pRc/CMV

The TI1/pBluescript plasmid, which contains the full length TI1 cDNA sequence was digested with the *Hind*III and *Apa*I enzymes which recognises sites within the TI1 cDNA. The *Hind*III enzyme cuts at nucleotide 46 of the TI1 cDNA which precedes the start codon, ATG, located at nucleotide 69. The *Apa*I site is located at nucleotide 894 bp, downstream of the stop codon, TAA, at nucleotide 849. Therefore, the resulting 848 bp *Hind*III/*Apa*I fragment contains the total TI1 cDNA coding for the open reading frame. The cDNA fragment was ligated by directional cloning into the *Hind*III/*Apa*I-digested pRc/CMV vector and a plasmid containing the 848 bp TI1 fragment was propagated (Figure 7.1). The identity of the TI1/CMV plasmid was verified by PCR, restriction digests and sequence analysis. Using primers designed to anneal 5′ and 3′ of the multiple cloning site of the pRc/CMV vector, the correct size PCR product of 950 bp was amplified from the TI1/CMV plasmid. An empty CMV plasmid yields a 202 bp PCR product following amplification with the CMV primers (Figure 7.2). Restriction digests of the plasmid with the *Kpn*I and the *Stu*I enzymes confirmed that the TI1 fragment had been ligated into the plasmid in the correct orientation (Figure 7.3). The TI1/CMV plasmid was sequenced in both directions using the CMV primers and compared to the original sequence published by Kallin et al., (1991) using GeneJockey, identifying the insert as TI1 (Appendix VIII).
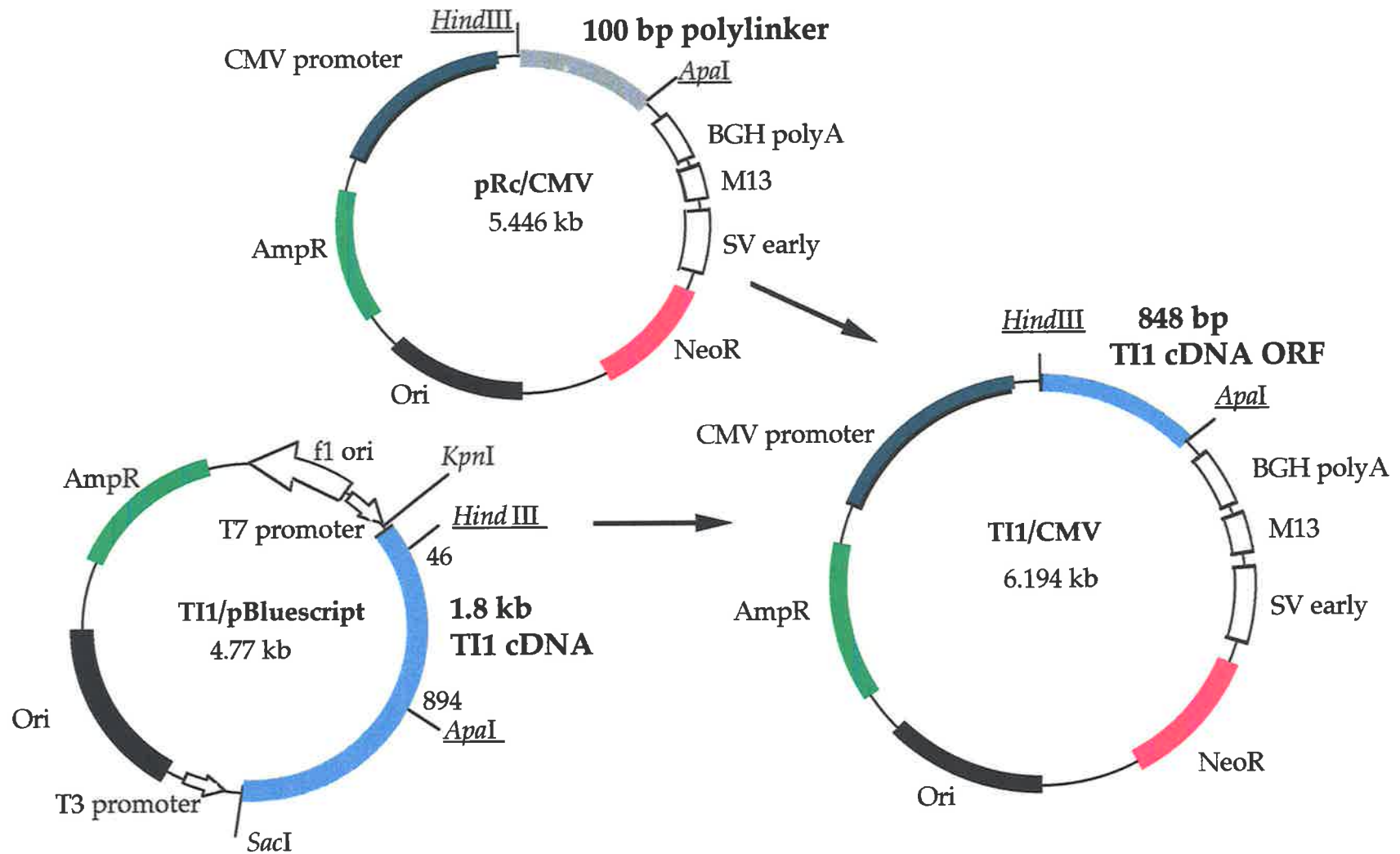
Figure 7.1
The pRc/CMV plasmid was digested with the *Hind*III and *Apa*I enzymes which removed 100 bp of the polylinker. The 848 bp *Hind*III/*Apa*I TI1 cDNA fragment, containing the open reading frame coding for the TI1 protein, was excised from the TI1/pBluescript plasmid and cloned into the *Hind*III/*Apa*I-digested pRc/CMV plasmid vector.

Figure 7.2

A.

Diagrammatic representation of the 950 bp TI1/CMV PCR product amplified from the TI1/CMV plasmid using the 5′CMV and 3′CMV primers, located 5′ and 3′ respectively of the pRc/CMV polylinker. Amplification of an empty CMV plasmid would yield a 202 bp product, as pRc/CMV contains an extra 100 bp of polylinker compared to the TI1/CMV plasmid.

B.

Amplification of TI1 cDNA from the TI1/CMV plasmid using 5′CMV and 3′CMV primers, confirming ligation of the 848 bp TI1 cDNA into the pRc/CMV vector. The PCR products were electrophoresed on a 1.5% agarose gel.

Lane 1: pRc/CMV plasmid
Lane 2: TI1/CMV plasmid
Lane 3: SPP1/*Eco*RI marker
Lane 4: negative control (no DNA)

A

950 bp TI1/CMV product

5' CMV   3' CMV

56 bp   848 bp TI1 cDNA   46 bp

TI1/CMV plasmid

B

1   2   3   4

← 950 bp

← 202 bp
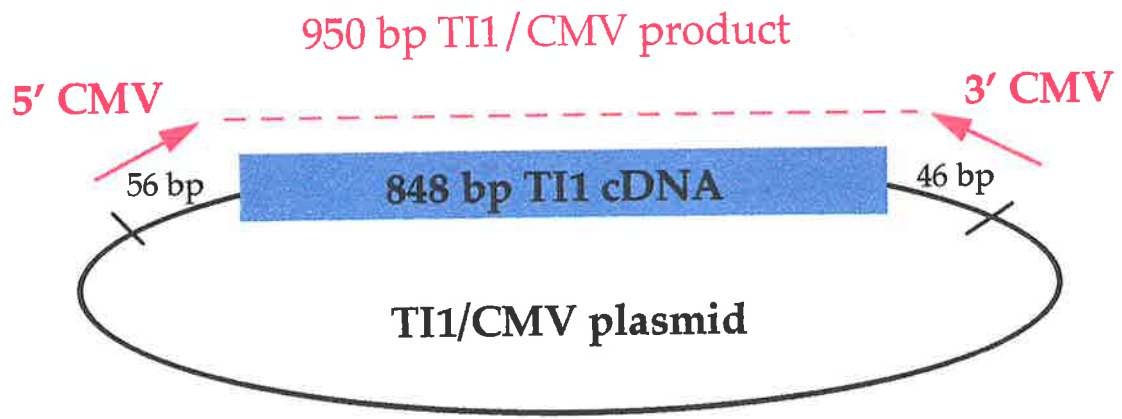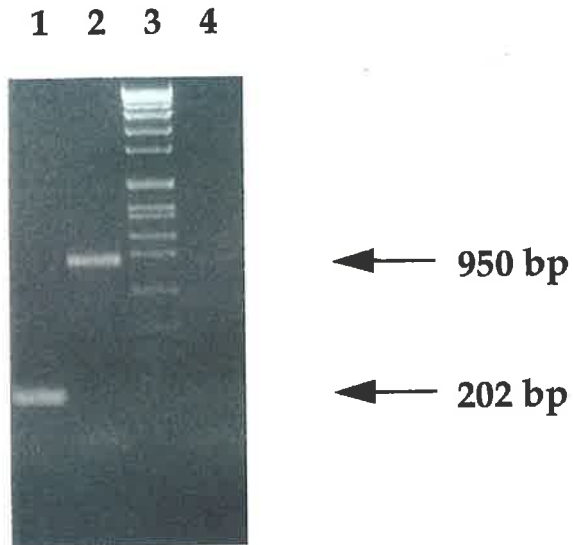
Figure 7.3

The TI1 cDNA was originally excised from the pBluescript plasmid using the *Hind*III and *Apa*I sites at locations 46 and 894 bp. The pRc/CMV vector was digested with *Hind*III and *Apa*I enzymes which removed 100 bp from the polylinker, therefore the 5446 bp vector became 5346 bp in length. With the addition of the 848 bp TI1 cDNA fragment, the total length of the TI1/CMV vector was 6194 bp. Restriction sites within the pRc/CMV vector are depicted in black and the sites within the TI1 cDNA are depicted in blue.

A.

Schematic diagram of the TI1/CMV plasmid showing the location of the *Kpn*I restriction enzyme sites. The *Kpn*I enzyme has two recognition sites within the 848 bp TI1 cDNA (450 and 816) and one site within the pRC/CMV plasmid (1732). If the TI1 cDNA has been inserted into the CMV plasmid in the correct orientation, the expected fragment sizes (red) after *Kpn*I digestion would be 5009 bp, 819 bp and 366 bp. Digestion of pRC/CMV alone with *Kpn*I would give two fragments of sizes 4611 bp and 835 bp as the *Kpn*I enzyme cuts within the polylinker at nucleotide 897, (removed by digestion with the *Hind*III and *Apa*I enzymes).

B.

Schematic diagram of the TI1/CMV plasmid showing *Stu*I restriction sites. The *Stu*I enzyme has one site within the 848 bp TI1 cDNA (675) and one site within the CMV plasmid (2070). If the TI1 cDNA has been inserted into the CMV plasmid in the correct orientation, the expected fragment sizes after *Stu*I digestion are 4896 bp and 1298 bp. Digestion of pRC/CMV alone with *Stu*I would linearise the plasmid to a size of 5446 bp.

C.

One μg of either the TI1/CMV or the pRC/CMV plasmid were digested with either the *Kpn*I or *Stu*I restriction enzymes for 1 hr at 37°C and run on a 1.2% agarose gel.

Lane 1: SPP1/*Eco*RI marker   Lane 4: pRc/CMV -*Stu*I
Lane 2: pRc/CMV -*Kpn*I   Lane 5: TI1/CMV -*Stu*I
Lane 3: TI1/CMV -*Kpn*I   Lane 6: pRc/CMV -undigested

A

KpnI   366 bp   KpnI

46   450   816   894

848 bp TI1 cDNA

891   991   819 bp

*Hind*III   *Apa*I

TI1/CMV plasmid
6194 bp

5009 bp   1732

*Kpn*I

B

*Stu*I

46   675   894

848 bp TI1 cDNA   1298 bp

891   991

*Hind*III   *Apa*I

TI1/CMV plasmid
6194 bp

4896 bp   2070

*Stu*I

C

1 2 3 4 5 6

5009 bp →   ← 4896 bp
4611 bp →

← 1298 bp

835 bp →
819 bp →

366 bp →
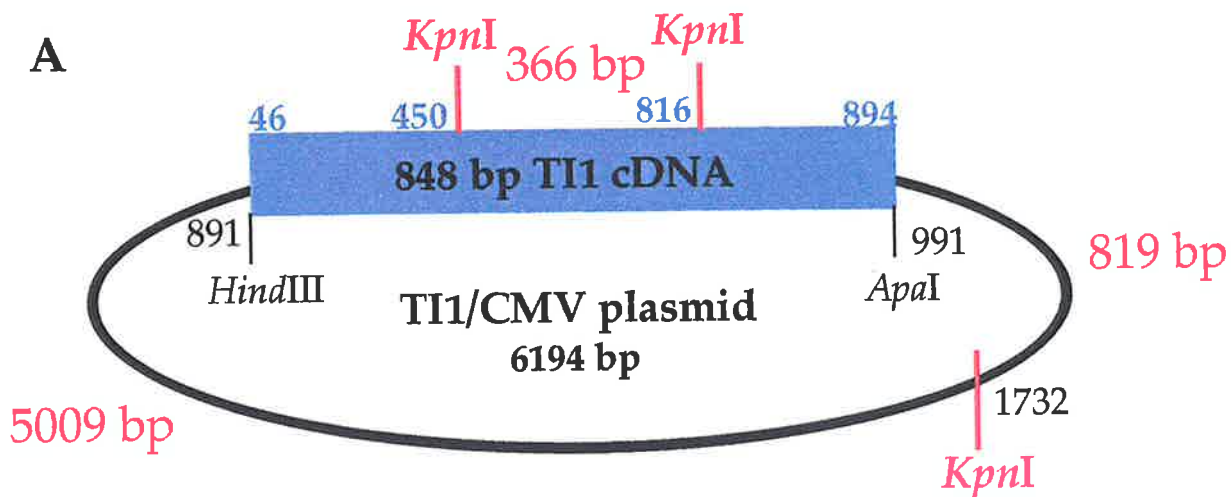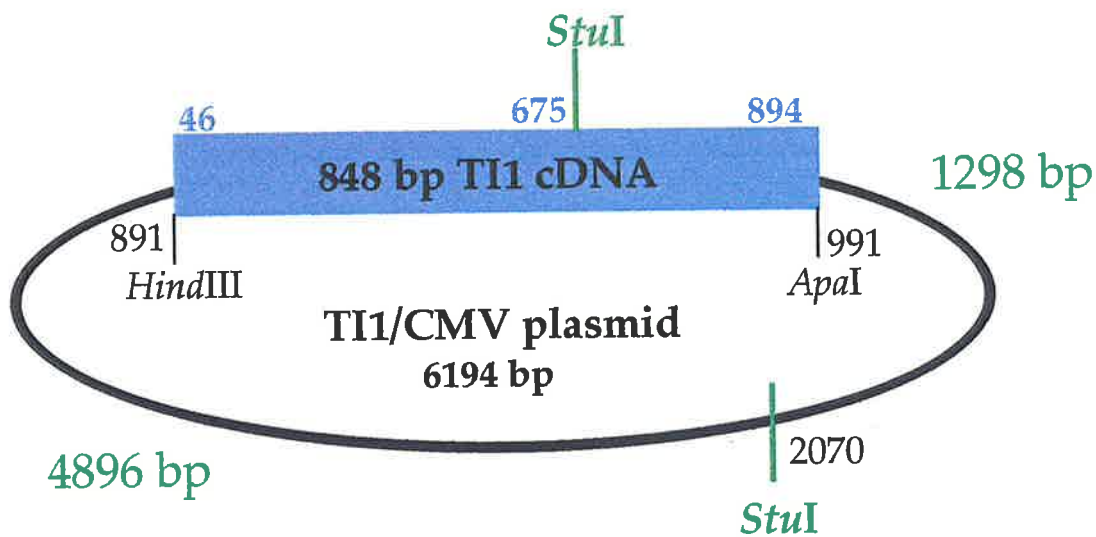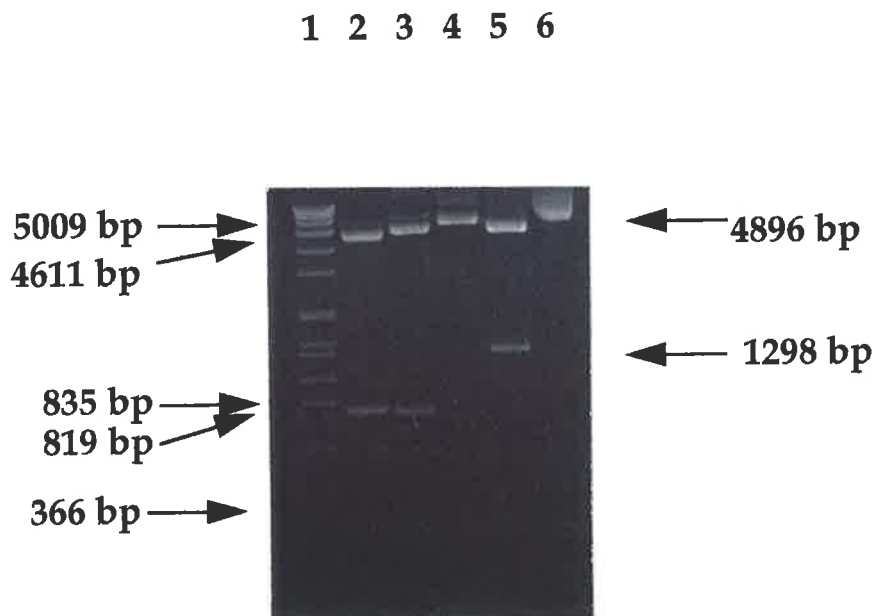
7.3.2 Overexpression of exogenous TI1 mRNA is antiproliferative to CCL64 cells

The mink TI1/CMV plasmid construct and a negative control, pRc/CMV vector were transfected into separate flasks of CCL64 cells. Unfortunately, the CCL64 cells grew very rapidly, despite increasing the concentration of G418 from 400 µg/ml to 1200 µg/ml, so that calculating colony numbers was not possible. The colonies were overlapping or touching each other so individual counts could not be determined. (A decrease in colony number from empty vector to TI1/CMV transfectants would indicate exogenous expression of TI1 was inhibiting growth.) Parental, untransfected CCL64 cells were G418-sensitive at concentration levels of 800 µg/ml and died within two weeks. After approximately two weeks of continual selection with 800 µg/ml G418, DNA was isolated from CCL64 cells transfected with either the TI1/CMV vector or the empty CMV vector. However, no TI1/CMV PCR product could be amplified from DNA isolated from the TI1/CMV cells, suggesting that the TI1/CMV plasmid construct was not present in these cells (Figure 7.4). A PCR product was obtained from the pRc/CMV transfected cells indicating that they did contain the pRc/CMV plasmid after selection and propagation. The pRc/CMV-transfected cells were also overgrown, but the plasmid was retained during selection and propagation.

DNA isolated from seven individual TI1/CMV colonies, examined for the presence of the exogenous TI1 gene by PCR using a CMV primer and a gene-specific primer, TM3, also failed to amplify TI1/CMV products (Figure 7.4). No exogenous TI1 transcript was detected in any of the seven clones, by Northern

Figure 7.4

A.

Diagrammatic representation of the positioning of the primers TM3 and 3' CMV used to detect the presence of the TI1/CMV plasmid in the CCL64 transfections.

B.

PCR analysis, using TM3 and 3'CMV primers, of DNA isolated from seven individual CCL64 clones transfected with the TI1/CMV plasmid and selected for two weeks with G418. The PCR products were run on 1.5% agarose gel. None of the seven clones amplified a TM3-CMV product, indicating that they did not contain the TI1/CMV plasmid.

Lane 1:   SPP1/*Eco*RI marker
Lane 2:   TI1/CMV clone 1
Lane 3:   TI1/CMV clone 2
Lane 4:   TI1/CMV clone 3
Lane 5:   TI1/CMV clone 4
Lane 6:   TI1/CMV clone 5
Lane 7:   TI1/CMV clone 6
Lane 8:   TI1/CMV clone 7
Lane 9:   positive control (TI1/CMV plasmid)
Lane 10:   negative control (no DNA)
Lane 11:   pUC19/*Hpa*II marker

A

B
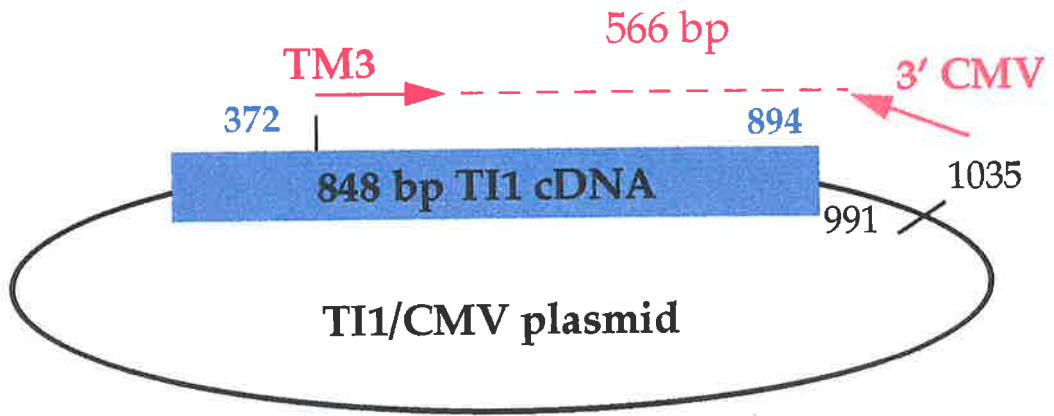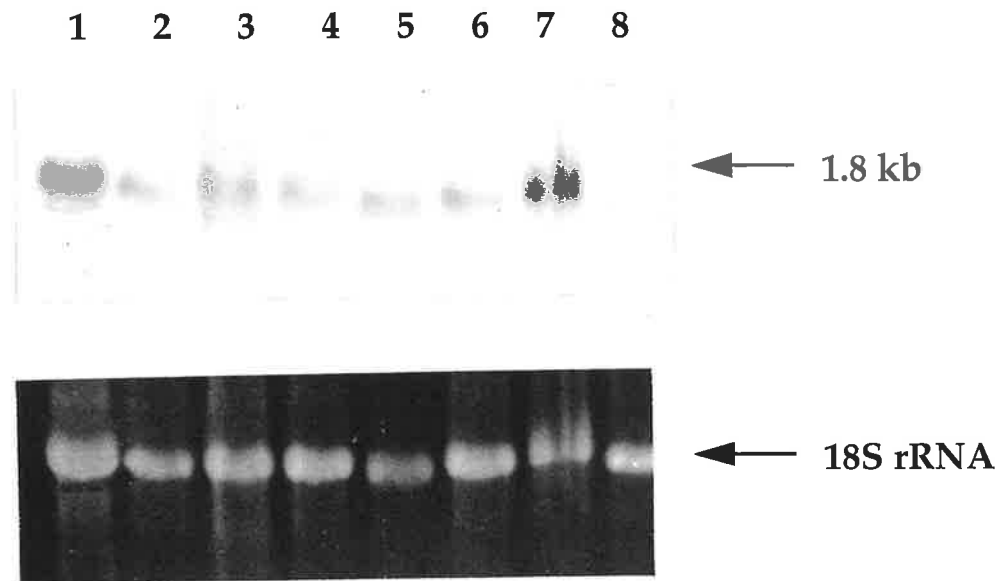
Figure 7.5

RNA was isolated from individual clones of CCL64 cells transfected with TI1/CMV. Northern analysis of these RNAs (upper panel) using TI1 cDNA as a probe detected endogenous expression of TI1 in all RNA samples, however no exogenous TI1 expression was detected. The endogenous TI1 transcript is 1.8 kb in length while any exogenous TI1 expression would be 1.1 kb in length. RNA in lane 1 was isolated from CCL64 cells transfected with the pRc/CMV vector only. RNA in lanes 2-8 was isolated from individual colonies transfected with TI1/CMV plasmid. In the lower panel is the parallel ethidium bromide-staining of the 18S rRNA as a control for loading.

analysis (Figure 7.5). The expected size of the exogenous TI1 transcript is 1.1 kb, compared to the size of the endogenous TI1 mRNA of 1.8 kb.

7.3.3 Expression of exogenous TI1 mRNA is antiproliferative in the 5637 cell line

The TI1/CMV construct was also transfected into the 5637 bladder carcinoma cell line. An empty pRc/CMV vector was transfected into duplicate cultures as a negative control. PCR amplification of the TI1/CMV construct, using CMV primers and also a 5' CMV primer with a gene-specific, ECD primer, from DNA isolated 2 days after transfection, revealed that the TI1/CMV construct was present (Figure 7.6). The 5637 cells grew too quickly to enable manual picking of individual clones during antibiotic selection. Therefore, DNA and RNA were initially isolated from the pooled clones after selection with G418 for two weeks, from either vector control or TI1/CMV transfectants. Expression of exogenous TI1 mRNA (1.1 kb) was detected in two duplicate TI1/CMV flasks by Northern analysis (Figure 7.7). PCR analysis detected the presence of the TI1/CMV plasmid in one of two duplicate flasks of cells (Figure 7.6).

The pooled clones from the TI1/CMV transfectants were then seeded sparsely at approximately 1000 cells per flask and the concentration of G418 increased to 800 µg/ml. A total of twelve individual clones were picked and propagated. Northern analysis of these twelve clones showed no exogenous expression of TI1 (Figure 7.7). Therefore, despite the TI1/CMV plasmid being present two days and also two weeks post-transfection in some of the 5637 cells, no stable TI1-

Figure 7.6

A.

Diagrammatic representation of the positioning of the 5′ CMV-3′CMV and 5′CMV-ECD primer pairs used to detect the presence of the TI1/CMV plasmid after transfection into 5637 cells. The positions of the 5′CMV and 3′CMV primers are based on the numbering of the pRc/CMV plasmid. The 848 bp TI1 cDNA was inserted into the pRc/CMV plasmid between nucleotides 891 and 991, the sites of the *Hind*III and *Apa*I enzymes. The position of the ECD primer is based on the numbering of the mink TI1 cDNA.

B.

DNA was isolated from each duplicate flask of the 5637 transfection for both the TI1/CMV and CMV transfectants after 2 weeks and also from the two day transfection. The 5′CMV and 3′CMV primers were used to amplify the TI1/CMV and CMV constructs. The PCR products were run on a 1.5% agarose gel. The expected size product for TI1/CMV transfected cells was 950 bp and for pRc/CMV was 202 bp.

Lane 1: SPP1/*Eco*RI marker
Lane 2: 5637 CMV (flask 1)
Lane 3: 5637 CMV (flask 2)
Lane 4: 5637 TI1/CMV (flask 1)
Lane 5: 5637 TI1/CMV (flask 2)
Lane 6: 5637 TI1/CMV (Day 2- post transfection)
Lane 7: Positive control (TI1/CMV)
Lane 8: Negative control (no DNA)

C.

DNA was isolated from each duplicate flask of the 5637 transfection for both the TI1/CMV and CMV transfectants after 2 weeks and also from the two day transfection. The 5′CMV and ECD primers were used to amplify the TI1/CMV construct and the PCR products were run on a 2% agarose gel. The expected size product for TI1/CMV transfected cells was 503 bp and no product was expected for the 5637 CMV DNA.

Lane 1: SPP1/*Eco*RI marker
Lane 2: 5637 CMV only (flask 1) -negative control
Lane 3: 5637 CMV only (flask 2) -negative control
Lane 4: 5637 TI1/CMV (flask 1)
Lane 5: 5637 TI1/CMV (flask 2)
Lane 6: 5637 TI1/CMV (Day 2- post transfection)
Lane 7: Positive control (TI1/CMV)
Lane 8: Negative control (no DNA)

A

503 bp

5'CMV   ECD

950 bp

5'CMV   3' CMV

46   492   894

834   891   **848 bp TI1 cDNA**   991   1035

TI1/CMV plasmid
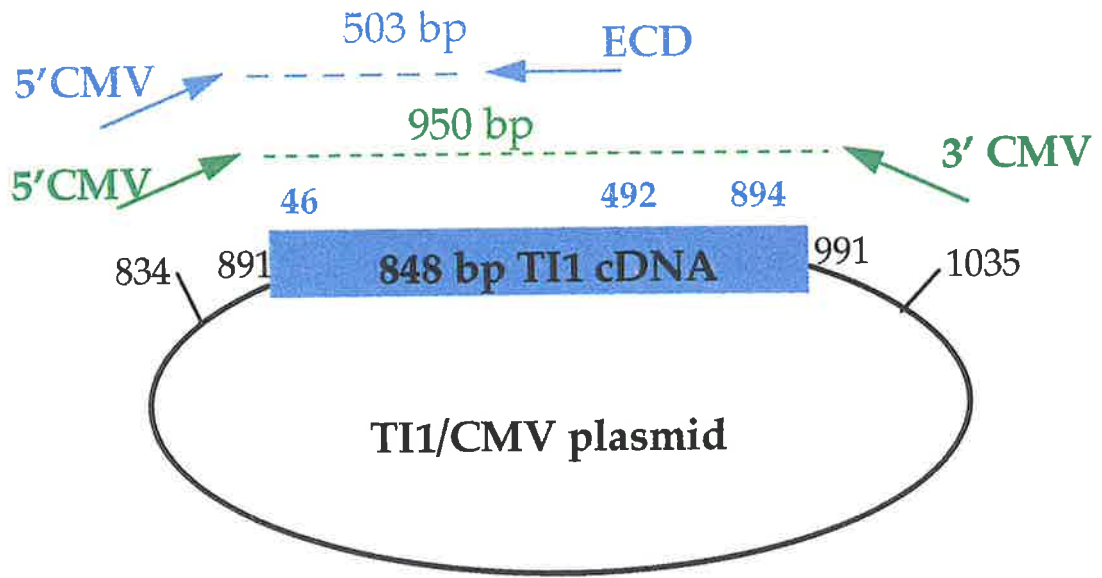
B

1 2 3 4 5 6 7 8

←— 950 bp

C

1 2 3 4 5 6 7 8

←— 503 bp

Figure 7.7
A.
Northern analysis (upper panel) of RNA isolated from 5637 cells transfected with TI1/CMV and hybridised with TI1 cDNA. Exogenous TI1 expression is detected in RNA isolated from duplicate flasks of pooled TI1/CMV transfectants (lanes 3 and 4). Lanes 1 and 2 contain RNA isolated from 5637 cells transfected with CMV only. Lane 5 contains RNA isolated from a transient 2 day transfection with TI1/CMV and a faint band is seen at 1.1.kb. Lane 6 contains RNA isolated from CCL64 cells, known to express endogenous TI1 at 1.8 kb. The exogenous transcript size of TI1 is 1.1 kb. In the lower panel is the parallel ethidium bromide staining of 18S rRNA as a control for RNA loading.

B.
Northern analysis (upper panel) of RNA isolated from individual clones of 5637cells transfected with TI1/CMV and hybridised with TI1 cDNA. No exogenous expression of TI1 mRNA was detected from RNA of any of the 12 clones, in lanes 2-13. Lane 1 contains RNA isolated from CCL64 cells, known to express endogenous TI1 (1.8 kb). The lower panel contains parallel ethidium bromide staining of 18S rRNA as a control for RNA loading.

A

1 2 3 4 5 6

← 1.8 kb

← 1.1 kb

← 18S rRNA

B

1 2 3 4 5 6 7 8 9 10 11 12 13

← 1.8 kb

← 18S rRNA
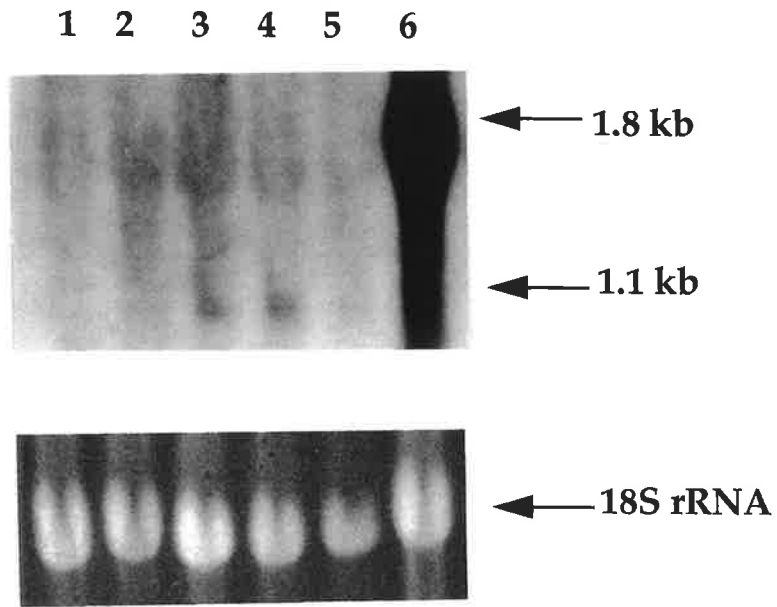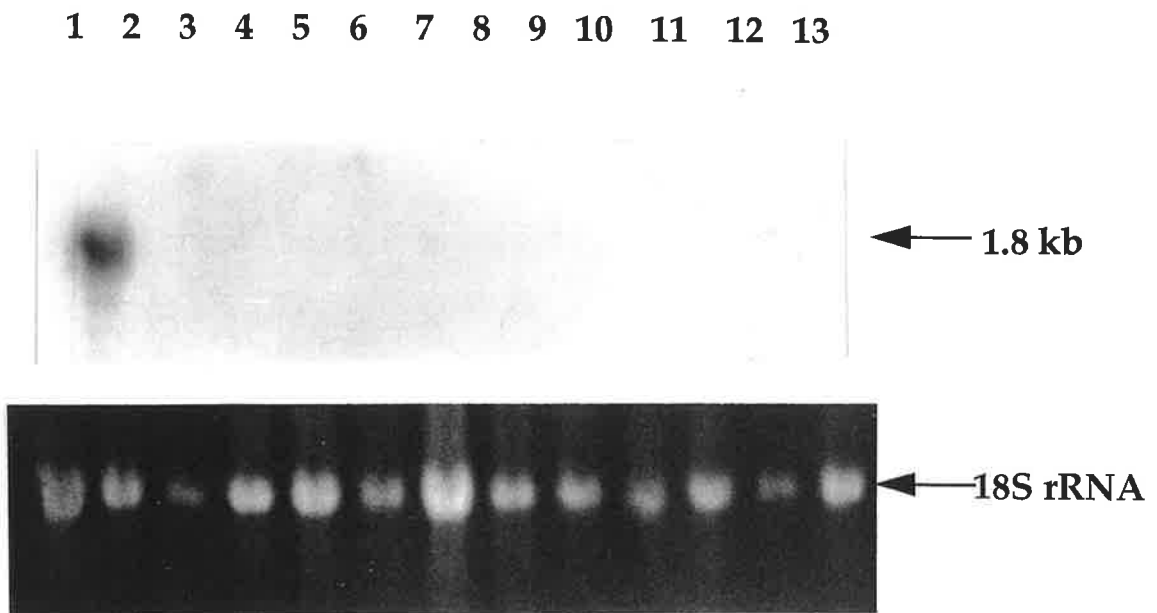
expressing clones could be propagated, suggesting continued expression of TI1 is inhibitory to the growth of 5637 cells.

7.3.4 Expression of exogenous TI1 mRNA is antiproliferative in the T24 cell line

Transfection of the TI1/CMV construct into duplicate flasks of T24 cells resulted in only a small number of colonies, eight and five, after two weeks of selection in G418 (400 µg/ml). In contrast, transfection of the empty pRc/CMV vector in duplicate flasks of T24 cells resulted in colony numbers of 58 and 51 (Table 7.1). This result represents a marked reduction of approximately eight-fold in colony number in those flasks transfected with the TI1/CMV plasmid.

Table 7.1 Number of colonies in duplicate flasks of a single transfection

Vector	Experiment 1	Experiment 2
pRC/CMV	58	51
TI1/CMV	8	5

A transient transfection of two days showed, by PCR, that the TI1/CMV plasmid was present in the T24 cells. After two weeks of selection, DNA was isolated from thirteen TI1/CMV individual clones. PCR analysis, using 5′ and 3′ CMV primers resulted in products of 950 bp for three of the clones. However, when using an internal 5′, TI1 primer, (TM3) and the 3′CMV primer, 566 bp products were amplified from six clones (Figure 7.8). Only three individual clones amplified a PCR product with both sets of primers, suggesting that the total

Figure 7.8

A.

Diagrammatic representation of the positioning of the 5'CMV-3'CMV and TM3'-CMV primer pairs in relation to the TI1/CMV plasmid construct. The positions of the 5'CMV and 3'CMV primers are based on the numbering of the pRc/CMV plasmid. The 848 bp TI1 cDNA was inserted into the pRc/CMV plasmid between nucleotides 891 and 991, the sites of the *Hind*III and *Apa*I enzymes. The position of the TM3 primer at nucleotide 372 is based on the numbering of the mink TI1 cDNA.

B.

PCR analysis using 5'CMV and 3' CMV primers to amplify DNA isolated from the T24 cells transfected with TI1/CMV (Clones 1-13). Lanes 16 and 17 contain PCR products from DNA isolated from T24 cells transfected with CMV only. DNA isolated on day 2 following transfection was positive for the TI1/CMVplasmid. Clones positive for the TI1/CMV plasmid were number 6, 9 and 13.

Lane 1:	SPP1/*Eco*RI marker	Lane 11:	Clone 9
Lane 2:	T24 TI1/CMV Day 2	Lane 12:	Clone 10
Lane 3:	Clone 1	Lane 13:	Clone 11
Lane 4:	Clone 2	Lane 14:	Clone 12
Lane 5:	Clone 3	Lane 15:	Clone 13
Lane 6:	Clone 4	Lane 16:	T24 CMV only (flask 1)
Lane 7:	Clone 5	Lane 17:	T24 CMV only (flask 2)
Lane 8:	Clone 6	Lane 18:	Positive control (TI1/CMV)
Lane 9:	Clone 7	Lane 19:	Negative control (no DNA)
Lane 10:	Clone 8	Lane 20:	SPP1/*Eco*RI marker

C.

PCR analysis using TM3 and 3'CMV primers to amplify DNA isolated from the T24 cells transfected with TI1/CMV. Clones positive for the TI1/CMV plasmid are clones 1, 6, 7 9, 11 and 13. DNA, isolated two days after transfection of TI1/CMV, also amplified the TM3-3'CMV product.

Lane 1:	pUC19/*Hpa*II marker	Lane 10:	Clone 9
Lane 2:	Clone 1	Lane 11:	Clone 10
Lane 3:	Clone 2	Lane 12:	Clone 11
Lane 4:	Clone 3	Lane 13:	Clone 12
Lane 5:	Clone 4	Lane 14:	Clone 13
Lane 6:	Clone 5	Lane 15:	T24 TI1/CMV Day 2
Lane 7:	Clone 6	Lane 16:	Positive control (TI1/CMV)
Lane 8:	Clone 7	Lane 17:	Negative control (no DNA)
Lane 9:	Clone 8	Lane 18:	SPP1/*Eco*RI marker

A

5'CMV

TM3 → 566 bp → 3' CMV

950 bp

5'CMV →

46 |372 894

891

834

848 bp TI1 cDNA

991

1035

3' CMV

TI1/CMV plasmid

B
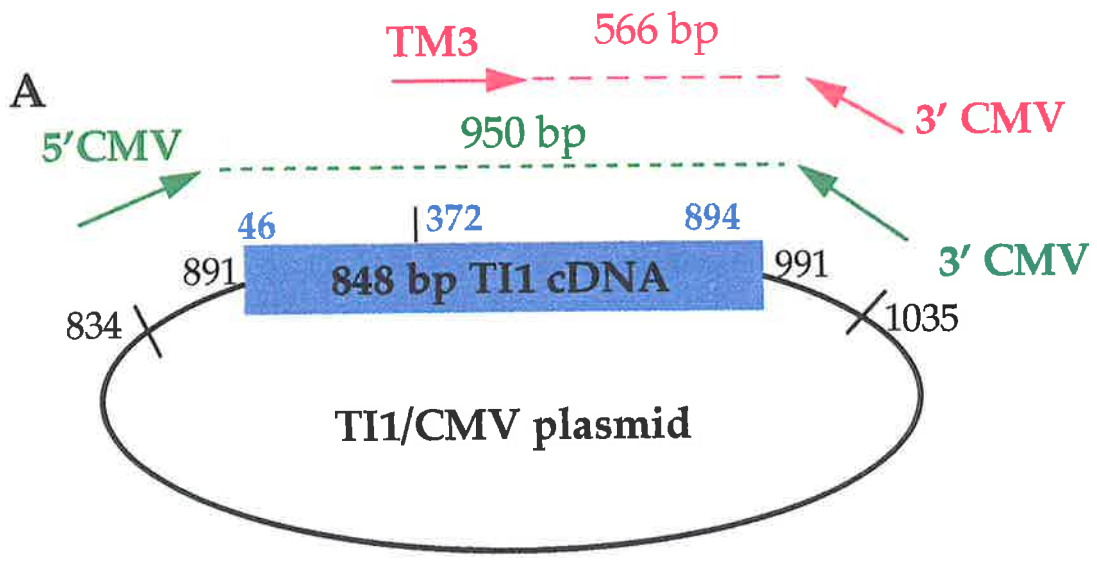
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

←950 bp

C

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

←566 bp

TI1/CMV plasmid was not present or was rearranged in the other three clones. The rearrangement must have removed the 5′ CMV primer site which implicates the loss of at least a region of the CMV promoter, where the 5′ CMV primer anneals.

Total RNA was extracted from each of the thirteen stable TI1/CMV transfectant cell lines after selection. Northern analysis, using the TI1/pBluescript plasmid as a probe, failed to detect expression of the exogenous TI1 mRNA in any of the clones (Figure 7.9). Therefore, although three clones did appear to contain the TI1/CMV plasmid as determined by using both sets of PCR primers, these clones did not express detectable levels of exogenous TI1 mRNA. The cells from the transient transfection of two days also showed no expression, although the cells were shown by PCR to contain the TI1/CMV plasmid.

7.3.5 Transfection of TI1/CMV into the NIH3T3 cell line

In initial experiments, the TI1/CMV construct and the pRc/CMV plasmid were transfected into the mouse fibroblast NIH3T3 cell line. At the time of these experiments, it was not known that the uroplakin Ib gene was the bovine homologue of the mink TI1 gene. Therefore, it seemed reasonable to use the 3T3 cell line to investigate the growth-inhibitory properties of TI1. Northern analysis of a number of resultant stable clones detected two clones which had exogenous expression of TI1 (Figure 7.10). The growth of these clones in comparison to the vector clones was not followed up, because it was subsequently reported that UPK1B is both epithelial- and bladder-specific (Yu et al., 1994.) and would not be expected to be biologically relevant in a cell of fibroblast lineage. However, the

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

← 1.8 kb

← 18S rRNA
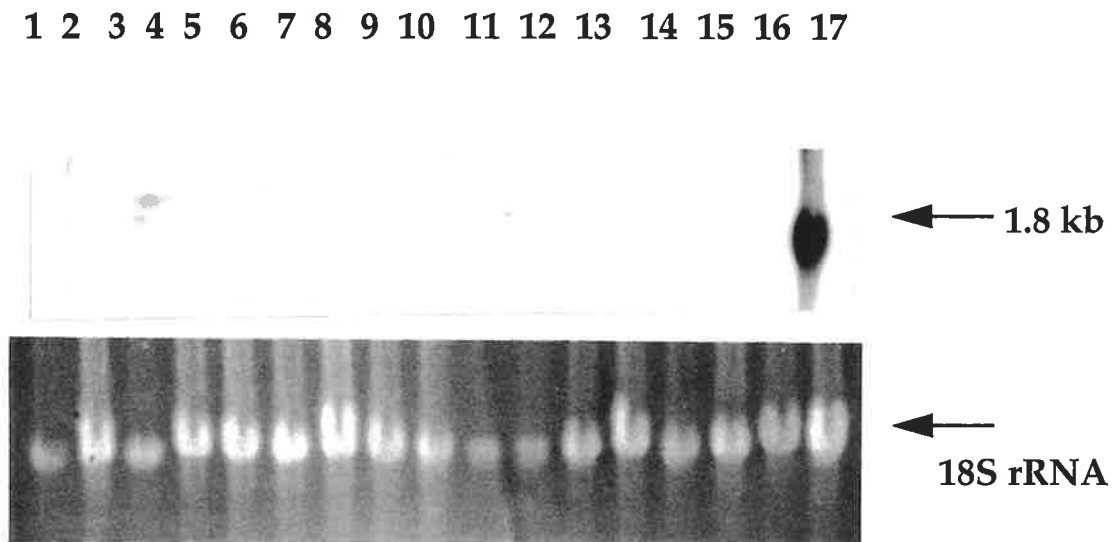
Figure 7.9
Northern analysis (upper panel) of RNA isolated from T24 cells transfected with either TI1/CMV or CMV only, hybridised with the TI1/pBluescript plasmid. Lanes 1-13 contain RNA isolated from 13 individual T24 clones transfected with TI1/CMV. None of the 13 clones express exogenous TI1. Lanes 14-15 contain RNA isolated from T24 cells transfected with CMV only as negative controls. Lane 16 contains RNA isolated from a transient two day transfection with TI/CMV. No exogenous expression was detected for the two day transfection. Lane 17 contains RNA isolated from CCL64 cells, known to express an endogenous 1.8 kb TI1 transcript. In the lower panel, the parallel 18S rRNA was stained with ethidium bromide to control for RNA loading.
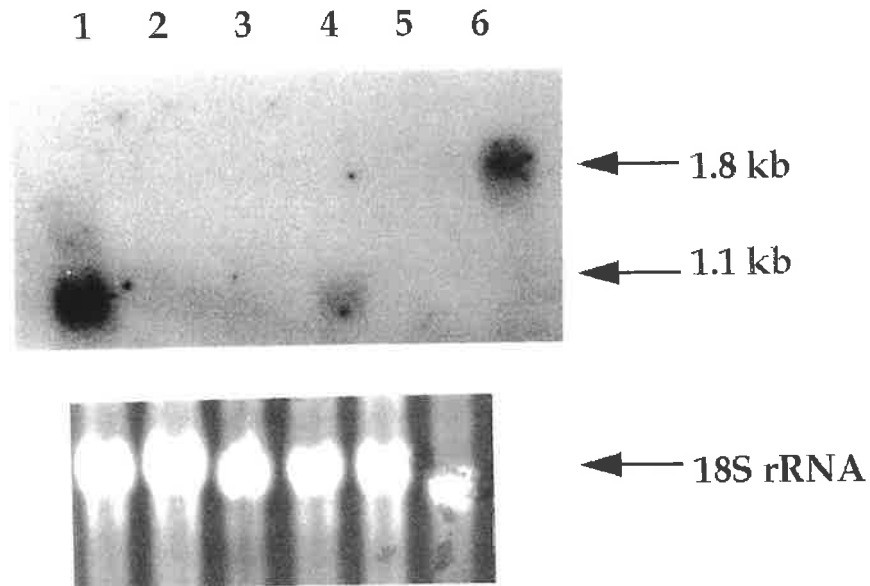
Figure 7.10
RNA was isolated from a number of individual clones of NIH3T3 cells transfected
with TI1/CMV, run on a Northern gel and transferred. The subsequent Northern
blot was hybridised with TI1 cDNA and exogenous TI1 expression was detected in
two clones, 1 and 4. Endogenous expression of TI1 was detected in RNA isolated
from CCL64 cells.

Lane 1: NIH3T3 TI1/CMV clone 1
Lane 2: NIH3T3 TI1/CMV clone 2
Lane 3: NIH3T3 TI1/CMV clone 3
Lane 4: NIH3T3 TI1/CMV clone 4
Lane 5: NIH3T3 TI1/CMV clone 5
Lane 6: CCL64 RNA

importance of these experiments is that TI1 cDNA inserted into the pRc/CMV construct is able to be transcribed and the mRNA is sufficiently stable to be detected by Northern analysis. The antiproliferative effect of exogenous TI1 in epithelial cell lines was not seen in the mouse fibroblasts, suggesting fibroblasts are not sensitive to the inhibitory effects of TI1.

7.3.6 Cloning of human UPK1B ORF cDNA into pTRE

The failure to successfully propagate stable clones expressing constitutive TI1, presumably due to the antiproliferative nature of the TI1 gene, lead to the cloning of the human UPK1B cDNA into an inducible vector, pTRE. The advantage of an inducible expression vector system is that it will allow stable clones to be propagated which then have the ability to express exogenous UPK1B on the addition of the inducer. The tetracycline, TET-ON, inducible vector system was therefore chosen to allow inducible expression of UPK1B.

The human UPK1B cDNA open reading frame was cloned into the pGEM-T plasmid as described in Chapter 3. As no suitable unique restriction enzyme sites were present both in the pTRE plasmid and the UPK1B/pGEM-T plasmid, the strategy used was to digest both plasmids and then use the Klenow enzyme, which adds on nucleotides to create blunt ends, to allow blunt-ended ligation of the UPK1B cDNA into pTRE (Figure 7.11). Initially, the UPK1B cDNA was excised from the pGEM-T vector with the *Sac*II and *Sac*I restriction enzymes and the pTRE plasmid linearised with *Sac*II. The overlapping single-stranded ends of both the vector and the insert were nullified using the Klenow enzyme and the pTRE vector and the UPK1B cDNA insert were then ligated.
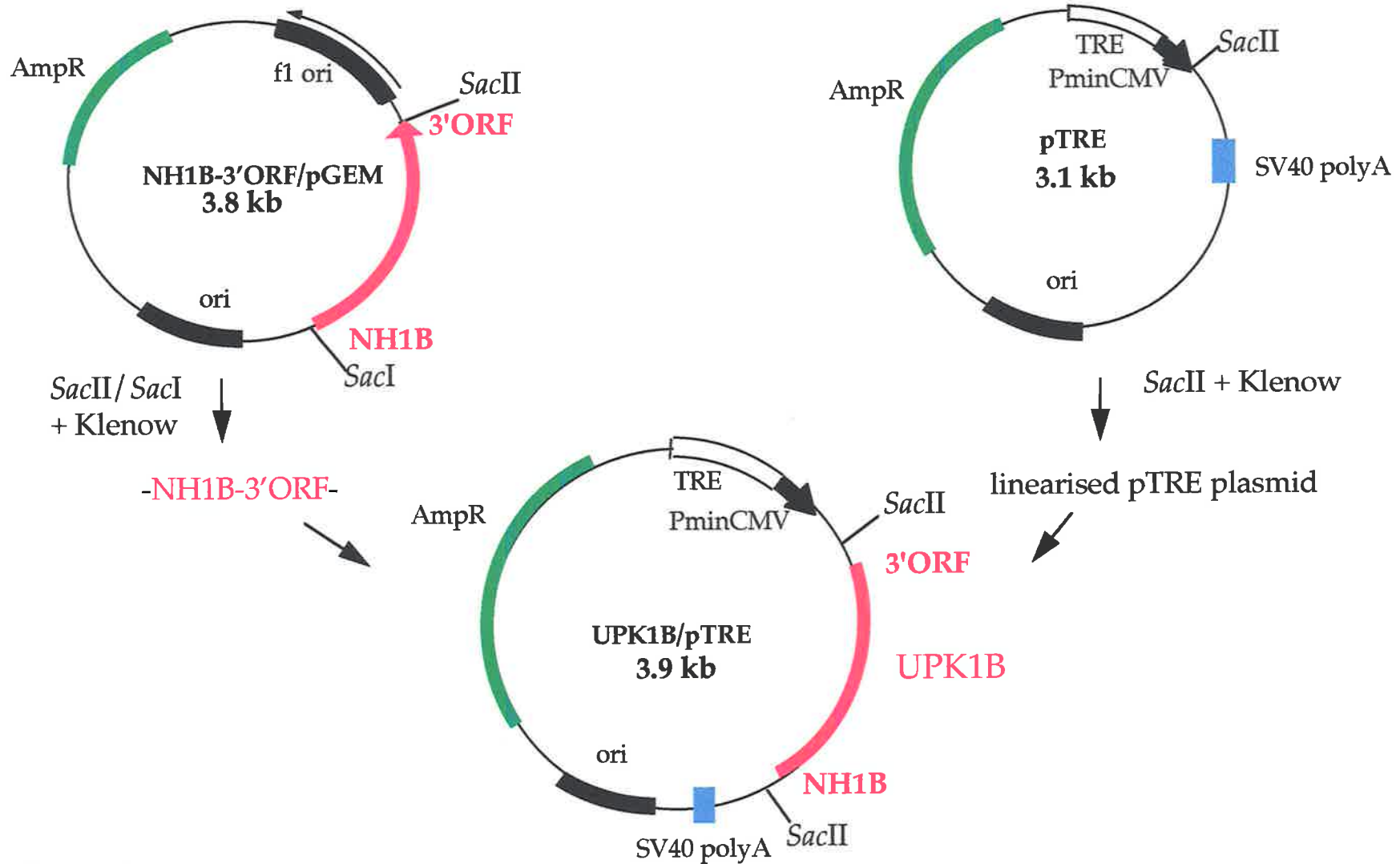
Figure 7.11

Ten µg of NH1B-3'ORF/pGEM plasmid was digested with *Sac*II and *Sac*I to release the NH1B-3'ORF fragment which was then treated with the Klenow enzyme to create blunt ends. The pTRE vector was linearised with *Sac*II and treated with Klenow enzyme to create blunt ends. The NH1B-3'ORF fragment was ligated into the "blunt-end" *Sac*II site of pTRE using T4 ligase.

The integrity and identity of the UPK1B/pTRE plasmid was confirmed by PCR, restriction digests and sequencing. The orientation of the plasmid was determined by using the following PCR primer combinations, 5'TRE/TM3, 5'TRE/ECD2 and TM3/3'TRE (Table 2.1). The combination of the 5'TRE and ECD2 primers produced the expected 640 bp fragment and the TM3/3'TRE primers produced the expected 577 bp fragment, indicating the UPK1B cDNA was cloned in the sense orientation with respect to the promoter of the pTRE plasmid. Similarly, no product was amplified from the UPK1B/pTRE plasmid using the 5'TRE and TM3 primers (Figure 7.12).

To confirm the UPK1B fragment had been cloned in the sense orientation, the UPK1B/pTRE plasmid was digested with *Eco*RI, which linearised the plasmid and with *Kpn*I, which digested the plasmid into four fragments of length, 2943, 571, 365 and 65 bps (Figure 7.13). Subsequent sequence analysis confirmed the identity and orientation of the UPK1B/pTRE plasmid (Appendix IX).

7.3.7 Transfection of pTET-ON and UPK1B/pTRE into the 5637 cell line

The tetracycline-inducible system requires two separate transfections. Firstly, the pTET-ON response plasmid was transfected into the 5637 cells and the transfected cells were selected for by the addition of 400 µg/ml of G418 antibiotic. Clones containing this plasmid were detected by PCR analysis using primers designed to anneal to the pTET-ON plasmid. A pooled, mass culture of clones and also two individual clones contained the pTET-ON plasmid (Figure 7.14). These cells were propagated and used for the second transfection of two co-transfected plasmids, UPK1B/pTRE plasmid (or an empty pTRE control) and pTK-HYG,

Figure 7.12

A.

Diagrammatic representation of the primer combinations used to confirm the presence of the UPK1B insert in pTRE and also to determine the orientation of the UPK1B insert with regard to the pTRE promoter. PCR primers used were vector-specific primers, 5'TRE and 3'TRE in combination with gene-specific primers, TM3 and ECD2.

B.

PCR analysis of the UPK1B/pTRE plasmid using the vector-specific and gene-specific primer combinations. If the UPK1B insert was cloned in the sense orientation, PCR products will be amplified from the 5'TRE-ECD2 and 3'TRE-TM3 primer combinations of sizes 640 bp and 577 bp respectively. No product will be amplifed from UPK1B/pTRE using 5'TRE and TM3 primers, if UPK1B has been cloned in the sense orientation with regard to the TRE promoter. The PCR products were electrophoresed on 1.5% agarose gels.

Lane 1:   pUC19/*Hpa*II marker
Lane 2:   UPK1B/pTRE (primers 5'TRE-ECD2)
Lane 3:   UPK1B/pTRE (primers 3'TRE-TM3)
Lane 4:   UPK1B/pTRE (primers 5'TRE-TM3)
Lane 5:   Negative control (no DNA)
Lane 6:   SPP1/*Eco*RI marker

A

Sense orientation
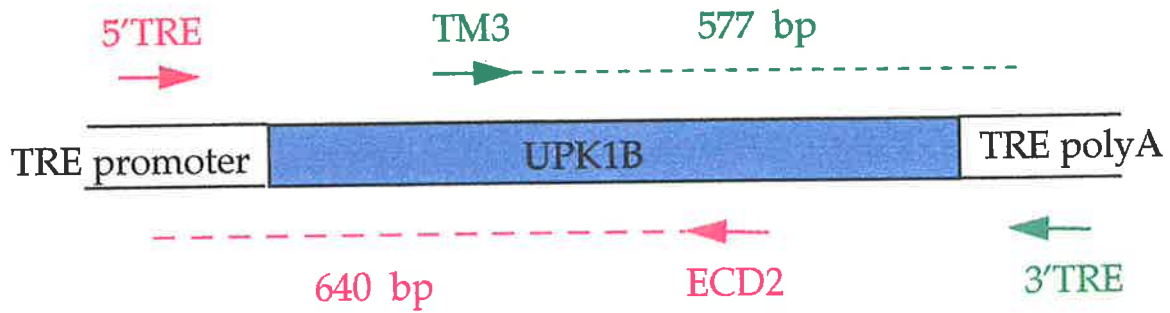
Primers	Expected product size (bp)
5' TRE/ECD2	640
TM3/3'TRE	577
5'TRE/TM3	No product

B
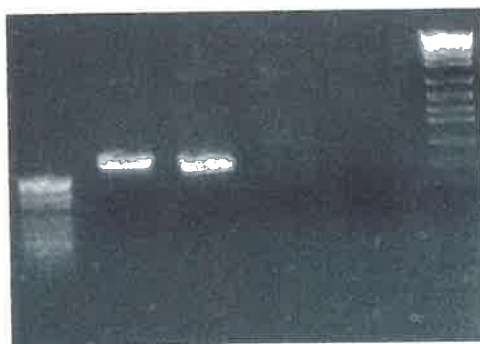
Figure 7.13

A.

Schematic diagram of the UPK1B/pTRE plasmid showing the location of *Eco*RI and *Kpn*I restriction enzyme sites. Digestion of the UPK1B/pTRE plasmid with the *Eco*RI site will linearise the plasmid (total plasmid size is 3944 bp). The *Kpn*I enzyme has two recognition sites in the pTRE plasmid and two in the UPK1B insert. If the UPK1B insert is cloned in the sense orientation, the expected fragment sizes are 2943 bp, 571 bp, 365 bp and 65 bp. If the UPK1B insert is cloned in the antisense orientation, the expected fragment sizes are 2943 bp, 422 bp, 365 bp and 214 bp.

B.

Five μg of UPK1B/pTRE plasmid was digested with either the *Kpn*I or *Eco*RI enzyme for 1 hr at 37°C and the resultant fragments electrophoresed on a 1.5% agarose gel. The fragment sizes confirmed that the UPK1B insert was present and was cloned in the sense orientation with regard to the pTRE promoter.

Lane 1:   SPP1/*Eco*RI marker
Lane 2:   UPK1B/pTRE   -*Eco*RI
Lane 3:   UPK1B/pTRE   -*Kpn*I
Lane 4:   pUC19/*Hpa*II marker

A

B
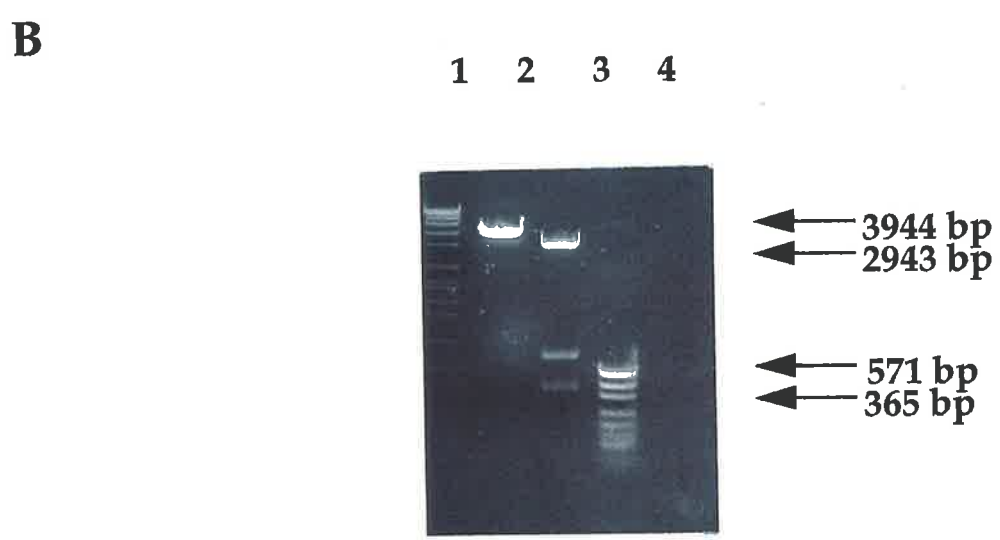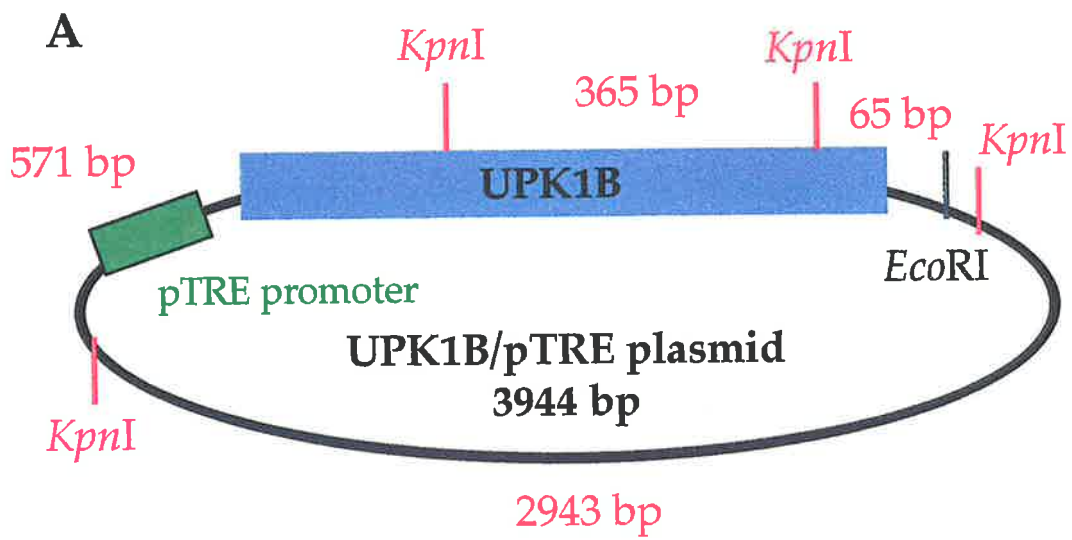
Figure 7.14

A.
Diagram of the positioning of the pTET-ON primers, located in the reverse tetracycline-responsive Transcriptional Activator (rtTA). The rtTA binds to the tet-responsive element (TRE) after transfection of the pTRE plasmid, activating transcription of the cloned gene in the presence of tetracycline.

B.
PCR analysis to detect the presence of the pTET-ON plasmid in DNA isolated fromindividual and pooled clones of 5637 cells transfected with the pTET-ON plasmid. The 5'TET-ON and 3'TET-ON primers amplify a 248 bp PCR product, run on a 2% agarose gel.

Lane 1:   pUC19/*Hpa*II
Lane 2:   5637 pTET-ON pooled clones
Lane 3:   Clone 1
Lane 4:   Clone 2
Lane 5:   Positive control (pTET-ON plasmid)
Lane 6:   Negative control (no DNA)

C.
PCR analysis to detect the presence of the pTET-ON plasmid in individual clones of 5637 cells transfected with the pTET-ON plasmid.

Lane 1:   SPP1/*Eco*RI marker
Lane 2:   Clone 3
Lane 4:   Clone 4
Lane 5:   Clone 5
Lane 6:   Clone 6
Lane 7:   Positive control (pTET-ON plasmid)
Lane 8:   Negative control

A

5'TET-ON

248 bp

3'TET-ON

NeoR

pCMV

rtTA

pTET-ON

SV40 polyA

AmpR
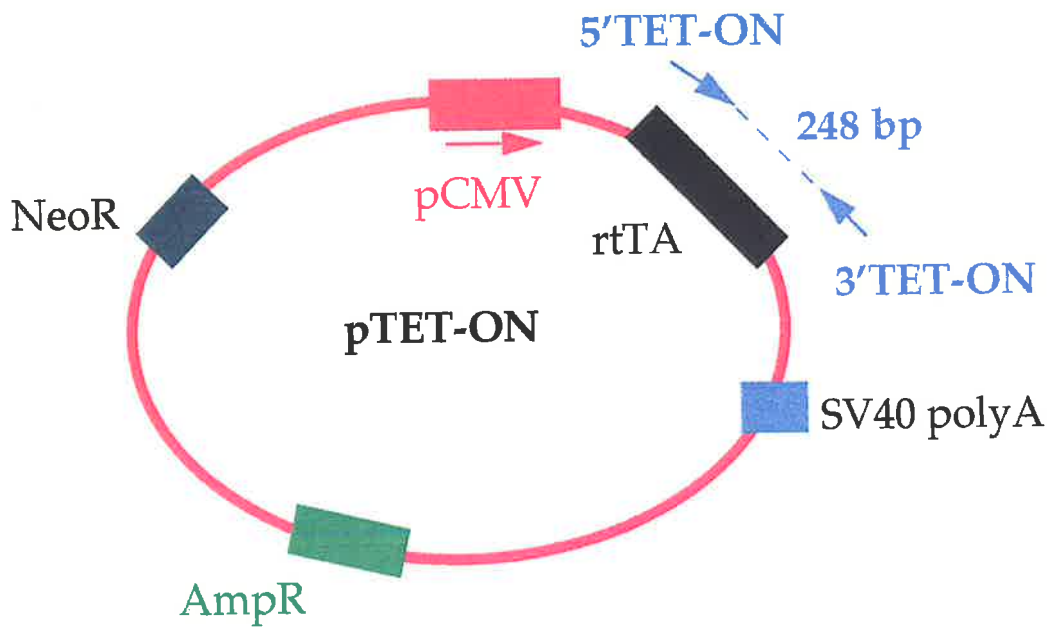
B

1 2 3 4 5 6

← 248 bp

C

1 2 3 4 5 6 7 8

← 248 bp

which contains a hygromycin-resistance gene. The transfected cells were grown in the presence of both G418 and hygromycin to ensure the continued selection for both of the transfected cells. Unfortunately, despite a number of attempts, no colonies were formed for either the cells transfected with UPK1B/pTRE or the negative control, pTRE, so UPK1B-inducible clones could not be analysed.

7.4   DISCUSSION

In Chapter 5, it was shown that expression of the human UPK1B gene was markedly reduced or lost in a high percentage of bladder tumours. Similarly, none of the bladder cancer cell lines analysed had any detectable expression of UPK1B by Northern analysis. The next questions to be answered are: Does this loss of expression implicate abnormalities in expression of UPK1B in the pathogenesis of bladder cancer? If the expression of UPK1B is reintroduced into these UPK1B-negative bladder cancer cell lines, can the transformed phenotype be reverted? Does the expression of UPK1B initiate or maintain growth arrest as suggested by the experiments in the CCL64 cell line by Kallin et al., (1991)? Could UPK1B have tumour-suppressive or metastasis-suppressive activity, as suggested by the frequent loss of expression of UPK1B in bladder cancer and by analogy with other tetraspan genes?

To begin to answer these questions, the TI1 cDNA was cloned into a pRc/CMV vector and transfected into the CCL64 cell line. The same construct was also transfected into the UPK1B-negative bladder cancer cell lines, T24 and 5637. The pRC/CMV vector was chosen because the cytomegalovirus promoter provides

strong constitutive expression of cloned genes. At the time of these experiments the total open reading frame of the human UPK1B had not been cloned, however the high degree of sequence homology between mink TI1 and bovine uroplakin Ib cDNA suggested mink TI1 would be biologically active in human cells.

The failure to propagate any transfectant clones expressing exogenous TI1 mRNA after selection in G418, in either the CCL64, 5637 or the T24 cell lines, suggests the cells that are not constitutively expressing TI1 mRNA have a growth advantage. Overexpression of the TI1 gene in the CCL64 cells may induce these cells to enter growth arrest or block entry into the cell cycle so they do not form colonies. Unlike the two bladder cancer cell lines, the CCL64 cells do have a basal level of endogenous expression of TI1. However, the continual expression of TI1 in all phases of the cell cycle, as opposed to the normal transient manner of TI1 expression during growth arrest, may disrupt the normal cell cycling, pushing cells into a permanent state of growth arrest. This would explain the absence of clones which express exogenous TI1 and why only G418-resistant colonies grew in culture and propagated. The cells must either retain the neomycin resistance gene of the pRc/CMV plasmid or spontaneously mutate to develop neomycin resistance to enable them to grow in the presence of the G418 antibiotic. The latter explanation is unlikely as flasks of control untransfected cells did die in the presence of G418.

Early transfections of TI1/CMV into the mouse fibroblast cell line, NIH3T3, resulted in the detectable expression of exogenous TI1 mRNA transcripts by Northern analysis. These experiments exclude the possibility of a problem with

either the construction of the TI1/CMV plasmid or the capability of the cytomegalovirus promoter to induce expression of TI1 mRNA, at least in fibroblast cells. Detection of expression of exogenous TI1 by Northern analysis in 5637 cells confirmed that the TI1/CMV plasmid was also able to transcribe exogenous TI1 mRNA transcripts in epithelial cells.

Transfection of the TI1/CMV plasmid into 5637 cells failed to produce colonies which stably expressed exogenous TI1 mRNA. Although transient transfections showed the presence of the TI1/CMV plasmid in the 5637 cells by PCR and the presence of mRNA expression by Northern analysis, after selection in G418 none of the clones either contained the plasmid construct or expressed any exogenous TI1. These results suggest that expression of exogenous TI1 is incompatible with the continued proliferation of the 5637 bladder carcinoma cells.

Expression of exogenous TI1 in the T24 bladder cancer cell line resulted in an eight-to-ten-fold decrease in the number of colonies propagated in comparison with those arising from cells transfected with the empty CMV vector. Therefore expression of TI1 in these cells appears to be growth-inhibitory. Northern analysis of the colonies that did grow when transfected with exogenous TI1 under the control of the constitutive CMV promoter revealed that none of them expressed TI1 mRNA. PCR analysis using the CMV primers showed that three of the TI1/CMV clones did contain the TI1/CMV plasmid. However, use of the gene-specific TM3 primer and the 3'CMV primer, yielded a PCR product from six clones, three of which also amplified a product from the CMV primers. The other three clones could possibly contain rearranged CMV sequences, preventing

the annealing of the 5'CMV primer to the CMV promoter. The three clones that did amplify the expected size products from both sets of primers did not show any exogenous TI1 expression by Northern analysis, suggesting that although they did contain a structurally intact transfected TI1 cDNA, the gene was still not able to be expressed. This apparent discrepancy may be explained by a low efficiency of transfection, so the expression is not able to be detected. Another explanation for the lack of detectable exogenous TI1 transcript may be that the CMV promoter or the polyA tail were deleted or rearranged which would either prevent the expression of TI1 or reduce its stability respectively. Spontaneous mutations of the TI1 cDNA or of a region of the CMV promoter or polyA region may account for the clone's ability to survive despite the presence of the TI1/CMV plasmid at the DNA level. Analysis of the integrity of the TI1/CMV plasmid of these clones by restriction digests and Southern analysis using regions of the TI1/CMV as probes may confirm these possibilities.

Differences in transfection efficiency between different flasks of cells was controlled for in these experiments by using duplicate flasks for both the control plasmid and TI1/CMV plasmid. To control for a possible difference in transfection efficiency of different plasmids, perhaps a scrambled TI1 cDNA cloned into the pRc/CMV vector could be used in future experiments. However, the same conclusions, that TI1 is antiproliferative, were reached from all experiments in three different cell lines, suggesting that the results were not due to differences in transfection efficiency. In agreement with the argument that differences in transfection efficiency of plasmids was not significant in these experiments, are the NIH3T3 experiments which involved the transfection of the

pRc/CMV and TI1/CMV plasmids. In this experiment, TI1/CMV-containing clones were isolated after selection which did express exogenous TI1. Therefore, both the pRc/CMV plasmid and the TI1/CMV plasmid were transfected to degrees of efficiency which allowed both to be propagated as stable clones, although colony numbers were not compared.

There are examples in the literature of other genes which fail to propagate colonies which express the gene of interest, when exogenously expressed in cells via the cytomegalovirus constitutive promoter. For example, exogenous expression of the p16 gene, directed by the cytomegalovirus promoter, inhibited the growth and propagation of the clones in culture unless the exogenous gene had been rearranged. The study found that there was a 10- to 20-fold decrease in the number of colonies produced from the cells which had been transfected with the exogenous p16 cDNA in comparison to those cells which had been transfected with the control plasmid. The authors conclude from these results that p16 inhibits the growth of bladder cancer cells when it is overexpressed, that is, p16 is inducing growth arrest in these cells (Wu et al., 1996a).

Another study also investigated the function of the p16 gene, using the cytomegalovirus promoter to drive exogenous expression of p16. A human esophageal carcinoma cell line (HCE-4) was transfected with p16 plasmid constructs and the colony-forming efficiency was reduced by 68% for p16 cloned in the sense orientation in comparison to antisense p16 cDNA plasmids. The ovarian cancer cell line, SK-OV-3 was also transfected with the p16 cDNA plasmid construct and isolated clones were expanded. Exogenous p16 mRNA

was transiently expressed at 48 hours after transfection but in the expanded cell population, no expression was detected. The expanded clones containing sense p16, also showed rearrangement of the gene (Okamoto et al., 1994). These results suggest that constitutive expression of exogenous p16 inhibits cell growth and that rearrangement of p16, leading to inactivation of the transfected gene, confers a selective growth advantage to the cell.

A recent study investigating the GAS1 gene used the pRc/CMV vector to provide constitutive expression of GAS1 in the GAS1-negative A549 lung adenocarcinoma cell line. Although expression of exogenous GAS1 was detected by Northern analysis 72 hr after transfection, expression was absent in individual clones after G418 selection for two weeks. There was also a 3 - 5 fold decrease in the number of colonies produced from cells transfected with exogenous GAS1 cDNA in comparison to those cells transfected with the control plasmid. The authors suggest the continued expression of GAS1 mRNA was antiproliferative to the A549 cells (Evdokiou et al., 1998).

As detailed in this chapter, colonies expressing exogenous TI1 mRNA could not be propagated in any of the cell lines tested, suggesting TI1 has an antiproliferative effect. From the results of the transfection of the constitutive TI1/CMV into the CCL64, 5637 and T24 cell lines, it became evident that in order to analyse stable clones expressing UPK1B, an inducible system was required. To verify the constitutive TI1 expression results and to study the potential growth-suppressive and tumour-suppressive functions of the UPK1B gene, it was necessary to be able to propagate and isolate stable clones. An inducible

expression vector system using tetracycline to induce gene expression in the pTRE plasmid, was then assessed as a possible method to allow analysis of TI1-expressing clones. The tet-inducible system (Clontech) was chosen, as the tetracycline antibiotic was claimed to have no effect on the growth rate of human cells. However, a recent study has suggested that tetracyclines inhibit tumour cell invasion and metastasis in melanoma cells through inhibiting matrix metalloproteinase (MMP) activity (Seftor et al., 1998). Other inducible expression systems, for example, dexamethasone, will inhibit the growth rate of cells, in particular epithelial cells. A preliminary experiment to study the effect of dexamethasone on CCL64 cells, showed an 80% suppression of growth in comparison with cells growing without the addition of dexamethasone (data not shown). Therefore, the dexamethasone-inducible system, pMAMneo, was not suitable for use in the epithelial cell lines.

The tetracycline system requires two separate transfections. Stable transfectants are selected which contain the first plasmid, pTET-ON and a second transfection is performed co-transfecting both the UPK1B/pTRE plasmid and the pTK-HYG plasmid, containing the hygromycin resistance gene. The reverse tetracycline-responsive Transcriptional Activator (rtTA) of the pTET-ON plasmid binds to the tet-responsive element (TRE) of the pTRE plasmid, activating transcription of the cloned gene (UPK1B) in the presence of tetracycline. The initial transfection of pTET-ON in the 5637 cell line was successful as the presence of the pTET-ON gene was detected by PCR, but the inducibility of the pTET-ON plasmid was not confirmed with the luciferase system. The second, co-transfection, of UPK1B/pTRE and pTK-HYG was not successful, as none of the cells grew in

hygromycin, although the hygromycin resistance gene was contained within the pTK-HYG plasmid. Unfortunately, due to time constraints these experiments could not be completed.

In summary, the results of the transfection studies using constitutive TI1 expression in CCL64, 5647 and T24 cell lines, suggest an antiproliferative role for the UPK1B gene product. To study the tumour- or metastasis-suppressive function of UPK1B, an inducible system of UPK1B expression needs to be developed to allow analysis of UPK1B-expressing clones. Nude mice studies could then be initiated to look for a suppression in tumour growth or metastases in those mice injected with UPK1B-expressing bladder cancer cells. The number of metastatic foci formed in other organs could be monitored to assess metastasis-suppressing properties of UPK1B. It may be hypothesised from the preliminary results described in this chapter that expression of UPK1B would reduce the growth rate of transplanted bladder tumours and possibly the formation of metastases in nude mice.

CHAPTER 8

GENERAL DISCUSSION

8.1 DISCUSSION

8.1.1 Introduction

There are genes preferentially expressed during G0 that are essential for the induction and maintenance of growth arrest. The preferential expression of these genes during G0 was demonstrated by the micro-injection of messenger RNA from growth-arrested cells into growing cells which induced the growing cells to undergo growth arrest (Pepperkok et al., 1988). The mink TI1 gene was originally isolated from the CCL64 epithelial cell line as a novel gene with preferential expression during growth arrest mediated by transforming growth factor β (Kallin et al., 1991). Levels of expression of TI1 mRNA in CCL64 cells were increased by serum-starvation and down-regulated upon serum stimulation, indicating a possible role for TI1 in the induction of growth arrest or its maintenance. In 1994, the bovine uroplakin Ib protein, specifically expressed as a differentiation product of bladder urothelium, was discovered to be the bovine homologue of the putative mink TI1 protein (Yu et al., 1994). Bovine uroplakin Ib is one of four uroplakin proteins expressed as differentiation products of the asymmetrical unit membrane (AUM) of the bovine bladder, believed to be important in strengthening the urothelial apical surface and maintaining the structure of normal bladder. TI1/UPKIb may signal for the growth arrest required for terminal differentiation. Differentiation and cellular proliferation are believed to be mutually exclusive events. Withdrawal from the cell cycle appears to be a prerequisite for differentiation and may be an early event in terminal differentiation (Philipson et al., 1991, Olson, 1992).

The TI1/UPKIb protein belongs to a family of proteins named "transmembrane 4 superfamily", "tetraspans" or more recently "tetraspanins" (reviewed in Maecker et al., 1997). All protein members of this tetraspan group are distinguished by the presence of four hydrophobic membrane-spanning domains, two extracellular domains and two short cytoplasmic domains. These transmembrane glycoproteins are found expressed in a range of mammalian species and also in *Drosophila*, *C.elegans* and *Schistosoma*. Many of the tetraspans have been implicated in growth regulation, signal transduction and cell activation in a wide range of cells. Some of the tetraspans may have a role in the pathogenesis of cancer as many tetraspans have altered patterns of expression in cancer and appear to act as tumour or metastasis suppressors.

8.1.2 Cloning and characterisation of the human uroplakin 1B gene

Prior to this study, the human homologue of the bovine uroplakin Ib and mink TI1 gene had not been cloned. The only insights available into the putative human homologue were inferred from studies of the bovine uroplakin Ib and mink TI1 genes. The possible role of the uroplakin 1B gene in cancer could only be ascertained by analogy with the roles of the other members of the tetraspan family of proteins. To discover any details regarding the human homologue of the bovine UPKIb and mink TI1 genes, human-specific uroplakin 1B cDNA and genomic sequences had to be cloned. Therefore, the first aim of this thesis was to clone the human uroplakin 1B gene. Assuming the high degree of sequence homology between mink TI1 and bovine UPKIb cDNA sequences would also be conserved in the human homologue, PCR primers were designed from mink TI1 and bovine UPKIb cDNA sequences. Using a PCR-cloning technique, partial

cDNA and genomic human UPK1B sequences were amplified, cloned, sequenced and analysed as described in Chapter 3. The open reading frame of the human uroplakin 1B gene was 783 bp in length and coded for a putative 260 amino acid protein. The amino acid homology between the human uroplakin 1B protein and the bovine UPKIb and mink TI1 proteins were 92% and 93% respectively. The putative human uroplakin 1B protein retained many of the conserved protein motifs observed in other tetraspan proteins, including four cysteine residues in the large extracellular domain. The cloning of the UPK1B cDNA open reading frame provided novel information regarding the structure of the human uroplakin 1B gene and its putative protein, as well as confirming the existence of the human homologue. A total of 2.5 kb of human uroplakin 1B genomic sequence was cloned using PCR and inverse-PCR methods. From Southern analysis using human uroplakin 1B cDNA probes, the UPK1B gene is estimated to be at least 17 kb in length. Further characterisation of the human uroplakin 1B gene led to the discovery of an intragenic *Taq*I restriction fragment length polymorphism.

One of the main hypotheses of this thesis was that the human UPK1B gene may be involved in the pathogenesis of bladder cancer. The chromosomal location of the human UPK1B gene was determined in order to discover if UPK1B was located in a region known to be deleted in bladder cancer, for example, chromosome 9q. Radioactive *in situ* hybridisation of two contiguous genomic UPK1B probes revealed that the human UPK1B gene was located on chromosome 3q13.3-21 (Chapter 4). This region of the genome is not frequently deleted in bladder tumours and therefore is not thought to harbour a tumour

suppressor gene for bladder cancer. However, only three independent studies have concentrated on chromosome 3q as a potential region of deletion in bladder tumours and none of them have closely examined the bands where UPK1B is located (Atkin et al., 1985, Knowles et al., 1994 and Li et al., 1996).

In situ hybridisation of the human UPK1B cDNA ORF probe on human chromosomes detected a number of sub-peaks in addition to the expected 3q13.3-21 peak. The extra sub-peaks are not likely to represent processed pseudogenes of the human UPK1B gene because the sub-peaks are not as strong as for other previously discovered pseudogenes. To date, there have been no reports of the existence of pseudogenes of other human tetraspan genes. However, two distinct loci in the mouse genome were discovered by the genetic localisation of the mouse CD63 gene. One of the *Cd63* loci mapped to chromosome 10 and the other to chromosome 18. The authors designated the loci on chromosome 10 as *Cd63* because of its synteny to human chromosome 12 where human CD63 had been mapped (Hotta et al., 1988). The other *Cd63* loci was designated Cd63-rs1 (Cd63-related sequence 1).

A partial mouse genomic Upk1b fragment was cloned using the TM3 and ECD primers which are almost 100% homologous in sequence between human UPK1B, mink TI1 and bovine UPKIb cDNA sequences. The mouse Upk1b probe was used to map the mouse Upk1b gene to mouse Chromosome 16B5-C2. This chromosome region maintains synteny with both human chromosome 3q and bovine chromosome 1. Therefore, the location of the uroplakin 1B gene has been conserved with neighbouring genes in all of these three species.

8.1.3 UPK1B and bladder cancer

Antibodies raised against individual bovine uroplakins have detected uroplakin protein expression in the asymmetric unit membrane of the bladder of many mammalian species including cattle, human, monkey, sheep, pig, dog, rabbit, mouse and rat (Wu et al., 1994). Antibodies to bovine uroplakin III detected expression of uroplakin III in the human bladder and in no other human tissue (Moll et al., 1995). Expression of the human uroplakin 1B and mouse Upk1b mRNAs were described in Chapter 5. Expression of human UPK1B mRNA was readily detected in normal urothelium by Northern analysis using a human UPK1B-specific cDNA probe. In mouse tissue, Upk1b expression was detected in the bladder by Northern analysis using either human UPK1B or mink TI1 cDNA probes, but also in the liver by RT-PCR.

The majority of patients, (70-80%), with transitional cell carcinoma present with non-invasive, superficial bladder cancer. Despite tumour removal by transurethral resection, two-thirds of these patients have tumour recurrence within five years and 20-30% of these cases will progress to muscle invasive disease (Fleshner et al., 1996). Approximately 30% of all patients with transitional cell carcinoma will develop metastases. Half of these patients will present with metastatic bladder disease at diagnosis while the other half will relapse after local treatment for invasive disease (Lamm et al., 1996). The current treatment of those patients with clinically-localised invasive bladder cancer is radical surgery. The 5 year survival rate or overall cure of invasive bladder cancer is approximately 50%, with patients dying from metastatic disease. It is therefore presumed that micrometastases undetectable at the time of initial cystectomy are

responsible for these therapeutic failures. Development of prognostic indicators based on tumour characteristics would allow early implementation of other therapies, with the hope of improving both disease free survival and cure rates for all bladder cancers.

The expression levels of UPK1B mRNA in normal urothelium were compared with transitional cell carcinomas and bladder cancer cell lines as reported in Chapter 5. Five bladder cancer cell lines analysed had no detectable UPK1B expression by Northern analysis. Eleven out of sixteen bladder cancers had either reduced or absent expression of UPK1B mRNA. This high percentage (68%) of tumours demonstrating a reduction or loss of expression of UPK1B suggests that loss of expression of UPK1B may be involved in the development of bladder cancer or its progression. The sample size of the bladder tumours analysed precludes correlations between levels of expression of uroplakin 1B and degree of invasion and tumour grade, however initial comparisons do suggest that those tumours without uroplakin 1B expression are often also invasive in nature. In summary, this is the first indication that the human uroplakin 1B gene has a role in the pathogenesis of bladder cancer. These results are in agreement with loss of expression of other uroplakins in transitional cell carcinoma. Loss of expression of uroplakin III protein was observed in 47% of invasive TCCs (Moll et al., 1995) and 60% of TCCs had lost expression of uroplakin II (Wu et al., 1998). Similarly, Ogawa et al., (1996) showed that, in chemically-induced carcinoma in the rat bladder, expression of uroplakins was absent from the usual location on the luminal surface and only a small amount of focal disorganised staining was observed in the intermediate cells. From these three studies of uroplakins and

the results of human uroplakin 1B, it is possible that loss of expression of uroplakins occurs as a later event during bladder carcinogenesis and may be related to the progressive loss of differentiation of advanced tumours. The uroplakins are terminal differentiation products of the bladder, so it is reasonable to propose that, as a tumour becomes less differentiated the uroplakins may be markers of this loss of differentiation. The uroplakins may be useful as prognostic markers of advanced bladder cancer and metastasis. A large prospective study needs to be done investigating patients with bladder tumours, according to their clinical outcome and expression levels of uroplakins to determine the value of uroplakins as prognostic markers.

Genes that are important in cancer progression often develop molecular alterations that result in loss of expression of the gene. Two approaches were undertaken to assess the integrity of the UPK1B gene in bladder cancer (Chapter 6). Southern analysis using UPK1B genomic and cDNA probes revealed identical banding patterns between DNA isolated from normal controls and bladder cancer patients. Three different restriction enzymes and two different UPK1B probes failed to detect altered banding patterns in five bladder cancer cell lines. Therefore, it is unlikely that gross gene rearrangements, deletions or amplifications are responsible for the frequently observed down-regulation of UPK1B mRNA expression in bladder cancer. To investigate allelic loss, two highly polymorphic microsatellite markers were chosen which were located on either side of the UPK1B gene on chromosome 3q13.3-21. The dinucleotide repeats were amplified from normal DNA and matched bladder tumour DNA and run on polyacrylamide gels to separate the alleles. No allelic loss was

detected in any of the fifteen matched pairs of normal and bladder tumour DNAs. In summary, no genetic alteration of UPK1B was detected as a cause of the down-regulation of UPK1B expression. Other molecular mechanisms which could result in loss or reduction of expression include transcriptional silencing by methylation or promoter sequence abnormalities. Loss of expression of UPK1B mRNA may also result from a lack of mRNA stability or the down-regulation of an upstream gene or transcription factor required by UPK1B for its expression. Point mutations, not examined in the current study, or deletions of UPK1B are not likely to prevent transcription of a gene unless they affect the binding of transcription factors in the promoter. However, it may be more beneficial to concentrate on looking for point mutations or deletions in those bladder tumours which do express UPK1B mRNA, as a sequence anomaly may result in a mutant or truncated UPK1B protein.

The inability so far to find a molecular mechanism to explain the loss of expression of human UPK1B mRNA in bladder cancer does not necessarily lessen the importance of UPK1B in the pathogenesis of bladder cancer. A recent paper by Sager (1997) argues that the emphasis of cancer geneticists should not only be on mutated cancer genes with tumour suppressor potential but also non-mutated genes whose expression is lost during cancer progression. Classical tumour suppressor genes do not account for all known cancer phenotypes and so far none are known to be involved in the important cancer processes of metastasis and invasion. Tumour suppressor genes could be grouped into two classes: class I genes, which are mutated and class II genes, which affect the phenotype by loss of expression. The author suggests that non-mutated genes with altered expression

(class II) are important in solving the intricacies of the cancer network and may provide targets for pharmacological intervention to induce expression of these genes. The reinduction of expression of these class II genes would cause inhibition of cell growth and induce terminal differentiation, therefore removing the cells from the cancer-promoting environment. The human uroplakin 1B gene whose expression is lost in bladder cancer but does not appear to be genetically altered, may belong to the class II type of tumour suppressor gene.

In Chapter 7, experiments were described involving the transfection of the mink TI1 cDNA ORF into the mink CCL64 cell line and the human bladder cancer cell lines, T24 and 5637. At the time of the experiments, the human UPK1B cDNA ORF was not available, but because of the high sequence identity between mink TI1 and human UPK1B it was assumed that the mink TI1 ORF would be functional in the human cells. The mink TI1 cDNA ORF was cloned into the pRC/CMV constitutive expression vector and transfected into the CCL64, T24 and 5637 cell lines. Stable TI1-expressing colonies could not be propagated in any of the cell lines indicating that those cells without exogenous TI1 expression had a selective growth advantage. Therefore, the addition of TI1 was inhibitory to the growth of the CCL64, 5637 and T24 cells, suggesting that the UPK1B/TI1 gene product has antiproliferative activity and can act as a growth suppressor.

The bovine UPKIb protein is specifically associated with uroplakin III but not uroplakins Ia or II (Wu et al., 1995). The UPKIb/UPKIII complex may reflect an important protein:protein interaction in the asymmetric unit membrane. UPKIb,

like all tetraspans, lacks a significant cytoplasmic domain as the largest hydrophilic loop of UPKIb, between the third and fourth transmembrane domains, extends into the extracellular domain (Yu et al., 1994). The putative human UPK1B protein contains a N-glycosylation site on its largest hydrophilic loop, suggesting its largest domain is also extracellular in location. An important consideration for elucidating the biological functions of the tetraspans is to determine how they signal to the nucleus with short cytoplasmic domains, lacking signalling motifs. The uroplakin III protein consists of a single transmembrane domain with a 189 amino acid extracellular domain and a cytoplasmic domain of 52 amino acids (Wu et al., 1993). The interaction of UPKIb with UPKIII, with the latter having a significant cytoplasmic domain, may be important for the functioning of UPKIb.

Many studies have reported the interactions of tetraspan proteins with other tetraspans as well as in association with integrins (reviewed in Hemler et al., 1996). The complexes of tetraspans and integrins may be important in the understanding of the mechanisms of tetraspan activity. Tetraspans may mediate their role in cellular activation, cell growth and cell motility through activation or regulation of integrin signalling. As is the case with other tetraspans, the human uroplakin 1B gene may form complexes, not only with human uroplakin III, but also with integrins. Integrins are involved in cell-cell adherence and motility and are thought to be involved in the metastatic process of tumour cells. Studies of integrin expression in bladder cancer showed that a disorder of expression of two integrins $\alpha2$ and $\beta4$, occurred in invasive bladder cancers (Mialhe et al., 1997). Over-expression of the $\alpha6\beta4$ integrin was detected in

invasive bladder cancer in conjunction with the loss of co-localisation of α6β4 with collagen VII (Liebert et al., 1994). The authors suggest that overexpression of α6β4 without collagen VII may allow cells to migrate. The role of human uroplakin 1B may be to maintain the structure of the bladder through interactions with uroplakin III and perhaps also with integrins in a multimolecular complex. The absence of expression of uroplakin 1B in a high proportion of bladder cancers (Chapter 5) may allow the integrin to play a role in the metastatic and invasive processes of bladder tumour cells by promoting cell motility. Alternatively, as in the case with α2 and β4 integrins, loss of expression of the integrin may be important in the progression of the tumour and the loss of differentiation, leading to subsequent loss of expression of uroplakin 1B. The significance of uroplakin 1B interactions with uroplakin III and potentially with integrins, will be important in determining the function of human uroplakin 1B and its role in signalling pathways.

The frequent loss of expression of UPK1B in bladder cancer may be a consequence of the loss of differentiation associated with tumour progression, therefore, UPK1B may be useful as a prognostic marker. Alternatively, loss of UPK1B may remove a growth-inhibitory control from the urothelial cell which results in the further development of the bladder tumour, loss of differentiation and ultimately metastatic formation. Expression of the UPK1B protein may normally trigger a signalling pathway which induces the withdrawal from the cell cycle required for the onset of terminal differentiation of the urothelium. Loss of expression of

UPK1B in bladder cancer may be one of the events leading to tumour or metastatic formation.

8.2 FUTURE DIRECTIONS

Further experiments which would extend the findings of this thesis, include the cloning of the total human UPK1B gene which could be achieved by screening a human genomic lambda or cosmid library with the partial human UPK1B genomic probes. Knowledge of the total human UPK1B genomic sequence would be useful in determining the genomic structure of UPK1B in regard to exon/intron boundaries. Exon/intron sequence data would allow the design of primers to amplify each exon for SSCP analysis, therefore providing an approach to detect point mutations in bladder cancer. Total UPK1B sequence data would enable primers to be designed to amplify the *Taq*I RFLP, therefore small biopsy samples of bladder cancer could be analysed to detect allelic loss of UPK1B. The presence of any CpG islands could be determined by the cloning of the UPK1B promoter region, also allowing detection of any methylation changes of the UPK1B promoter in bladder cancer, which could lead to loss of expression of UPK1B.

The findings reported in this thesis indicate that UPK1B has a role in the pathogenesis of bladder cancer. Further study is required to determine if there is a correlation between loss of expression of UPK1B and the tumour grade and stage. The preparation of antibodies raised against human UPK1B could be used to study archival bladder tissues and the expression status of UPK1B could be linked to bladder tumour progression. It would be of interest to analyse non-

malignant bladder tumours to determine if they express UPK1B mRNA. The use of an antibody directed against human UPK1B in Western blotting would determine if there is a correlation between levels of expression of UPK1B mRNA and protein in bladder cancer tissues.

The constitutive expression of TI1 in UPK1B-negative bladder cancer cell lines suppressed the growth of the cells, therefore no colonies expressing TI1 could be propagated. An inducible expression vector system could be employed to allow stable UPK1B-expressing colonies to be propagated and analysed by growth curves and invasion assays. A human UPK1B antibody could detect UPK1B protein in transfectant cell lines by Western blot analysis. To assess if UPK1B has tumour-suppressive activity, the UPK1B-expressing bladder cancer cells could be injected into nude mice and the growth of their tumours compared to nude mice injected with bladder cancer cells transfected with vector alone. The formation of metastatic foci in other organs would be monitored to assess the metastasis-suppressive activity of UPK1B.

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ERRATA

p29: To be included after the first paragraph

"The authors explain their contradictory results by suggesting that high migratory potential in primary tumours with CD9 expression may enhance local tumour cell dissemination whereas reduction of CD9 expression may be required for metastasis formation. The study by Cajot et al., (1997) uses only two sets of human colon cancer cell lines, derived from matched primary and metastatic cell lines to assess CD9 expression and migration potential of colon cancer cells. The authors admit that for one set of cell lines there is only a slight difference in metastatic potential between the primary tumour cell line and its matched metatastatic cell line. Another consideration for the discrepancy in results is that the *in vitro* system used to assess the role of CD9 on cell migration is not truly reflective of the cellular environment found in the the original tumours. However, the studies by Miyake et al. (1996) and Higashiyama et al. (1995) investigate human tumours. Further studies are required to determine the role of CD9 in tumour cell migration and metastasis formation.

p29: Replace line 12 of paragraph 2 with the following sentence:

"Later, it was discovered that CD82 provides a co-stimulatory signal in the CD3/T cell receptor pathway leading to strong IL-2 production and T-cell differentiation (Lebel-Binay et al., 1995)."

p32: OX-44 was initially discovered by Paterson et al., (1987), Journal of Experimental Medicine 165:1-13 and not Bell et al., (1992).

p33: Add after second paragraph

"The CD81-deficient mice results do not rule out a possible function for CD81 in T cell development. There are likely to be many factors involved in T cell development, for example, the pre-TCR complex, which also affects T cells at a similar stage of development to CD81 (Boismenu et al. 1996). Loss of CD81 may be compensated for by other molecules to allow normal T cell development. In an isolated cell culture system, CD81-transfected cells allowed development of immature thymocytes (Boismenu et al. 1996) but in an *in vivo* system, other molecules may also be able to provide the trigger for T cell development.

p34: Add after second paragraph.

"The membership of L6 in the tetraspan family has been disputed (Wright et al., 1994, Maecker et al., 1997). Although L6 has four hydrophobic domains, common to all tetraspan members, it does lack the conserved cysteine motifs (CCG) observed in the extracellular domain of other tetraspans. The L6 protein also lacks the charged amino acids conserved in many tetraspans, including asparagine (N) residue in transmembrane domain 1 and the glutamate (E) or glutamine (Q) residues in transmembrane domain 3 and 4. These

observations suggest that L6 is perhaps a distant relative of the tetraspan family.

p36: Paragraph 2, line 12

Delete the following sentence : "A single CCG triplet is conserved in almost all tetraspan proteins in the large extracellular domain of each tetraspan protein"

p39: Add after second paragraph

"The IL-TMP protein may be only a distant relative of the tetraspan family because IL-TMP lacks some of the conserved motifs of the tetraspan proteins. Although the IL-TMP protein shares between 20-50% amino acid homology with other tetraspan family members, (Wice et al., 1995), it does not have the highly conserved CCG motif in its extracellular domain or some of the highly conserved charged residues."

p172: Add after first paragraph

"There may be other explanations for the failure of propagation of clones expressing exogenous UPK1B, besides UPK1B having an antiproliferative effect on the cell lines. It is possible that the CMV promoter of the pRC/CMV vector is not active in the bladder cancer cell lines because it is not known if they express transcription factors required by the promoter. However, a previous study has shown expression of retinoblastoma in 5637 cells under the direction of a

CMV promoter, indicating that the promoter is active in at least the 5637 bladder cancer cell line (Zhou et al., 1994). Expression of exogenous UPK1B would have to be detected at least transiently in the CCL64 and the bladder cancer cell lines to prove that UPK1B has an antiproliferative effect. It would also be necessary to confirm that overexpression of exogenous UPK1B mRNA is suppressing cell growth by inducing the growth arrest phase of the cell cycle and not causing a non-specific toxic effect. Induction of growth arrest could be determined by measuring DNA synthesis by incorporation of 5′-bromo-deoxyuridine. From the experimental results in Chapter 7, it is premature to strongly suggest that UPK1B expression has an anti-proliferative effect on human bladder cancer cell lines. Additional experiments are required to prove this hypothesis correct and analysis of stable UPK1B-expressing clones could be accomplished by using the inducible expression vector system."

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APPENDICES

Appendix I (a)
Raw sequencing data of the NH1B-ECD2/pGEM plasmid, primed with the 5′ pGEM primer. The presence of the ECD2 primer sequence (underlined) indicates that the NH1B-ECD2 insert was cloned in the reverse orientation with respect to the 5′ pGEM primer.

NCGATNTATACGATCCTATAAGGCGATTGGGCCGACGTCGCATGCTCCCGGCCGCCATGGCCGCGGGATTTGCCAGTCTGACGGGCCATTTACGCCACAGCAATTGTCCTGGAGCATGAGCCTGTCCCAGGTT
10 20 30 40 50 60 70 80 90 100 110 120 130

TTGGTGACTCCATTGTTTTTCCACTGGTCATCATTGTTTGGAGGGCTGTTGTTTTGGTACCTCTCTANCATCTGCTTCAGGAAGAGGTTGGGTGTGAAAAAGTCTCGTTGTGTTGCTGCTGTGA
140 150 160 170 180 190 200 210 220 230 240 250

ATACAAGATGCCACTTCAAAGGCATATACTATAAACATCAGAATGAAATACGCCAGAAGAATTTTCCTGCTGGACTTCATGATGCCTACAATGCCTANAACAGACAGGCAGAAC
260 270 280 290 300 310 320 330 340 350 360 37

GAAGCAGATGCCCACAAATATGCCGATCCAGGCAGCCCCATANATGTCATCGTTGTCNGTGGCTTCAAACAATGGGTTAAAGCTGTGTTGGTCCATNCCA
70 380 390 400 410 420 430 440 450 460

AGAAAATGCACTCCGCANTCAGGGCAATGCCGGCAACAACCAATAATCACATTTCCAAAAATCAGCAAGGCCTGGAAACCACNAACAATTGAATTGTCTTTCNCCA
470 480 490 500 510 520 530 540 550 560 570

Appendix I (b)
Raw sequencing data of the NH1B-ECD2/pGEM plasmid, primed with the 3′ pGEM primer. The presence of the NH1B primer sequence (underlined) indicates that the NH1B-ECD2 insert was cloned in the reverse orientation with respect to the 5′ pGEM primer.
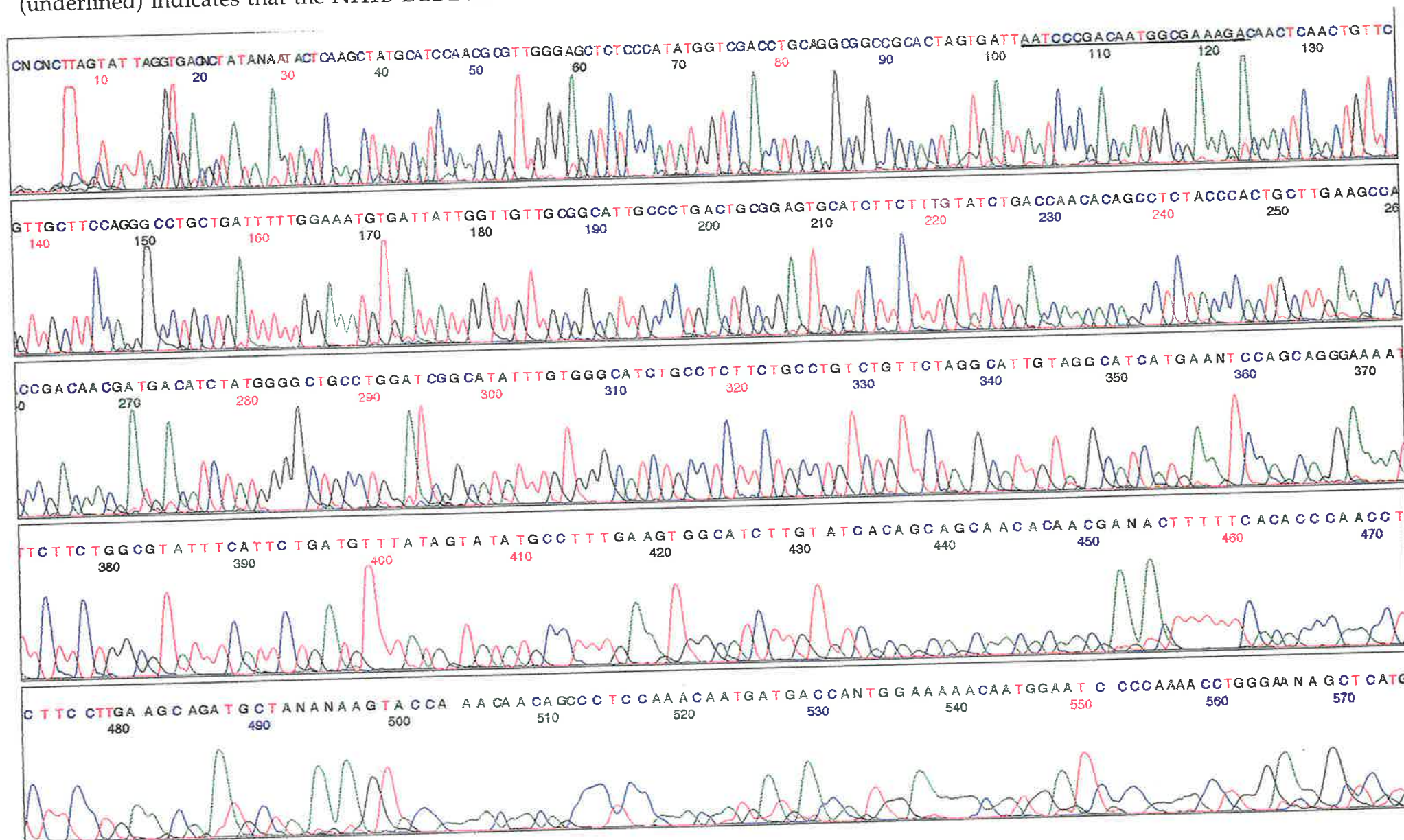
Appendix I (c)

Raw sequencing data of the TM3-3'ORF/pGEM plasmid, primed with the 5' pGEM primer. The presence of the 3' ORF primer sequence (underlined), indicates that the TM3-3'ORF insert was cloned in the reverse orientation with respect to the 5' pGEM primer.
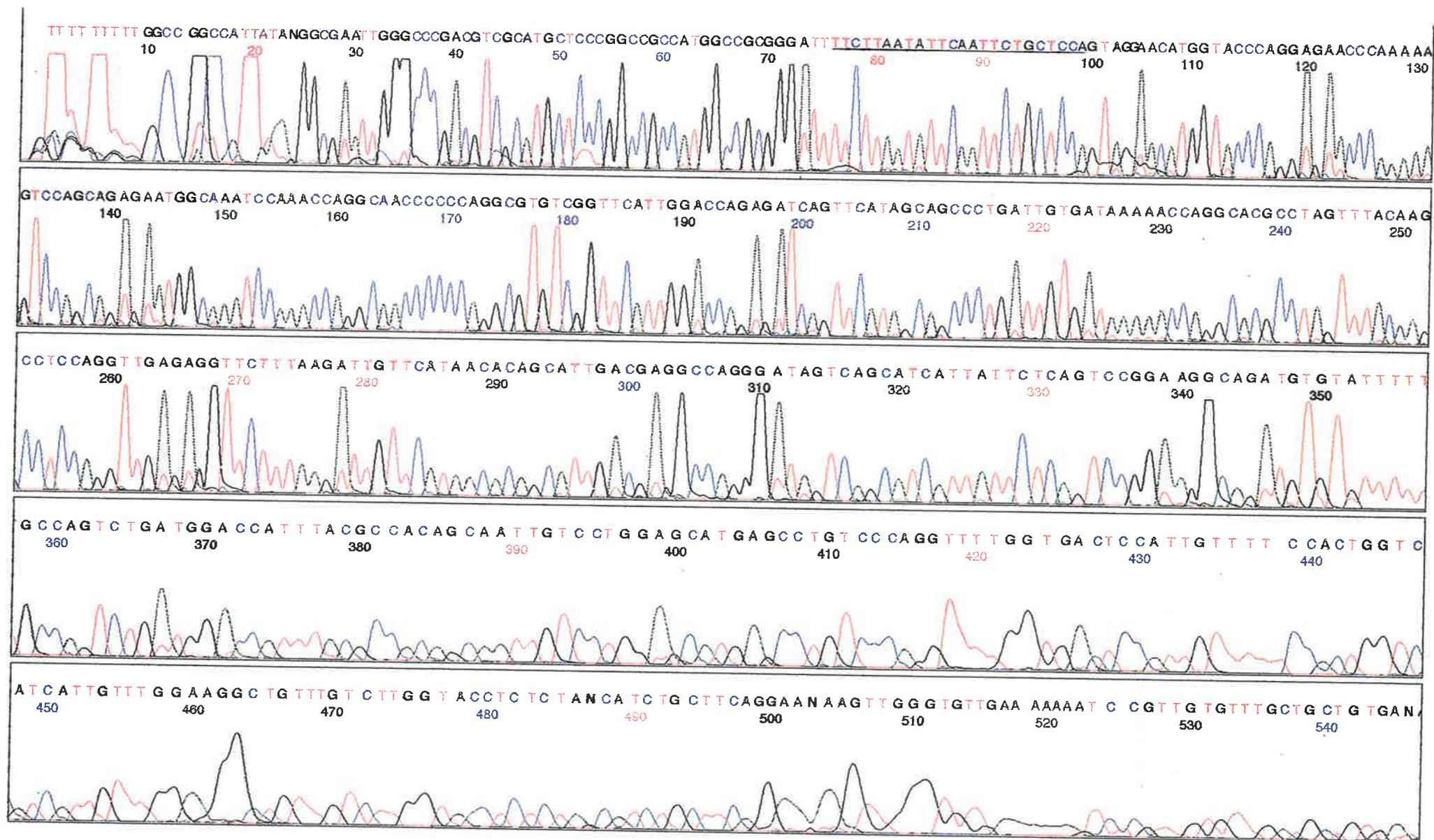
Appendix I (d)

Raw sequencing data of the TM3-3'ORF/pGEM plasmid, primed with the 3' pGEM primer. The presence of the TM3 primer sequence (underlined), indicates that the TM3-3'ORF insert was cloned in the reverse orientation with respect to the 5' pGEM primer.

Appendix II (a)

Raw sequencing data of the NH1B-3'ORF/pGEM plasmid, primed with the 5' pGEM primer.

GNNNNNNNNNNNNNGA TTGTA T ACGA TCA TATAGGGCGA TTGGGCCCGACGTCGCATGCTCCCGGCCGCCA TGGCCGCGGGG ATTTTCTTAAT ATTCAATTCTGCTCCAGT AGAACATGGT ACCCAGGAGA ACC
10   20   30   40   50   60   70   80   90   100   110   120   130

CAAAAAGTCCAGCAGAGAATGGCAAATCCAAACCAGGCAACCCCCCAGGCGTGTCGGTTCATTGGACCAGAGAT CAGTTCATAGCAGCCCTGATTGTGATAAAAACCAGGCACGCCTAGTTT
140   150   160   170   180   190   200   210   220   230   240   250

ACAAGCCTCCAGGTTGAGAGGTTCTTTAAGATTGTTCATAACACAGCATTGACGAAGCCAGGGATAGTCAGCATCATTATTCTCAGTCCGGAANGCAGA TGTTTTTTTTTG
260   270   280   290   300   310   320   330   340   350   360

CCAGTCTTGATGGACCATTTACGCCCACCAGCAATTGTCCTTGGANCATTGACCTTGTCCCANGTTTTTGGGTGA TCCATTGTTTTTCCACTGGTCATCATTGTTTNG
370   380   390   400   410   420   430   440   450   460   470

AAGGCTGTTTGTTTTTGGTACTCTCTTACATCTGCTTCNGGAAAAGGTTTGGTTTTTNAAAAATTCNCTTTGNNNTTTGCTGCCGTTGAATACNAAATGCCCTTTCCNAAGGC
480   490   500   510   520   530   540   550   560   570   580

Appendix II (b)

Raw sequencing data of the NH1B-3'ORF/pGEM plasmid, primed with the 3' pGEM primer.

AG TCATTTANGT GACACT AT AG A AT ACT CAAGCT ATG CAT CCA A CG CG T TG GG AGCT CT CCCA TA TGG T CGA CCT G CAGG CG GCCGCACT AGT GATT AAT CCCGA CA ATGG CG AAAG ACA ACT CAA CTG T TCG T
10   20   30   40   50   60   70   80   90   100   110   120   130

TG CT TCCAGGG CCTGCTGA TTTTTTGGAAATGTGATTAT TGG T TGT TG CGG CAT TGCCC TGA CTG CGG AGTG CAT CT TCT TTG TAT CTGA CCAA CAC AGC CTCT ACC CACTG CT TGA AGC CACCG
140   150   160   170   180   190   200   210   220   230   240   250

ACAA CGA TGA CAT CT AT GGGG CT GCCT GGA T CGG CAT ATT TGT GGG CAT CT GCCTCT TCT GCCT GT CT GT TCT ANG CATTGT AGG CAT C AT GA AGT C CAGC AGGA AAAT TC TT
260   270   280   290   300   310   320   330   340   350   360   370

C T G GCG TTAT TTC ATTC TGA T GT TTT TAT A GT TAT AT GC CT T TGAAA TTTGG CAT CT T GT T T CC CCA GCCAGC A ACA CA AC G AAA CT T TTT CCACA CC C AA CTC T TC CT GA
380   390   400   410   420   430   440   450   460   470   480

ACCAGAAT GCT A AAAA A GTTACCCAA A ACA ACA N CC T CCC AAA CAATG AA TGAA CATTTGG AAA A ACA TGG A TT C CCCCAAA A CCT GGG AAAA GCT CCAT GCT CC C AAG AAAANTG TGT GGGT
490   500   510   520   530   540   550   560   570   580   590   600

Appendix III (a)
Raw sequencing data of the 784 bp genomic UPK1B/pGEM plasmid, primed with the 5' pGEM primer.

GGTTNGTA T ACGACT CACT AT AGGGCGA ATT GGGCCCGACGT CGCA TGCT CCCGGCCGCCA TGG CCGCGGG ATT TCCA TTGG TCATCA TTG TTT GGAGGG CTG T TG TTT TGG TACCTC TCT AGCA
10 20 30 40 50 60 70 80 90 100 110 120

TCTG CTT CAGGA AG AGG TTGGG TG TGAACT AT AGT AGG AAG AGG CA ACACA AG AGGG AGAGA TAG CACG CTAA TTTT CAAGG AG AGG AAAAT AT TTT TC TC TG TC TTT TCCT GGGG AC
130 140 150 160 170 180 190 200 210 220 230 240

ACTGA ACA TTCTCTCCT GG AGCA AAACT GTA CT CAG AGG CACCAAGT ACAG CTGG CTGAGG T TGG CA TAAGT CCCCA TG CAGAGA AG AGACTCTCA TCT TAAAAG
250 260 270 280 290 300 310 320 330 340

A AAT CAC TA T GAGGC CAGG G GAAACA CA TAC TG T AGGGT GGC T TAGGC TA NGA AAA CAGA GA ATGG GAGAGA AAGA AAGA TGT TAGA AT TGAGA GCAA
350 360 370 380 390 400 410 420 430 440

T CT TANCT GAAGT ANA ACAGAAAT CCT CCC A TTG GAAA GGC CA TGGCCA TGG CAGA TTGG TGAANCCC CAGGG GATT ACCAACTTGGGCCCTGGGA TTCA G
450 460 470 480 490 500 510 520 530 540

Appendix III (b)

Raw sequencing data of the 784 bp genomic UPK1B/pGEM plasmid, primed with the 3' pGEM primer.

NNNGG GG TTCAAGNNATTT ACGT GACACT AT AGA AT ACT CA AGCT ATG CA TCCAA CG CG T TG GG AGCT CT CCCA TA TGGT CG ACCT G CAGG CG GCCGCA CT AGT GA TTG AAG TGGCA TCT TG TA

10   20   30   40   50   60   70   80   90   100   110   120

TCACAG CAG CA ACACAA CG AG ACTTTG TGAGT ACAACCT CAAA AAG CAAA AT AAT ATGAATT ATCAT TAT TACAACAA CCAACA TG TATTAACCCCT TACT ATG TG CCCAGG CAA TC

130   140   150   160   170   180   190   200   210   220   230   240

CAA TA TG CACAG TAA TAA TA CAT C TAT CA TTT TACT TAAT CCT CA TA AAT C TATG AAAT AGG CAT TA TCACT CT GGT CATTT GAG AAAGG AAAAA AAAT GAGA

250   260   270   280   290   300   310   320   330   340

CAG GGAAGG T AAAAT GA CT TGT C TA AAGT GA CAT AGT TGG CAACT GA AAGA GCA AGA ATTC T GA ACCC AGA TGGCCT GA CTC CCAGGG CCCAAGT

350   360   370   380   390   400   410   420   430   4

TTGT AATC CC CT GT GGGT TTC AC CA CTC TGC CAT GGCCA TGGGCCT TC CAAT GG GAAG AT CTCTGTTC TACC TC AGCT AA ANT T GCTC TC AATTC TACA

40   450   460   470   480   490   500   510   520   530

Appendix IV (a)

Raw sequence data of the 1.4 kb genomic UPK1B/pGEM plasmid, primed with the 5′ pGEM primer.

ATT GGGCCCGNCGT CGCANG CT CCCGGCCGCCATGG CCGCGGG A TTT GCTG TG ATA CA AG ATG CCA CT T CAAAG G CA TA TA CT AT A A A CAT CAGA ATG A AAT ACTGG A AGGGA AAT AAATG AGA
10   20   30   40   50   60   70   80   90   100   110   120

TAAAAG AAT TA CA T T T ACC CG T CAAAG AGCG A CT TG CCCAA A TA AT TA TG T TTT T T CAG CA AA T T TA TCA TA TA A T CA TG T AA TCA CT CA TG T AG T TA T T CAA CA AA CCC TCA T TGA CCAG
130   140   150   160   170   180   190   200   210   220   230   240

TGG CT TGG A TTGAGG CT TGG TGC TAG T CAGG T CAA T T CT CTC CT T T T CTC CAGAGA ACA AA TAGC CAT TG AA TA CAGA CCT TC CT CAG T CCAG T T TCC TGAG T T TCTA TT TTAAC TGGG AA
250   260   270   280   290   300   310   320   330   340   350   360

A A ACA AGT A CA AG A GA ATGA A CTGG TGT GT ATG TGT G CACA CA CACA TG T GAGCA T T TACA TG CT CA T T TACA TGT GT GCA TT TACA TG CA TG TTG CA TGC TCAG T GA GT GGGCA AGAGAG TCT T
370   380   390   400   410   420   430   440   450   460   470   480   490

A GTGT ATCACA TA CAGT A TAGGGCCT CA TAA A TC CC CT T T AAT T TT G GAA T T T TGG AGAAA TTT CCT T TAC AA TTT G CTG AAAA T T TA GA T CCC GGAGT TCAGA A A AAGANG A CT GG A TAA T
500   510   520   530   540   550   560   570   580   590   600   610

Appendix IV (b)

Raw sequence data of the 1.4 kb genomic UPK1B/pGEM plasmid, primed with the 3' pGEM primer.

NCTCTGGNCCNAACGNATTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCACTAATGATTCTCCAAACAATGATGACCGNTGGAAAAACAATGGAGTCACCAAAACCTGGGACAGGC
10 20 30 40 50 60 70 80 90 100 110 120

CTCATGCTCCAGGTAAGACCTGCGGTCTTGGGGAGATGCCATTTTTATTTACTGGTGGGAGTGGGTGTCATACACATGACTCAGTGGGACCCTATATTTTATTTCNGGGCCATGATGATTAG
130 140 150 160 170 180 190 200 210 220 230 240

CTTACATTTGTTGGAATGATTTGTCAANAAACTTAGGTTAATCATACACTCTCCCTCTTAGAACTCAGGTATCATTTACATTAAAAGAAAAATATTTTCTCTACCCCACAGTGAAACTCGA
250 260 270 280 290 300 310 320 330 340 350 360

ATCNCCACCCCTGAAATAACCCTTTTATTCAAATGCTGCAGGGACCTCAGTTCCCCCTTTATAGAATGAGTCCAGCAATGTNANGTGCTTGTTAATTTACTCTTCCTTTTAACTAACCAC
370 380 390 400 410 420 430 440 450 460 470 480

CTGTCCTGGGTTCCAATGGAATTACAAACATTTACNTTCTCTGGTTCCTTANTGTNCCTTAATTCCAGGGCANAATTCCCNCTTTTGGTGGGTGGGGGAAAGGAATCCGGAAAAANGGAAAGI
490 500 510 520 530 540 550 560 570 580 590 600

Appendix V (a)

Raw sequence data of the 700 bp UPK1B genomic PCR product, primed with the hTI1A primer.

N A TTCCCT TTATAGAATGA N CCAGCAATGN AAGGNGCT TGN TAGT TTACT CTTCCT TTTAACTAACCACTGTCCTGGT TCCAATGGATTACAGAACATTTACATTCTCTGGT TCCTTAGTGTGCG
10   20   30   40   50   60   70   80   90   100   110   120

TAAGN TCCAGGG CAAA AGT ACC CACT TTTGGTGGGNGGGG AAAGG AAT ACAGG AAAAAGGG ANAAG ANACACAGGNGN TCCTAA AG ACAAATTCACTTGCATCCCAGCCTCACTTTGGGCCAGC
130   140   150   160   170   180   190   200   210   220   230   240

CATG TN CATGCCTGA CAACCAGG A NA ACT GAGCTGGGGAAGCAA AGGGGCT AA AGG AAGA AACAC CTCACT GCAT ACCTC CTTTCTGGA CC TTCTTTTAC CAAGAACCCACAAA C
250   260   270   280   290   300   310   320   330   340   350   360

TCCTTGGCCAAGAAACAANGNCA CCANCTTTACACCNGGAAAAAAAT TTTT CTTTGGGGCCCCCTAAAG TTTCCCCCCCCT TTTT AAATGTTNCCCCCAACNTTTTT CCCC GGGTGA
370   380   390   400   410   420   430   440   450   460   470

TTTTTTTAAGGGCA CACAAAAGTTTTTTTCT CCCAAAAAACNCCCCCTNCCCCNA AAAAANAAAATTTT CCCCCCCCC TCCCCCCNTAAAANTAACAAANTNCCCCNCCNCCCCAA
490   500   510   520   530   540   550   560   570   580   590

Appendix V (b)

Raw sequence data of the 700 bp UPK1B genomic PCR product, primed with the UPK1B-1 primer.

GNNNNNT TTTANTGC TGNGCATGCT CAGTG AGNGGCAAG AGA T CTTAGTGTATCACATACAGTAT AGGGCCTCATAAATCCCCTTTAATTTTGGAATTTTGGAGAAATTTTCTTTACAATTTGC
10 20 30 40 50 60 70 80 90 100 110 120

TGAAAATTTAGAATCACGG AGTTCAGAAAAAGATGAACTGGATAATGTTGAACATCTAGGGTCCTTCCTAACAAGCATCCAGTCTCATCAGAGG AGAGGCAGGCTCCAAGTGCACAGCATTTG
130 140 150 160 170 180 190 200 210 220 230 240

TGGGTGTTCTTTCACACGCACCCACACTCAAAGTACATTGGTTCAGTTTGAGTGAAATGGCATTTGCTGTGTGTGGTATTGGTNGGTTTGTGTGCNTTNAACATGGGCACATGGGTT
250 260 270 280 290 300 310 320 330 340 350 360

GCGTTGTTGCACATGATGCCTCCACATGACAGTGATGCTATTTCTGGCTGGAAAAATATCCGAAACAGCCAAATGAACTCCTGAATCTGGANTGGGGAAANGTTTGCCA
370 380 390 400 410 420 430 440 450 460 470

AAATTTCCACTTTTGGCTTCCCCCCAGGGTTGCNCACNCCCCTGGAAAAAAGGGGNCCGTNNGGTCNTNTTCCNGGGGTGGGNGGGAAAAANGTCCCCCCCCTGGGGGG
480 490 500 510 520 530 540 550 560 570 580

Appendix VI (a)
Raw sequence data of the mouse TM3-ECD/pGEM plasmid, primed with the 5' pGEM primer.

NNNNNNNN CNNNNNNNNNG NTTGTTTACGAC CA T N AGGGCGAA TGGGCCCGACGT CGCAT GCTCCCGGCCGCCATGG CGGCCGCGGG AAATTCGA TTTCCATTGG TCATCATTG TTTGGAGGACTGTTGTTTTG
10   20   30   40   50   60   70   80   90   100   110   120   130

GATACCTCATCAG CATCTG CTTCAGGA AGAGG TTGG TTGTGAACTG CAGGGG TGAG AATG ACACGAGAGAGTCAG CACACCTG CTCCCT AG AAAG GCCAA ATAG CACCTG TTG CTCTAT AAACT
140   150   160   170   180   190   200   210   220   230   240   250   26

G CACATTCT CTTGG AG GG AAGCT AAACT CAGGG ACGCTG CACTCATTTGG CTGGGG CTGCTTTGGG TTTCTG TATAAGA AGGA AACACTG CATGA CTGGCTGT TTTAAGGATG
270   280   290   300   310   320   330   340   350   360   370

GTGT ANATTAGG GA AACAG CGA GA AACGGG GA CA AGA AAGA TTCTGA CTTATGT CTTC CACGA NG CA NAAACATGC AGAAAGGCC ATGG TGTTGGA CGAAATGGCA   4
380   390   400   410   420   430   440   450   460   470

GT C AGTGCC GG CCGG GA TTCC A CT TGGG CATTGG GAATCA GCTTCCTTCC A CTCC AAAAT CCCTGCTGT NAACTGT CA CCCAT CCT CCCTA AAAACATTCCTTA CCTTTTA
80   490   500   510   520   530   540   550   560   570   580   59

Appendix VI (b)

Raw sequence data of the mouse TM3-ECD/pGEM plasmid, primed with the 3′ pGEM primer.

NNNNNNNNNNNNN CNNNNNN TINNN TTT T G TCAT GGCCGT TA A CTATAGAATACT CA NG CTATG CATC CAA CG CG TTGGG AGCT CTCCCATA TGGT CGACCTG CAG G CG GCC GCG AATT CA CTAGTG ATTG AAG T
10   20   30   40   50   60   70   80   90   100   110   120   130

GG CATCT TG TAT CACAG CAG CAA CACAACG CGA CT TTG TG AG TATAG T CCCCAAT G CAT GG AG CAA AAAT G ACACT CCAG CAA CT GA CATG CTT CGA CCC CTC GCT GTG TGCT CG TCG TG CCA
140   150   160   170   180   190   200   210   220   230   240   250

AT ACACA CAC TAT TAAT ACC CA TG CAGT AT CGGG A AG CC TTG CTT GT CG TTT CTAAA TT AAAAG AGA AAGG CACT AAA CGC TA AGA AATG TATC TAGGA ATGA ATG G CTGA
260   270   280   290   300   310   320   330   340   350   360

CAGG TC A CAG CAGG A ATTC T GA GT C GA AGA AGC C TGA TTC CCA ATG C CC A AGC T GGG AT C CC G G CC GG CA CTGA CT G CCA TTC GTC CACACC A TG G CC T TTC
370   380   390   400   410   420   430   440   450   460   47

T GC ATGT T C T GCC TCCGT G GA AA AA CAT AA T TCAGA ATC TTT C TTGT CCCCG TTTC TCC CT GTTT CC C TAATC T ACACCATC C T T NAAA CAGCC A T CCATGCA TGTTT CC C
0   480   490   500   510   520   530   540   550   560   570   580

Appendix VII (a)
Raw sequence data of the 415 bp human UPK1B cDNA/pGEM plasmid, primed with the 5' pGEM primer.

TCNTGA T NA T AT ANGG CCA T TGGGC CGA CGT CGCA TGCT CCCG GCCGCCA TGGCCG CGGG A TGCCAGT CTG A CGGGCCA TT T TA CG CCA CAG CAA TT G T CCT GG AGCA TG AGCCTG T CCCAGG T T T T
10　20　30　40　50　60　70　80　90　100　110　120

GG TG ACT CCA TTG T T T T T CCA CTGG T CAT CA TTG T T TGG AGGG CTG T TG T T T TGG T ACCT CT C TAGCA T CTG CT T CAGG A AN AGG T TGGG T G TG AAAA AGT CT CG T TG TGT TG CTG CTG TGA T ACA
130　140　150　160　170　180　190　200　210　220　230　240　250

AGA TGCCACT T T TAA AGG CAT A TACT A TAAA CA T CAGA AT AAAA TA CGC CAGA AGA ATT T TC CTG CTG GACT T CA TGA TGCC TACA A TGCC TA NA ACA GA CAGG CAGA AG ANG CAGA TGCC CAC
260　270　280　290　300　310　320　330　340　350　360　370

A AA TA TGCCGA TC CAGG CAGCCC CA T AGA TGT CAT CG T TG T CGG TGG CT T CA AN CAA TGGG T A AAA GCT GTG T T GG T CANA TACGA AGAANA TGCACT C TG CA A T CA CT AA TTG
380　390　400　410　420　430　440　450　460　470　480

CGG CCGCCT G CAAGT CNA CCA TA TGGGAA AACT CCCCAA C CCN TTG GA TGCA TA CT TGA TT A TTCTA TAA TTGTT C N CTAAA TA C TTNGC TTA T CA TGG TCATA C
90　500　510　520　530　540　550　560　570　580　590

Appendix VII (b)

Raw sequence data of the 415 bp human UPK1B cDNA/pGEM plasmid, primed with the 3' pGEM primer.

GGGGGGGGTGGTN NTTTATGTGACCTA CCTA TA TCAAGCTATGCATCCAACG CGT TGGG AGCTCTCCCA TATGGT CGACCTG CAGGCGGCCGCACTAGTG ATTG CAGAGTG CATCTTCTTCGTATCTGA
10 20 30 40 50 60 70 80 90 100 110 120 13

CCAACACAGCCTCTACCCACTG CTTG AAGCCACCGACAACGATGA CATCTATGGGG CTGCCTGGATCGGCATATTTGTGGGCATCTGCCTCTTCTGCCTGTCTGTTCTAGGCATTGTAGGCATC
30 140 150 160 170 180 190 200 210 220 230 240 250

CATGAAGTCCAGCAGGAAAATTCTTCTGGCCG TATTTTATTCTGATGTTTATAGTATATGCCTTTAAAGTGGCATCTTGTATCACAGCAGCAACACAACGAGACTTTTTCACACCCACCT
260 270 280 290 300 310 320 330 340 350 360 370

TCTTCCTGAAAGCAGATGCTAGAGANGTACCAAAACAACAGCCCTCCAAAACAATGATGACCAGTGGGAAAAACAATGGAGTCACCAAAACCTGGGACAGGCTCATGCTCCCAAG
380 390 400 410 420 430 440 450 460 470 480

ACAATTGCTGTGGCGTTAAATGGGCCGTCAGACTGGCATCCCCGCCGGCCA TGGCGGGCCGGGGAACATGCGACNTCCGGCCCAATTCCCCCTATAAT GAATTCNTAT
490 500 510 520 530 540 550 560 570 580 590

Appendix VIII (a)

Raw sequence data of the mink TI1/CMV plasmid, primed with the 5' CMV primer.

NC GAA TTA TACGACT CACTAT AGGG AG ACCCAAG CTTGTGCCAAAT CCCG ACAATGG CG AAAAG ATGACT CCT CTG TT CGTTG CTT CCAGGG C CTG CT GATTTTT GGAAAT GTGATT GTTG GTATG TGCC

10   20   30   40   50   60   70   80   90   100   110   120   13

G CAT CGCC CTG ACCGCAG AGTG CATC TTCTTCG TATCTGACCAGCA CAGCCTCTACC CATTGCTTG AAG CCACCGA CA ACGATG ACATCTACGG GG CAGCCTGGATTGGCATGTTTGT CGG CA

0   140   150   160   170   180   190   200   210   220   230   240   250

TCTGCCTC TTCTGT CTGTCCGTTCTAGGCATTGTAGG CATCATGAA TCCAAC AGGAAAAT CTTC TGGCGTTATTTCATTC TGATGTTTTATAGT ATA TGGGCTTTGAAGTTGGCATC

260   270   280   290   300   310   320   330   340   350   360   370

TTGTTATCACAGCCAGCAACAC AACNA ANT TCTTCCAGCCCCA CCTC TTCCCTGAAANCAAAT CT GGA AAA AGGTTT CCCCAAAAC AAAA CCCCNCCCCAA CCA TGAATNAACCA

380   390   400   410   420   430   440   450   460   470   480

TINGGA AAAA TAATGGA TTT CCCCCAAANA TT GGAAA AAATCC TTCT CCCAN AACANGG TNNTGGT TTTTTAATGG CCCCTCCCNAAA GGGGNAAAAAAAACTTCTTCCCTTCCCGGAATGGC AAAA

490   500   510   520   530   540   550   560   570   580   590   600   610

Appendix VIII (b)

Raw sequence data of the mink TI1/CMV plasmid, primed with the 3' CMV primer.

GACCTCATAGAATAGGGCCCAGAGTCAACTGGANGAAGATGGTGTGGTGGTGACACTTTGTTCTTAATATTCAATTCTGCTCCAGTAGAACATGGTACCCAGGAGAACCCAAAATGTCCAGCAGAGA
10   20   30   40   50   60   70   80   90   100   110   120

ATGGCAAATCCAAACCAGGCAACCCCCCAGGCGTGTCGGTTCATGGGTCCANANATGAGTTCATACCACCCCTCTTTGTGATAGTACCCGGGCACTCCTAGCTTGCAGGCCTCCACATTGAGAG
130   140   150   160   170   180   190   200   210   220   230   240   250

GTTCTTTCAGACTGTTCATCACACAGCACTGACGAGGCCAGGGATAGTCGGCATCATTATTCGCAGTCCGGAAGGCAGATGTTTTATCTCTGCCAGTCCTGACGGGCCCATTGACACC
260   270   280   290   300   310   320   330   340   350   360   3

ACAGCAGTGGTCCCTGNAACATGATTCTGTCCCAANTCTTGGTGACTCCCATTATTTTCCCATTGGTCCNCCATTGTTTTGGAAGGCTATTGTTTTTGGGTACTCCCCCACA
70   380   390   400   410   420   430   440   450   460   470   480

TCTGCTTCAGAAAAGGTTNGGCGTTAAAAAAATCCCCTTTNTTTTTGCCGCNGTNAAAAAAAATCCCCTTCCAACCCTNTNTTTNACCTCCAAATAAANCCCAAAAAATT
490   500   510   520   530   540   550   560   570   580   590

Appendix IX (a)

Raw sequence data of the human UPK1B/pTRE plasmid, primed with the 5′ TRE primer.

GNNNNNTNNCTGGGAGACCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCGATCCAGCCTCCCTCCCATATGGTCGACCTGCAGGCGGCCGCACTAGTGATTAATCCCGACAATGGCGAAA
10 20 30 40 50 60 70 80 90 100 110 120 13

AGACAACTCAACTGTTCGTTGCTTCCAGGGCCTGCTGATTTTTGGAAATGTGATTATTGGTTGTTGCGGCATTGCCCTGACTGCGGAGTGCATCTTCTTTGTATCTGACCAACACAGCCTC
30 140 150 160 170 180 190 200 210 220 230 240 250

TACCCACTGCTTGAAGCCACCGACAACGATGACATCTATGGGGCTGCCTGGATCGGCATATTTGTGGGCATCTGCCTCTTCTGCCTGTCTGTTCTAGGCATTGTAGGCATCATGAAGTCCA
0 260 270 280 290 300 310 320 330 340 350 360 370

AGCAGGAAAATTCTTCTGGCGTATTTCATTCTGATGTTTATAGTATATGCCTTTGAAGTGGCATCTTGTTTCACAGCAGCAACACAACGANACTTTTTCACACCCAACCTCTTCCTGAACA
380 390 400 410 420 430 440 450 460 470 480 490

GATGCTAGANAAGTTACCAAAACAACACCCCTCGAAACAATGAATGAACCAGTGGGAAAAAACAATGGAATTCCCCCAAAANCTTGGGGAACAGGCTCCATGCTCCCCAAGAANAATTGCTTGTT
500 510 520 530 540 550 560 570 580 590 600 610

Appendix IX (b)

Raw sequence data of the human UPK1B/pTRE plasmid, primed with the 3' TRE primer.

CT CCAT CAAT GT ATCT TATCAT GT CT GGAT CCT CT AG ANG ATCCC CGGG T ACCG AN CT CG AAT T CGGGG CGGG ATT TT CT TAAT AT T CAAT T CT GCT CCAG T AG A ACAT GG T ACCCA A
10 20 30 40 50 60 70 80 90 100 110

G AGA ACC CAA A A AGT CCAN CAG ANA ATGG CAA AT CCAA A CCANG CA ACC CC CCAN GCG TGT CGG T TCAT TGG ACCA NA T AT CNGT TCA TAG CAN CC CTGA TT GT GA TA A A A AC CANG CAC
120 130 140 150 160 170 180 190 200 210 220 230

GCCT AN TT TACA AN CCTC CANG T TG ANANG TTCT T TAANA TTGT TCA TAA CACAN CAT TG ACG AGG CCAGGG A TACT CAGCA TCNT T TATTC TCAN TCCGGA AGGCA TATGT GT ATT TT TGC
240 250 260 270 280 290 300 310 320 330 340 350 3

CACT CT GA TGG ACCAT T TACGC CACAG CAAT TGT CCT GGAN CATGA CCT GT CCCANGT TT TGG TGA CT CCAT TGT TT TT CCACTG GTC NT CATT GT TT TGG AAGGCT GT T GT TT TGG TA CT
60 370 380 390 400 410 420 430 440 450 460 470

T CC CCT ACN T CTGCCT NCAGA A AAGTTT GGGT T TTAAAAAT C CC TTG T T TT GCN GCCGT NAAA C A AN CC CC TCC AAGN TT TCT TT AN TCCAATA ATC CCN AA AAT TCCCGCN GA ATCCN A GCCC
480 490 500 510 520 530 540 550 560 570 580 590 600