Regulation of the 25-Hydroxyvitamin D$_3$ 1 Alpha Hydroxylase and 24-Hydroxylase Gene Promoters

A THESIS SUBMITTED TO THE UNIVERSITY OF ADELAIDE
FOR THE DEGREE OF DOCTOR
OF PHILOSOPHY

By

Xiu-Hui Gao B.Sc. & M.Sc

Department of Molecular Biosciences
University of Adelaide
Adelaide, South Australia

March 2003

CONTENTS

CHAPTER TWO: MATERIALS AND METHODS

CHAPTER THREE: BASAL AND PARATHYROID HORMONE INDUCED EXPRESSION OF THE HUMAN 25-HYDROXYVITAMIN D 1α-HYDROXYLASE GENE PROMOTER IN KIDNEY AOK-B50 CELLS.

CHAPTER FOUR: REGULATION OF RAT *CYP24* GENE EXPRESSION IN KIDNEY AND OSTEOBLAST CELLS: ROLE OF SP1 AND CCAAT BINDING PROTEINS IN BASAL AND 1,25(OH)$_2$D$_3$ MEDIATED INDUCTION OF THE PROMOTER.

CHAPTER FIVE: CALCITONIN STIMULATES EXPRESSION OF THE RAT 25-HYDROXYVITAMIN D3-24 HYDROXYLASE GENE PROMOTER IN HEK-293T CELLS EXPRESSING CALCITONIN RECEPTOR: IDENTIFICATION OF SIGNAL PATHWAYS.

CHAPTER SIX: SUMMARY AND CONCLUDING DISCUSSION.

THESIS SUMMARY

1,25(OH)$_2$D$_3$ plays important roles in many process such as calcium homeostasis. The vitamin D hydroxylase enzymes, vitamin D 1-hydroxylase (*1α(OH)ase*) and vitamin D 24-hydroxylase (*CYP24*) are the key rate limiting enzymes responsible for the bioactivation and degradation of 1,25(OH)$_2$D$_3$ respectively. Several physiological factors including PTH, 1,25(OH)$_2$D$_3$ and calcitonin interact to regulate the gene expression of the two enzymes resulting in regulation of serum and tissue levels of 1,25(OH)$_2$D$_3$.

The aim of the study in this thesis was to understand the molecular mechanism by which PTH regulates expression of the human *1α(OH)ase* gene promoter in the kidney and also the molecular mechanisms by which 1,25(OH)$_2$D$_3$ and calcitonin regulate expression of the rat *CYP24* promoter in kidney and osteoblast cells.

The up-regulation of the gene for *1α(OH)ase* by PTH under hypocalcemic conditions is fundamentally important for the maintenance of calcium and phosphate homeostasis. The molecular mechanism that underlies this hormonal response has been investigated in the present study by transfection analysis of the human *1α(OH)ase* promoter in kidney AOK-B50 cells. It has been shown that the first 305 bp of promoter sequence can be induced by PTH in transient transfection assays and also within a chromatin environment when stably integrated. Mutagenesis of possible transcription factor binding sites within this promoter length has shown that three sites clustered within the region from −66 to −135 contribute to basal expression. A likely GC box and a CCAAT box site are particularly important for basal expression although these sites are not likely to functionally cooperate in a major way. Mutagenesis of the CCAAT box site consistently reduced PTH induction although mutagenesis of the GC box, EBS and other possible binding sites in the 305 bp of promoter had no significant effect on the level of PTH induction. Other experiments showed that PTH induction

but not basal expression was sensitive to the protein kinase inhibitor H89. I have therefore identified for the first time the sites in the human $1\alpha(OH)ase$ promoter responsible for basal expression and provide evidence for the role of a CCAAT box binding protein in a PTH mechanism of induction that involves an H89 sensitive step.

$1,25(OH)_2D_3$ functions in kidney and osteoblast cells to induce *CYP24* gene expression and enzyme activity, representing an important feedback mechanism to avoid toxicity that may result from a high level of $1,25(OH)_2D_3$. In the rat promoter, a GC box (-114/-101) and a CCAAT box (-62/-51) were found to strongly contribute to basal and $1,25(OH)_2D_3$ mediated induction in kidney HEK-293T cells and osteoblast UMR106 cells. The present study has focused on characterising the two sites and related signalling systems. EMSA using antibodies for Sp1 and NF-YB provided evidence for the binding of Sp1 to the GC box and the binding of NF-Y to the CCAAT box. Over expression of Sp1 and NF-Y in *Drosophila* SL3 cells revealed that Sp1 functions at least partially through the GC box and NF-Y acts through the CCAAT box. Use of the mammalian two-hybrid assay provided evidence for an interaction of Sp1 with Ets-1 and also of Sp1 with RXRα. Over expression of a dominant negative NF-YAm29 resulted in reduced basal expression but did not lower the fold $1,25(OH)_2D_3$ mediated induction, suggesting the NF-Y is important for basal expression but not for induction by $1,25(OH)_2D_3$. A model for induction is proposed that involves contributions of Sp1 and NF-Y to basal expression with the NF-Y protein being replaced with another unknown protein in response to $1,25(OH)_2D_3$.

The results in HEK-293T cells, using pharmacological inhibitors (H89, calphostin C, PD98059 and SB203580) demonstrated that PKA, PKC and ERK1/2/5 MAPK pathways are critical for $1,25(OH)_2D_3$ mediated induction of the *CYP24* promoter. Evidence is presented that the CCAAT and GC boxes are likely to be target sites for the cAMP/PKA cascade. In addition,

there is evidence that the PKC isoform, PKCζ is important for induction of the promoter by 1,25(OH)$_2$D$_3$ but not for basal expression and that this action of PKCζ occurs through the GC box. Hence, it can be proposed that basal expression is driven by Sp1 and NF-Y, both of which may be phosphorylated by PKA. In the presence of 1,25(OH)$_2$D$_3$, NF-Y is replaced by another protein perhaps following phosphorylation of NF-Y by PKA. At the GC box, Sp1 in the presence of 1,25(OH)$_2$D$_3$ could be phosphorylated by PKCζ.

Studies were undertaken to determine whether calcitonin could lower circulating 1,25(OH)$_2$D$_3$ levels, through induction of *CYP24* in kidney cells. It was found that the promoter was stimulated by calcitonin in transiently transfected HEK-293T cells that stably expressed calcitonin receptor. A GC box at -114/-101 and a CCAAT box at -62/-51 in the transiently transfected –298bp promoter predominantly accounted for calcitonin induction. Mutagenesis of either the GC or CCAAT box resulted in the almost totally loss of induction in stably transfected in these cells. Over expression of NF-YAm29 in these cells strongly inhibited basal expression of the *CYP24* promoter and moderately inhibited calcitonin mediated fold induction. ERK1/2 signalling pathways were not involved in the calcitonin-mediated induction as shown by studies with the pharmacological inhibitor PD98059 and a dominant negative of ERK1/2 (ERK1K71R). However, both PKA and PKC pathways are involved in the calcitonin induction mechanism as determined by the inhibitory action of the PKA and PKC inhibitors. PKCζ contributes 50% to calcitonin induction, but not basal expression of *CYP24* promoter expression as shown by over expression of a dominant negative PKCζK281M. Cotransfection of a dominant negative form of Ras (Ras 17N) resulted in calcitonin mediated induction being reduced by about 50%. Therefore a Ras-PKCζ signalling pathway was proposed which acts through the GC box.

Calcitonin and $1,25(OH)_2D_3$ synergistically induced *CYP24* gene promoter activity in both transient transfected and stably transfected cells. This synergy was almost abolished when the two VDREs were mutated, but synergy was still evident when the GC and CCAAT boxes were mutated. The findings have been extrapolated to the *in vivo* situation where it is suggested that induction of renal *CYP24* by calcitonin under hypercalcemic conditions could contribute to the lowering of $1,25(OH)_2D_3$ levels.

DECLARATION

This thesis contains no material that has been accepted for the award of any other degree or diploma by any university. To the best of my knowledge it contains no material that has been previously published by any other person, except where due reference has been made in the text.

I consent to this thesis, when deposited in the university library, being available for photocopying and loan.

Signature:

Date:....12 / 03 /2003

Xiu-Hui Gao

ACKNOWLEDGMENTS

I would like to express my sincere thanks to:

- Dr. Brian May, my supervisor, for his permission to undertake the Ph.D. study in the lab, and for his continuous encouragement, guidance and innovative supervision in the field.

- Dr. Prem Dwivedi, with whom I worked closely and happily, for his great supervision, valuable discussion and big patience.

- Josef, the Honest Aussie Boy, for his generous help and discussion (Small arc can make a big picture!).

- Tania, with whom the whole lab is cheering up, for her warm friendship and help.

- Dr. Howard Morris, for his careful review of some chapters.

- Other past and present BKM members, Tim, Satish, Ingrid, Ben, Senali, Andy, Paul, Diseree, Babara, Ana and Oly, for their great chatting and friendship.

- All the departmental staff and postgraduates, especially the support staff in TC, for the nice working environment they are making and maintaining.

Finally I would like to thank dear XJ and Cheryl for their understanding, love and support, and also to my parents and brother for their endless love and encouragement.

+ +
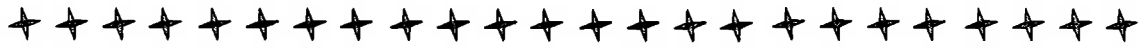
CHAPTER 1

Introduction

+ +
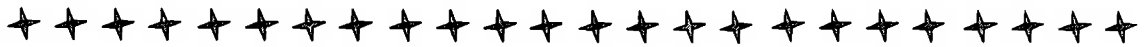
1.1 Overview of vitamin D metabolism

The biologically active form of vitamin D, $1,25(OH)_2D_3$, has many important pleiotropic functions involved in calcium and phosphate homeostasis, bone mineralization, cell proliferation and differentiation (Jones *et al.*, 1998), immune modulation (Becker *et al.*, 2002; Deluca and Cantorna, 2001), and bile acid metabolism (Makishima *et al.*, 2002). Figure 1.1 shows the flow chart for $1,25(OH)_2D_3$ metabolism. The first step for $1,25(OH)_2D_3$ synthesis begins with vitamin D_3, which is taken up from the diet or synthesized from 7-dehydrocholesterol in the basal epidermal layer of the skin through exposure to ultra-violet light (270nm-300nm) (Carlberg and Polly, 1998; Jones *et al.*, 1998). Vitamin D3 is then transported to the liver as a complex with vitamin D_3 binding protein (DBP) (Lowe *et al.*, 1992). In the liver, vitamin D3 is hydroxylated by vitamin D_3-25-hydroxylase at carbon atom 25 to produce $25(OH)D_3$, which is transported to the kidney. The 25-hydroxylation in the liver appears to be only loosely regulated. The kidney is the major site of production of circulatory $1,25(OH)_2D_3$. In the kidney the metabolic activation of $25(OH)D_3$ to $1,25(OH)_2D_3$ is tightly controlled by the 25-hydroxyvitamin D3 1α-hydroxylase (*CYP27B1* or *1α(OH)ase*), which hydroxylates the substrate at carbon atom 1. Further hydroxylation by 25-dihydroxyvitamin D3 24-hydroxylase (*CYP24*) initiates degradation of $1,25(OH)_2D_3$ through the C-23/C-24 oxidation pathway (Figure 1.1). Since *1α(OH)ase* and *CYP24* catalyze the rate limiting steps of $1,25(OH)_2D_3$ formation and degradation respectively (Omdahl *et al.*, 2002), they are the two crucial enzymes required for regulating ambient levels of serum and cellular $1,25(OH)_2D_3$.

Circulatory $1,25(OH)_2D_3$ can regulate calcium homeostasis through enhancing calcium uptake by the intestine, stimulating calcium reabsorption by the kidney and increasing calcium resorption from bone. Besides $1,25(OH)_2D_3$, parathyroid hormone (PTH) and calcitonin are the other two major hormones involved in calcium metabolism. This chapter will serve to introduce the physiological roles of $1,25(OH)_2D_3$, the key proteins involved in vitamin D_3
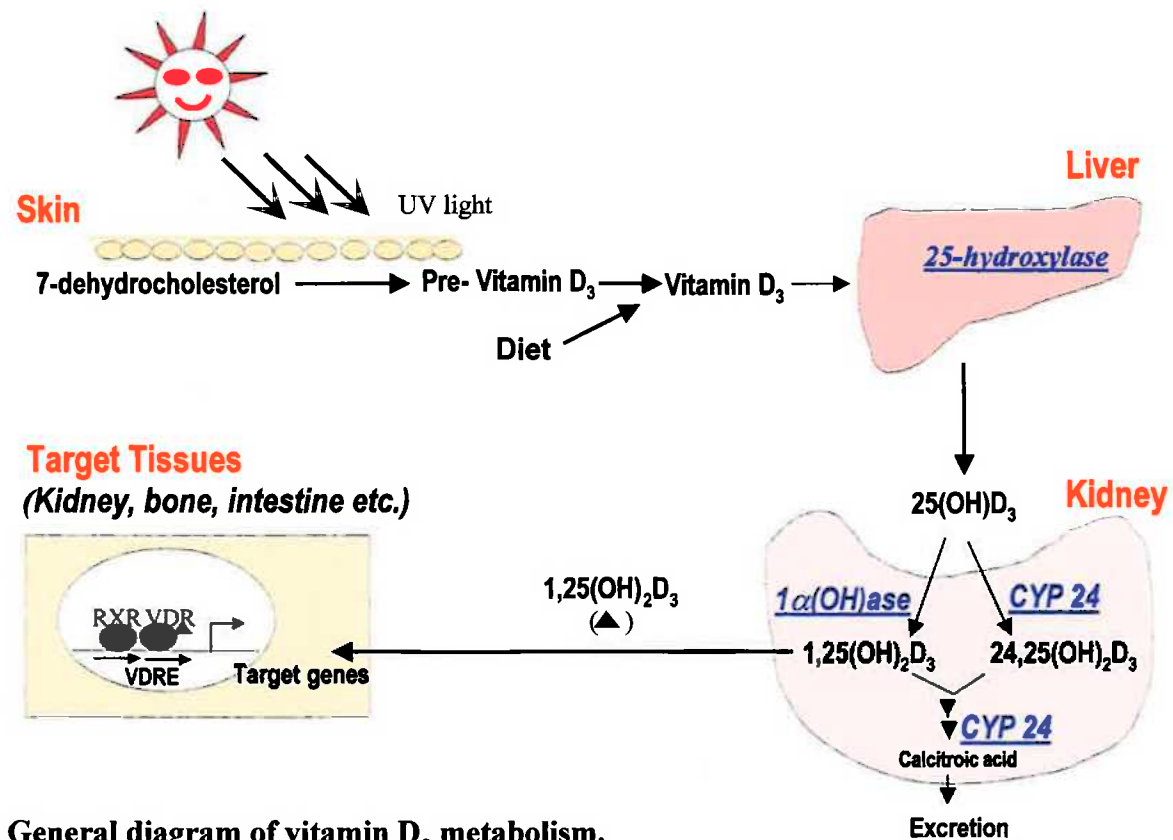
Figure1.1 General diagram of vitamin D$_3$ metabolism.

A flow diagram describes the biosynthesis and metabolism of vitamin D$_3$ and its major metabolites, and the cellular action of 1,25(OH)$_2$D$_3$. Parental vitamin D$_3$ from sunlight action on the epidermis or dietary sources is hydroxylated in the liver by 25-hydroxylase and activated in kidney by *1α(OH)ase* to give 1,25(OH)$_2$D$_3$, which acts on renal genes or enters the circulation to act on target tissues (intestine, bone, parathyroid). *CYP24* plays a dual role to remove 25(OH)D$_3$ from the circulation and excess 1,25(OH)$_2$D$_3$ from kidney. Circulating 1,25(OH)$_2$D$_3$ is also be removed by other tissues, notably the intestine (Omdahl et al., 2002).

metabolism (DBP; 25-hydroxylase; $1\alpha(OH)ase$; $CYP24$), the molecular actions of $1,25(OH)_2D_3$, PTH and calcitonin, and the possible mechanisms by which the three hormones modulate $1,25(OH)_2D_3$ levels by regulating the enzymes $1\alpha(OH)ase$ and $CYP24$.

1.2 Actions of $1,25(OH)_2D_3$

Classically, $1,25(OH)_2D_3$ has been considered to be predominantly involved in calcium and phosphate homeostasis and bone mineralization. However more recent studies have indicated that $1,25(OH)_2D_3$ exhibits antiproliferative and prodifferentiating properties, and also plays a role in the central nervous system, immune responsiveness and reproduction. Very recently Makishima et al., (2002) showed that $1,25(OH)_2D_3$ is also involved in secondary bile acid catabolism in a protective role against colon cancer. These properties of $1,25(OH)_2D_3$ will now be discussed.

1.2.1 Regulation of calcium homeostasis

$1,25(OH)_2D_3$ is one of the primary regulators of calcium and phosphate homeostasis. Traditionally, $1,25(OH)_2D_3$ acts to enhance calcium and phosphate uptake by the intestine, to increase calcium reabsorption by the kidney, to inhibit renal calcium excretion and to enhance calcium mobilization from bone to maintain the concentration of these ions (reviewed by Jones et al., 1998; Brown et al., 1999b). Rickets and hyperparathyroidism in VDR null mice can be prevented by a high calcium diet (Li et al., 1998; Amling et al., 1999), suggesting that $1,25(OH)_2D_3$ at least plays a role in intestinal calcium absorption. $1,25(OH)_2D_3$ induces the calcium-binding proteins, Calbindin-D9K and Calbindin-D28K, involved in the translocation of calcium in mammalian intestinal and kidney cells respectively (Jones et al., 1998; Walters, 1992; Hoenderop et al., 2002). $1,25(OH)_2D_3$ may also stimulate calcium reabsorption in the distal nephron by increasing expression of the epithelial calcium channel protein (EcaC2), which is involved in active calcium transport (Hoenderop et al., 2002). There is evidence that

1,25(OH)$_2$D$_3$ also stimulates the calcium transport protein (CaT1) that acts as a gatekeeper for calcium entry into enterocytes in the intestinal cells (Fleet *et al.*, 2002). The expression of EcaC2 and CaT1 is strongly VDR-dependent and consistently down-regulated in two VDR knockout strains of mice (Van Cromphaut *et al.*, 2001).

1.2.2   Action of 1,25(OH)$_2$D$_3$ on bone

A deficiency of vitamin D causes rickets in growing children and osteomalcia in adults but rickets can be treated either with a supplement of vitamin D or by exposure to ultraviolet light (Kato *et al.*, 2002). In addition, *1α(OH)ase* and VDR null mice also develop skeletal abnormalities (Dardenne *et al.*, 2001; Panda *et al.*, 2001a; Yoshizawa *et al.*, 1997). Therefore, 1,25(OH)$_2$D$_3$ functions in normal bone growth and bone mineralization.

1,25(OH)$_2$D$_3$ enhances calcium and phosphate uptake by the intestine and the kidney and this is important for bone mineralization. Bone remodeling throughout life is a balance of continuous bone formation by osteoblasts and bone resorption by osteoclasts. Vitamin D receptor (VDR) is present in osteoblast cells and osteoclast precursor cells (Aubin and Heersche, 1997) and direct action by 1,25(OH)$_2$D$_3$ on bone has been extensively studied. 1,25(OH)$_2$D$_3$ has a complex effect on bone formation. 1,25(OH)$_2$D$_3$ plays distinct roles at different maturation stages of osteoblasts. It stimulates differentiation of osteoblasts from immature proliferating cells to differentiated, nondividing cells that produce matrix protein for bone mineralization (Aubin and Heersche, 1997). It has been found that 1,25(OH)$_2$D$_3$ enhances the synthesis of osteocalcin by osteoblasts (Skjodt *et al.*, 1985; Demay *et al.*, 1990) and also increases expression of type I collagen synthesis, a major protein of the extracellular matrix (Franceschi *et al.*, 1988). Several other gene products including osteopontin, alkaline phosphatase and matrix gla proteins, which are mature osteoblast markers, are also up-regulated by 1,25(OH)$_2$D$_3$ (Noda *et al.*, 1990; Pols *et al.*, 1986; Aubin and Jeersche, 1997 and references therein).

Additionally, $1,25(OH)_2D_3$ is a potent inducer of osteoclast bone resorption by stimulating both osteoclastogenesis and accelerating mature osteoclast resorption activity. The action of $1,25(OH)_2D_3$ - enhanced osteoclastogenesis and osteoclast activity is indirect. $1,25(OH)_2D_3$ increases the expression of receptor activator of NF-κB ligand (RANKL), a transmembrane ligand, in committed preosteoblastic cells (Kitazawa R and Kitazawa S, 2002; Anderson *et al.*, 1997; Lacey *et al.*, 1998). Through a cell-cell interaction, RANKL stimulates its specific receptor RANK on the surface of the osteoclast progenitors (Anderson *et al.*, 1997; Kong Y *et al.*, 1999) promoting activation of osteoclasts and hence calcium mobilization.

Over all, the actions of $1,25(OH)_2D_3$ on bone are complicated and the response to $1,25(OH)_2D_3$ may be dependent on the stage of proliferation and differentiation of osteoblasts. The total effect of $1,25(OH)_2D_3$ on bone would be the balanced outcome resulting from regulating both osteoblast and osteoclast activities.

1.2.3 Inhibition of cell proliferation and induction of cell differentiation

Several studies have shown that $1,25(OH)_2D_3$ is involved in increasing cell differentiation while reducing cell proliferation in various cell types including osteoblasts, macrophages, keratinocytes, prostate cancer cells and pancreatic ß cells (Brown *et al.*, 1999b).

$1,25(OH)_2D_3$ and keratinocytes

$1,25(OH)_2D_3$ inhibits proliferation and stimulates differentiation of keratinocytes. $1,25(OH)_2D_3$ and analogs (especially, MC903) have been employed in the treatment of psoriasis, a hyperproliferative disease of the epidermis (Bikle *et al.*, 1992; Morimoto *et al.*, 1985). It has been shown that $1,25(OH)_2D_3$ stimulates the assembly of functional adherent junctions in keratinocytes resulting in cell differentiation (Gniadecki *et al.*, 1997). The locally synthesized $1,25(OH)_2D_3$ in the skin promotes the differentiation of keratinocytes through a calcium-dependent process that involves phospholipases C and D and protein kinase C (PKC) (Bikle *et al.*, 2001; Bollinger Bollag and Bollag, 2001).

Recent studies have shown that the alopecia in the VDR null mice is not due to an intrinsic defect in keratinocyte proliferation or differentiation, but rather to an abnormality in initiation of the hair cycle (Sakai and Demay, 2000). Interestingly alopecia is not observed in $1\alpha(OH)ase$-null mice (Panda et al., 2001a). Therefore VDR is important for prevention of alopecia in a $1,25(OH)_2D_3$ independent manner (Sakai and Demay, 2001).

1,25(OH)₂D₃ and cancer cells

Epidemiological studies have shown that the incidence of cancer is related to low $25(OH)D_3$ levels (Ylikomi et al., 2002). Generally $1\alpha(OH)ase$ expression decreases with tumor progression (Whitlatch et al., 2002; Hsu et al., 2001). In addition, high expression of *CYP24* in target cells may result in a complete insensitivity to $1,25(OH)_2D_3$ and *CYP24* was identified as a potent oncogene in breast carcinogenesis (Albertson et al., 2000). VDR has been also detected in particular hormone-related cancers such as prostate cancer, breast cancer and colon cancer (Ylikomi et al., 2002). The VDR mRNA is expressed in a relatively high level in well-differentiated cells and thus has been proposed as a useful marker in predicting the clinical outcome in patients since it correlates with the degree of differentiation in human colon cancer cells and breast cancer cells (Ylikomi et al., 2002).

For the purpose of cancer treatment, $1,25(OH)_2D_3$ also functions as an antiproliferative, prodifferentiating, and proapoptotic agent in cancer cells. Studies using cell culture and animal models have shown that $1,25(OH)_2D_3$ significantly inhibits the proliferation of prostate cancer cells and promotes the differentiation of these cells (Moffatt et al., 2001; Skowronski et al., 1993; Lokeshwar et al., 1999). Similar results are also observed in other cancer cells, such as leukemia cells and breast cancer cells (Suda et al., 1989; Hisatake et al., 1999; James et al., 1996).

The molecular mechanism by which $1,25(OH)_2D_3$ inhibits the proliferation of cancer cells has been extensively studied. The antiproliferative effects of $1,25(OH)_2D_3$ are predominantly

through a G1/S phase block of the cell-cycle, which is involved in the down regulation of cyclin-dependent kinase (CDK)2 and CDK6 kinase activity (Scaglione-Sewell *et al.*, 2000; Wang Q *et al.*, 2000). In addition, 1,25(OH)$_2$D$_3$ has an inhibitory effect on cell growth by inducing the expression of cyclin-dependent kinase inhibitors p21$^{WAF1/CIP1}$ (James *et al.*, 1996; Jensen *et al.*, 2001) and p27^{KIP1} (Wu *et al.*, 1997, Campbell *et al.*, 1997) in breast cancer cells. Moreover, 1,25(OH)$_2$D$_3$ could reduce the c-myc protooncogene transcription in breast cancer cells (Jensen *et al.*, 2001; Bortman *et al.*, 2002). 1,25(OH)$_2$D$_3$ has anti-cancer actions in stimulating tumor suppressor BRCA1 gene expression (Campbell *et al.*, 2000), inhibiting telomerase activity (Hisatake *et al.*, 1999) and inhibiting angiogenic activity (Majeski *et al.*, 1996). Furthermore 1,25(OH)$_2$D$_3$ suppresses cell growth also by regulating growth factors (IGF-1 signaling pathway), parathyroid hormone related protein (PTHrP) and androgen receptor (Feldman *et al.*, 2000; Omdahl *et al.*, 2002), which all play important roles in the development of prostate cancer cells. 1,25(OH)$_2$D$_3$ promotes the differentiation of colon carcinoma cells by the induction of E-cadherin (a tumor suppressor gene) and the inhibition of β-catenin (a protooncogene encoding a cytoskeleton-associated protein) signaling (Palmer *et al.*, 2001). 1,25(OH)$_2$D$_3$ has also been shown to induce apoptosis in numerous cancer cell types by regulating members of the Bcl-2 family (Ylikomi *et al.*, 2002; James *et al.*, 1996). 1,25(OH)$_2$D$_3$ up regulates proapoptotic proteins (bak) and down regulates anti-apoptosis proteins (Bcl-2 and Bcl-X$_L$) (Guzey *et al.*, 2002; Diaz *et al.*, 2000). Overall a sustained 1,25(OH)$_2$D$_3$ level may play a role as a deterrent to cancer onset and progression.

Though 1,25(OH)$_2$D$_3$ has the potential to prevent growth of cancer cells, pharmacological doses of 1,25(OH)$_2$D$_3$ also lead to hypercalcemia *in vivo* (Thys-Jacobs *et al.*, 1997). Thus, a number of 1,25(OH)$_2$D analogs have been synthesized which have enhanced antiproliferative activity and reduced the tendency to cause hypercalcemia and hypercalciuria (Leeuwen and Pols, 1997). Such analogs of 1,25(OH)$_2$D$_3$ (eg. EB 1089, a dimethyl side-chain analog (Lokeshwar *et al.*, 1999)) may be useful in the treatment of cancer (van den Bemd and Chang, 2002). The possibility of combination therapy has also been suggested since the

6

antitumor effect of 1,25(OH)$_2$D$_3$ can be modified by interaction with growth factors and other steroid hormones (Ylikomi *et al.*, 2002).

1.2.4 Modulation of the Immune system

1α(OH)ase is present in peripheral macrophages, precursor monocytes and granulomatoses, and *CYP24* is present in peripheral monocytes (Zehnder *et al.*, 2001; Overbergh *et al.*, 2000; Dusso *et al.*, 1997). VDR is detected in the T lymphocyte, peripheral macrophage, precursor monocytes and thymus cells (DeLuca and Cantorna, 2001; Omdahl *et al.*, 2002). 1,25(OH)$_2$D$_3$ has been shown to have actions on the immune system, especially on T cell-mediated immunity. It is evident in several animal models that 1,25(OH)$_2$D$_3$ prevents autoimmune diseases, extend graft survival and can down regulate immune responses in general (Lemire, 1997; Brown *et al.*, 1999b). 1,25(OH)$_2$D$_3$ has an inhibitory effect on T-cell proliferation and also has been reported to suppress the expression of cytokines (i.e. IL-2 and interferon γ) (Manolagas *et al.*, 1994; Bhalla *et al.*, 1986; Rigby *et al.*, 1987). In addition, it stimulates transforming growth factor (TGF) β1 and interleukin 4 (IL-4) production, which may in turn suppress inflammatory T cell activity (DeLuca and Cantorna, 2001).

1,25(OH)$_2$D$_3$ plays a significant role as a selective immunosuppressant, as shown in animal models of autoimmune diseases including encephalomyelitis, rheumatoid arthritis, systemic lupus erythematosus, type I diabetes and inflammatory bowel disease (DeLuca and Cantorna, 2001). The results suggest 1,25(OH)$_2$D$_3$ has a clinical potential in treatment of these autoimmune diseases in humans.

1.2.5 Functions in the nervous system

Accumulating evidence suggests that 1,25(OH)$_2$D$_3$ is involved in brain function (reviewed by Garcion *et al.*, 2002). Vitamin D$_3$ 25-hydroxylase, *1α(OH)ase* and *CYP24* enzymes were detected in the central nerve system (CNS) (Zehnder *et al.*, 2001; Naveilhan *et al.*, 1993),

suggesting that both the biosynthetic and the degradative pathways for $1,25(OH)_2D_3$ exist in the brain. It is suggested that $1,25(OH)_2D_3$ may have a neuroprotective effect by modulating the production of neurotrophins, inhibiting the synthesis of inducible nitric oxide synthase and increasing glutathione levels (Wang Y *et al.*, 2000; Garcion *et al.*, 1998; Shinpo *et al.*, 2000). $1,25(OH)_2D_3$ may also be useful for treatment of multiple sclerosis, autoimmune and inflammatory disease of the CNS (i.e., *Cerebral ischemia*) (Garcion *et al.*, 2002).

However in the VDR or *1α(OH)ase* knock out mice and in patients suffering from rickets, no obvious nervous dysfunction has so far been observed which suggests that $1,25(OH)_2D_3$ related functions might overlap with other signaling pathways (Garcion *et al.*, 2002).

1.2.6 Involvement of secondary bile acid metabolism

More recently it has been shown that activation of VDR by its ligand $1,25(OH)_2D_3$ induces expression *in vivo* of CYP3A, a cytochrome P450 enzyme that catabolizes the secondary bile acid lithocholic acid (LCA) in the liver and intestine. LCA is hepatotoxic and a potential enteric carcinogen (Makishima *et al.*, 2002). Therefore $1,25(OH)_2D_3$ plays a protective role against colon cancer in a novel mechanism related to secondary bile acid catabolism.

1.3 Vitamin D binding proteins (DBP)

DBP, the transport protein in the plasma, is a 58-kDa-plasma alpha globulin. DBP is primarily synthesized in the liver, and DBP mRNA is also found in other tissues such as kidney, placenta, and testis (McLeod and Cooke, 1989). DBP is able to bind the principal vitamin D metabolites including the major circulating metabolite $25(OH)D_3$ and the most active metabolite $1,25(OH)_2D_3$. One of the major functions of DBP is the binding, solubilization and serum transport of these vitamin D metabolites (White and Cooke, 2000). $1,25(OH)_2D_3$ in the serum is almost entirely bound to DBP. It seems that there is only a single binding site for all identified natural vitamin D_3 metabolites on each DBP molecule. The order of DBP binding

affinity for vitamin D_3 metabolites is as follows: $25(OH)D_3=24,25(OH)_2D_3>$vitamin $D_3>1,25(OH)_2D_3>1,24,25(OH)_3D_3$ (Cooke and Haddad, 1989; White and Cooke, 2000). DBP binding vitamin D_3 metabolites have limited access to the target cells (Cooke and Haddad, 1989). DBP seems to having a buffer role in preventing the free form of vitamin D_3 entering the target cells (Mendel., 1989) and therefore prolongs the half-life of vitamin D_3 and metabolites in the circulation (Brown *et al.*, 1999b). The role of DBP to maintain stable serum stores of vitamin D_3 metabolites was also supported from the DBP null mice (Safadi *et al.*, 1999). DBP null mice were found to be normal except that there were lower serum levels of $25(OH)D_3$ and $1,25(OH)_2D_3$. When maintained on a vitamin D deficient diet for a brief period, the DBP null mice developed secondary hyperparathyroidism and the accompanying bone changes associated with vitamin D deficiency (Safadi *et al.*, 1999).

1.4 Hepatic 25-hydroxylases of vitamin D_3

The liver is considered as the major site for vitamin D_3 25 hydroxylation, although 25-hydroxylase activity has also been found in other tissues such as skin, intestine, and kidney (Gascon-Barré *et al.*, 2001). The enzyme catalyzes the first step in the activation of vitamin D_3 through hydroxylation of carbon 25. Two forms of 25-hydroxylase in the liver (a microsomal and mitochondria form) have been implicated in this process. The microsomal enzyme uses as substrates, vitamin D_3, 1 alpha vitamin D_3, vitamin D_2 and 1 alpha vitamin D_2 and accepts electrons from the NADPH-cytochrome P450 reductase (Armbrecht *et al.*, 1992). The pig microsomal 25 hydroxylase has been purified and cloned, and since it shares 70-80% homology with members of the CYP2D subfamily in primary structure it has been designated CYP2D25 (Wikvall *et al.*, 2001). The mitochondrial vitamin D_3 25-hydroxylase (*CYP27A1*) accepts electrons from NADPH-ferredoxin reductase and ferredoxin (Figure 1.2) (Armbrecht *et al.*, 1992). Recombinant human *CYP27A1* has been shown to catalyze multiple reactions related to vitamin D_3, including 25-hydroxylation of vitamin D_3 (Sawada *et al.*, 2000). Of the

Figure 1.2   Cytochrome P450 system for the CYP27A1 enzyme.

Illustrated is the sequential electron transfer from the NADPH source, through ferredoxin reductase and ferredoxin to the terminal CYP27A1, which directs the 25-hydroxylation of substrate molecules in mitochondria.

two forms of 25-hydroxylase, the microsomal CYP2D25 functions primarily at the physiological substrate concentration in the liver while the mitochondria 25-hydroxylase plays a minor role in contributing to the circulating $25(OH)D_3$ pool (Omdahl *et al.*, 2002). The microsomal enzyme appears to be the important enzyme *in vivo*, since serum $25(OH)_2D_3$ is not altered in patients with cerebrotendinous xanthomatosis (CTX) resulting from *CYP27A1* deficiency (Bjorkhem and Moberg, 1995) or for mice whose *CYP27A1* gene is inactivated (Rosen *et al.*, 1998). However a human microsomal 25-hydroxylase has not yet been identified (Omdahl *et al.*, 2002).

Liver *CYP27A1* synthesis is not considered to be as tightly controlled as *CYP27B1* (*1α(OH)ase*), which is discussed later, and the product of $25(OH)D_3$ reflects the amount of vitamin D status.

1.5   Synthesis of $1,25(OH)_2D_3$ by *1α(OH)ase*

1.5.1   Catalytic properties

1α(OH)ase is a member of the cytochrome P450 family and is a heme-containing protein designated as *CYP27B1* by the Cytochrome P450 Gene Superfamily Nomenclature Committee. It is localized on the inner mitochondrial membrane as part of an electron transport chain that performs hydroxylation reactions using molecular oxygen (Figure 1.3) (Henry, 1997). *1α(OH)ase* catalyzes the C-1 hydroxylation of 25 hydroxylated vitamin D_3 including $25(OH)D_3$ and 24, $25(OH)_2D_3$. The rate of $1,25(OH)_2D_3$ synthesis is greater than that of $1,24,25(OH)_3D_3$ synthesis due to the 10-fold higher concentration of $25(OH)D_3$ compared with $24,25(OH)_2D_3$ though the latter is the preferred substrate for *1α(OH)ase* (Omdahl *et al.*, 2002).

Figure1.3 Cytochrome P450 system for *1α(OH)ase*.

Illustrated is the sequential electron transfer from the NADPH source, through ferredoxin reductase and ferredoxin to the *1α(OH)ase*, which directs 1 α hydroxylation of substrate molecules in mitochondria.

1.5.2 Tissue distribution

It was generally considered that $1\alpha(OH)ase$ is mainly expressed in the kidney proximal convoluted tubule cells (PCT). However, Zehnder *et al.* (1999) have shown that renal expression of $1\alpha(OH)ase$ is not restricted to the PCT, and mRNA and protein are abundantly expressed in more distal areas of the nephron, including the distal convoluted tubule, cortical collecting ducts, medullary collecting duct, thick ascending loop of Henle and papillary epithelium. It is also clear that $1\alpha(OH)ase$ activity and expression are also detected in extrarenal cells including bone cells, intestine, prostate cells, macrophages, keratinocytes, liver, parathyroid gland, pancreas, placenta, colon and lung cancer cells (Segersten *et al.*, 2002; Panda *et al.*, 2001b; Armbrecht *et al.*, 1992; Miller and Portale., 2000; Zehnder *et al.*, 2001). However, the physiological significance of $1\alpha(OH)ase$ in extrarenal tissue and cells remains unclear. Local synthesis of $1,25(OH)_2D_3$ may fulfill autocrine or paracrine functions, such as regulation of cell growth and differentiation.

1.5.3 Molecular cloning

While $1\alpha(OH)ase$ activity was detected in the early-1970s (Fraser and Kodicek, 1973), a cDNA clone was not isolated until the second half of 1997 because little $1\alpha(OH)ase$ protein is expressed in the kidney. The $1\alpha(OH)ase$ cDNA was first isolated from the kidney of mice lacking VDR where the synthesis of $1\alpha(OH)ase$ is increased ten-fold (Takeyama *et al.*, 1997). At the same time, three other independent research groups reported the isolation of rat, human and pig $1\alpha(OH)ase$ cDNA clones (Shinki *et al.*, 1997; Fu *et al.*, 1997; Monkawa *et al.*, 1997; Yoshida T *et al.*, 1999). The human $1\alpha(OH)ase$ gene has been isolated and contains nine exons and eight introns spanning about 6.5 kb with a 1.4kb 5'-flanking region (Monkawa *et al.*, 1997; Kong XF *et al.*, 1999).

1.5.4 Knockout mice and related rickets

As mentioned above $1\alpha(OH)ase$ is the crucial enzyme for controlling the formation of $1,25(OH)_2D_3$. To investigate the *in vivo* actions of $1,25(OH)_2D_3$, a mouse model deficient in $1\alpha(OH)ase$ has been developed and studied (Dardenne *et al.*, 2001; Panda *et al.*, 2001a). In $1\alpha(OH)ase$ knockout mice, no circulating $1,25(OH)_2D_3$ could be detected. In addition, altered non-collagenous matrix protein expression, reduced numbers of osteoclasts and rachitic bone lesions were evident. The $1\alpha(OH)ase$ knock out studies also reveal that $1,25(OH)_2D_3$ is important in reproductive physiology and the immune function (Panda *et al.*, 2001a). After weaning, mice developed hypocalcemia, secondary hyperparathyroidism, retarded growth, and the skeletal abnormalities, which are similar to those in patient with the genetic disorder vitamin D dependent rickets type I (VDDRI, also known as pseudo-vitamin D deficiency rickets) (Dardenne *et al.*, 2001; Panda *et al.*, 2001a). By linkage analysis, mutations in the human $1\alpha(OH)ase$ gene were correlated with the VDDRI since both the $1\alpha(OH)ase$ gene and the VDDRI disease locus were mapped to the chromosomal region 12q13.1-q13.3 in the human genome (St-Arnaud *et al.*, 1997; Fu *et al.*, 1997). Furthermore mutations in the coding regions of the $1\alpha(OH)ase$ gene have been identified in VDDRI patients (Fu *et al.*, 1997; Kitanaka *et al.*, 1998; Yoshida *et al.*, 1998; Wang *et al.*, 2002). Therefore rickets in VDDRI can be treated by replacement therapy with $1,25(OH)_2D_3$ (Glorieux and St-Arnaud, 1998).

1.6 Degradation of $1,25(OH)_2D_3$ by *CYP24*

1.6.1 Tissue distribution

CYP24 is also a member of the cytochrome P450 family (heme containing proteins) localized on the inner mitochondrial membrane where as a member of an electron transport chain it performs hydroxylation reactions (Figure 1.4) (Omdahl and May, 1997).
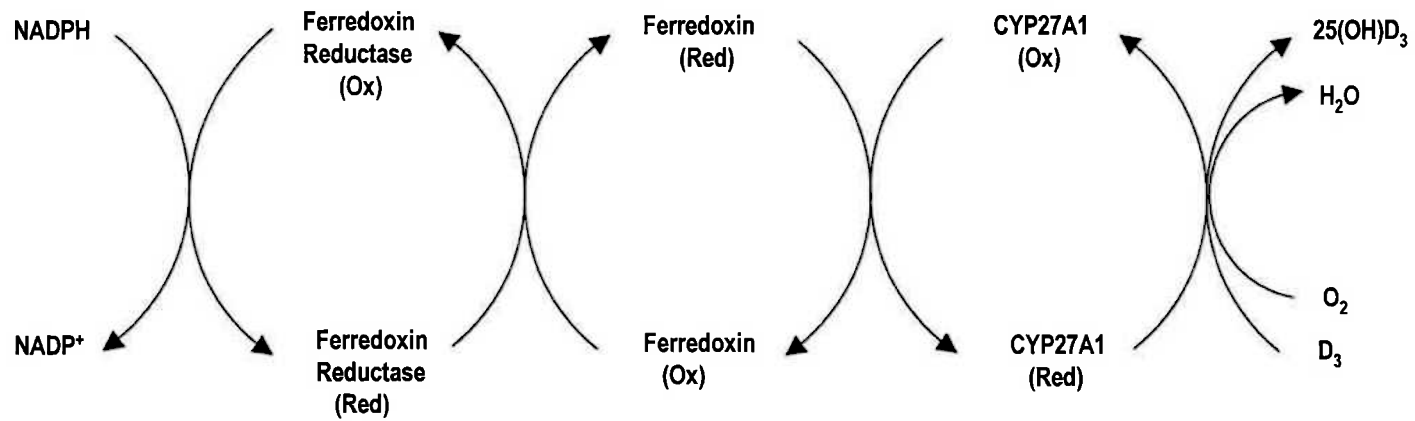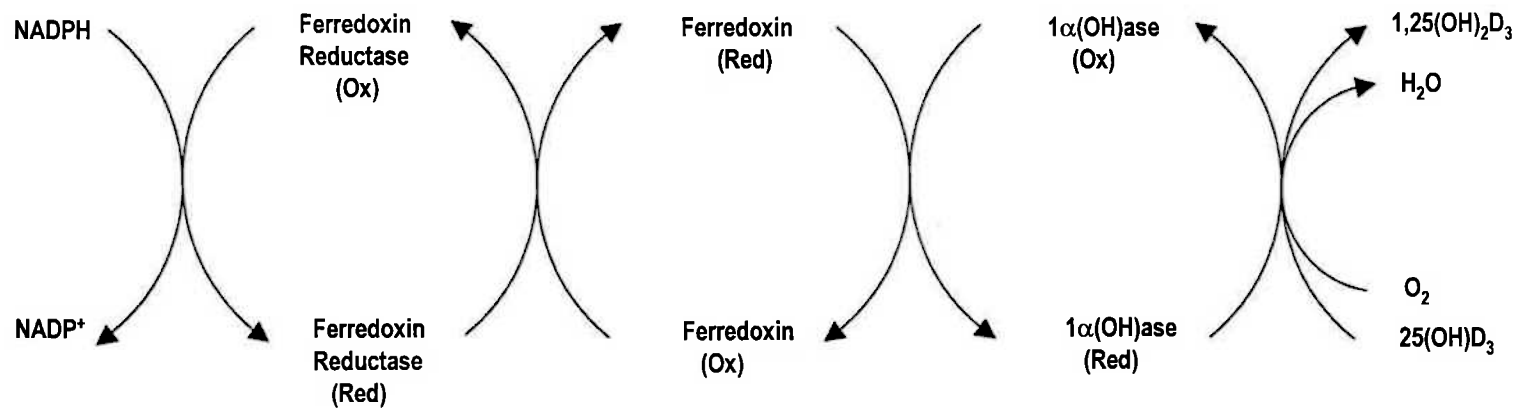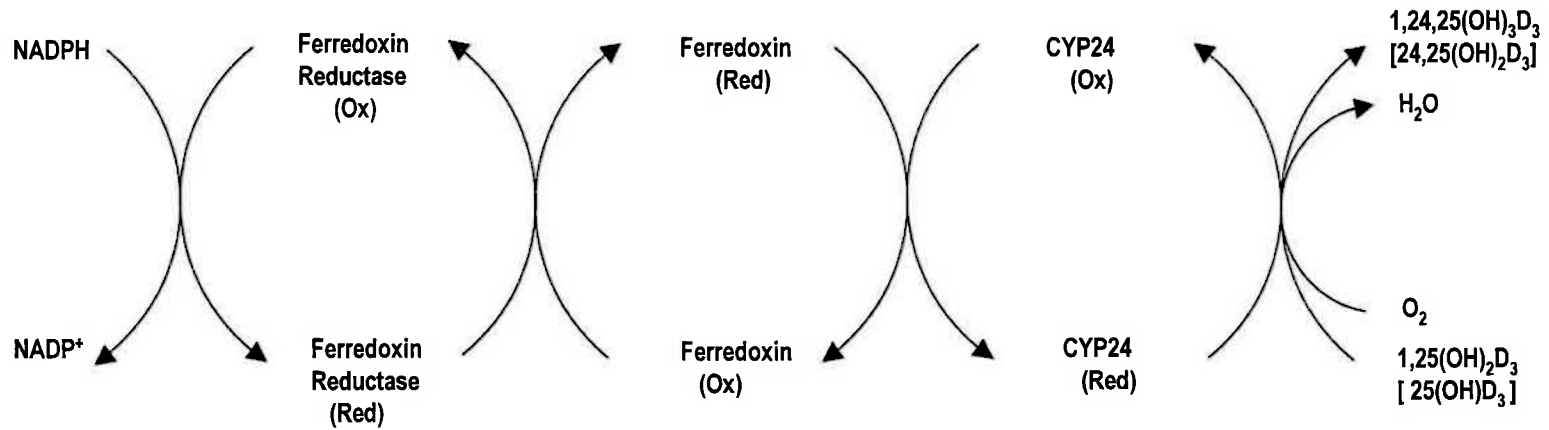
Figure 1.4 Cytochrome P450 system for *CYP24*.

Illustrated is the sequential electron transfer from the NADPH source, through ferredoxin reductase and ferredoxin to the terminal *CYP24* enzyme, which directs 24-hydroxylation of substrate molecules in mitochondria.

The major site of renal *CYP24* activity is the proximal convoluted tubule (PCT) as shown using microdissected nephron segments (Kawashima *et al.*, 1981). Yang *et al.* (1999) has also found *CYP24* expression in the distal nephron. While the highest level of *CYP24* expression occurs in the kidney, *CYP24* is also found to be widely distributed and may be present in every cell and tissue that contains VDR including intestine, bone, skin, thymus, and heart (Akeno *et al.*, 1997). The widespread distribution of *CYP24* suggests that the enzyme plays an important role in regulating the local concentration and action of $1,25(OH)_2D_3$ as well as serum levels.

1.6.2 Catalytic properties

CYP24 is a multicatalytic enzyme that catalyzes the side-chain oxidation of 25-hydroxyvitamin D metabolites such as $25(OH)D_3$ and $1,25(OH)_2D_3$. However, the K_m value of the enzyme for $1,25(OH)_2D_3$ is one-tenth of that for $25(OH)D_3$ (Akiyoshi-Shibata *et al.*, 1994), implying that the former is the real substrate for *CYP24*. The production of $24,25(OH)_2D_3$ is expected to be higher than $1,24,25(OH)_3D_3$ due to the nearly 1000-fold higher concentration of $25(OH)D_3$ relative to $1,25(OH)_2D_3$ (Omdahl and May, 1997).

1.6.2.1 Catabolism of $1,25(OH)_2D_3$ by *CYP24*

The catabolic process performed by *CYP24* is initiated by side chain hydroxylation of $1,25(OH)_2D_3$ at C-23 or C-24. The C-24 pathway is the main activity of the rat enzyme while for the human and guinea pig enzymes, the C-23 pathway is preferred (Omdahl *et al.*, 2002). It is now well established that the C-24 oxidation pathway initiated by *CYP24* plays an important role in inactivating $1,25(OH)_2D_3$ in such target tissues as the kidney, intestine, keratinocytes, bone, cartilage and placenta (Jones *et al.*, 1998; Brown *et al.*, 1999b). The initial step of $1,25(OH)_2D_3$ 24-hydroxylation results in the production of $1,24,25(OH)_2D_3$, a less active hormone with reduced affinity for VDR. The subsequent steps involve oxidation to

C24-OXO (1,25(OH)$_2$-24-OXO-D3), 23-hydroxylation to C23-OH/C24-OXO (1,23,25(OH)$_3$-

24-OXO-D$_3$) (Figure 1.5) (Akiyoshi-Shibata *et al.*, 1994), side-chain cleavage to the C23-

alcohol, and finally oxidation to the C23-carboxylic acid (Calcitroic acid) (Makin et al, 1989).

Carbon 23(C-23) oxidation pathway is initiated by hydroxylation at C-23, by recombinant

human *CYP24* to yield 1α,23S,25(OH)$_3$D$_3$ (Siu-Caldera *et al.*, 1995; Beckman *et al.*, 1996).

The subsequent steps are 26-hydroxylation, formation of 26,23-lactol and finally 25VD-

26,23-lactone (Omdahl *et al.*, 2002).

1.6.3   *CYP24* null mice and *CYP24* transgenic rats

The genes encoding the rat (Ohyama *et al.*, 1992) and human *CYP24* (Chen and DeLuca,

1995) have been isolated. The rat gene spans about 15kb and contains 12 exons (Ohyama *et

al.*, 1992). The human *CYP24* gene is localized at the chromosomal locus 20q13 (Omdahl and

May, 1997). Apparently no disease that implicates a genetic or acquired disorder has been

related to the *CYP24* gene (Omdahl *et al.*, 2002). However homozygous mice null for *CYP24*

exhibit a high level of circulating 1,25(OH)$_2$D$_3$ (St-Arnaud, 1999). This suggests that *CYP24*

performs a protective role when the level of 1,25(OH)$_2$D$_3$ becomes high and potentially toxic.

CYP24 null mice have impaired intramembranous bone formation indicating *CYP24* functions

in bone development and homeostasis (St-Arnaud, 1999). *CYP24* also converts 25(OH)D$_3$

into 24R,25-dihydroxyvitamin D (24,25(OH)$_2$D$_3$) (Figure 1.1) and it has been suggested that

24,25(OH)$_2$D$_3$ may be important for bone growth, development and repair (St-Arnaud, 1999;

Ono *et al.*, 1996; Seo *et al.*, 1997; Seo and Norman, 1997). However, St Arnaud *et al.* (2000)

showed that the bone abnormality could be rescued by crossing the *CYP24* null mice with

VDR null mice demonstrating that elevated 1,25(OH)$_2$D$_3$ and not lack of 24,25(OH)$_2$D$_3$ leads

to the impaired bone formation.

Recently Kasuga *et al.* (2002) generated a line of transgenic rats constitutively expressing

CYP24 to study the biological role of *CYP24 in vivo*. Surprisingly the transgenic rats showed
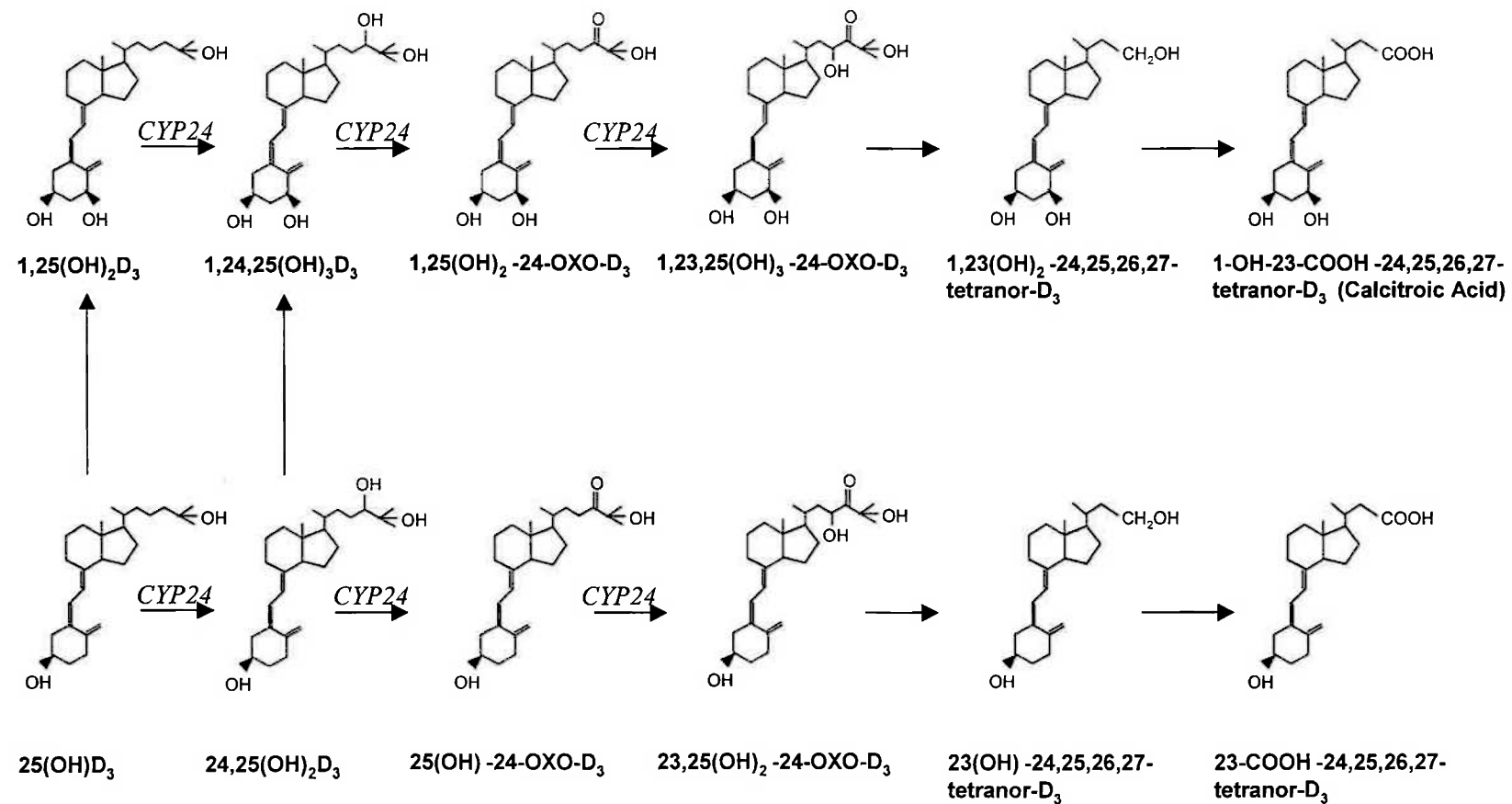
Figure 1.5   The C-24 oxidation pathways.

Two pathways are illustrated for $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ metabolism to side chain cleaved 3-COOH end products.

a low-plasma level of $24,25(OH)_2D_3$ and the mechanism for this reduction is not clear. These rats also unexpectedly developed albuminuria and hyperlipidemia shortly after weaning, suggesting that *CYP24* is involved in functions other than the regulation of vitamin D metabolism.

1.6.4 Another catabolic pathway for $1,25(OH)_2D_3$----C-3 epimerisation pathway

Analysis of *CYP24* knockout mice showed that half the mutant progeny were unaffected by the *CYP24* deficiency (St-Arnaud *et al.*, 2000). Therefore an alternative pathway of $1,25(OH)_2D_3$ catabolism was suggested to exist to regulate circulating levels of the hormone *in vivo*.

A new metabolic pathway, the C-3 epimerisation pathway, has been revealed in human keratinocytes (Reddy *et al.*, 1994), in rat osteosarcoma cells (UMR-106 and ROS17/2.8 cells) (Siu-Caldera *et al.*, 1999), in human colon carcinoma cells (Caco-2 cells) (Messerlian *et al.*, 2000) and in bovine parathyroid cells (Brown *et al.*, 1999a). It seems that C-3 epimerisation pathway is an alternative pathway to the classic C-23/C-24 oxidation pathway to inactivate $1,25(OH)_2D_3$. The new pathway is initiated by epimerisation of the hydroxyl group at carbon 3 of the A-ring from the β to α position. The product is $1\alpha,25$-dihydroxy-3-epi-vitamin D_3 which has a much lower affinity for VDR and less biological activity than $1,25(OH)_2D_3$ (Astecker *et al.*, 2000). It seems that *CYP24* is not involved in the process since ketoconazole, a strong inhibitor of vitamin D hydroxylases and most of the cytochrome P450 enzymes, did not inhibit the production of $1\alpha,25$-dihydroxy-3-epi-D_3 in keratinocytes (Astecher *et al.*, 2000). Thus the enzyme responsible for the process remains unclear. However, *CYP24* seems to be involved in further metabolizing $1\alpha, 25$-dihydroxy-3-epi-D3 (Astecker *et al.*, 2000).

1.7 Regulation of *1α(OH)ase* and *CYP24*

Since *1α(OH)ase* and *CYP24* are crucial enzymes for 1,25(OH)$_2$D$_3$ formation and degradation respectively, regulation of the two enzymes is coordinated through the action of physiological factors to control the ambient serum level of 1,25(OH)$_2$D$_3$. These factors include 1,25(OH)$_2$D$_3$ itself, PTH, calcitonin, serum calcium and phosphate, acidosis, insulin and IGF-I (Henry,1997; Omdahl *et al.*, 2002 and references therein), dexamethasone (Akeno *et al.*, 2000) and TGFβ (Pedrozo *et al.*, 1999). However the major regulators are considered to be PTH and 1,25(OH)$_2$D$_3$. How *1α(OH)ase* and *CYP24* are regulated by PTH, 1,25(OH)$_2$D$_3$ and calcitonin will be described in more detail in later relevant sections. Here I will focus on introducing how the two enzymes are regulated by serum calcium and phosphate levels.

1.7.1 Regulation of *1α(OH)ase* and *CYP24* by serum calcium

Classically serum calcium levels modulate the production of 1,25(OH)$_2$D$_3$ mainly through regulating PTH secretion by the parathyroid glands (Akizawa and Fukagawa, 1999). The process involves a calcium sensing receptor (CaR) (Brown and MacLeod, 2001). CaR is a member of the G protein-coupled receptor family and is located in several cell types including parathyroid cells, kidney cells and thyroid C cells (Brown, 2000). Hypocalcemia stimulates the synthesis and secretion of PTH at transcriptional and posttranscriptional levels (Moallem *et al.*, 1998; Brown and MacLeod, 2001). PTH increases 1,25(OH)$_2$D$_3$ levels by up-regulating *1α(OH)ase* and down-regulating *CYP24* in the kidney (Bland *et al.*, 2001; Omdahl *et al.*, 2002). In hypercalcemia, the secretion of PTH is inhibited and the secretion of calcitonin is stimulated (Jones *et al.*, 1998).

In vitro studies have indicated a direct effect of calcium on *1α(OH)ase* expression in kidney and cell lines independent of PTH (Fukase *et al.*, 1982; Bland *et al.*, 1999; Brown and Pollak, 1998). The direct effect of calcium on *1α(OH)ase* in human proximal tubule HKC-8 cells has been studied by Bland *et al.* (1999). In this study, cells grown in low calcium medium showed

a 4.8-fold induction of *1α(OH)ase* enzyme activity and cells grown in a high level calcium medium exhibited significant inhibition of $1,25(OH)_2D_3$ production. However, the molecular mechanism of this action remains unclear, but may involve CaR. It is known that CaR is coupled to several downstream signaling pathways (PKC and MAPK) (Kifor *et al.,* 1997; Kifor *et al.,* 2001), which may be involved in regulation of *1α(OH)ase* gene expression. *CYP24* enzyme activity was also unexpectedly increased in the presence of low calcium, but remains to be clarified (Bland *et al.,* 1999 and 2001). It remains unclear as to whether there is a direct effect of calcium on these hydroxylases *in vivo.*

1.7.2 Regulation of *1α(OH)ase* and *CYP24* by phosphate

The expression and activities of *1α(OH)ase* and *CYP24* in the kidney are also tightly controlled by phosphate. The synthesis of $1,25(OH)_2D_3$ by *1α(OH)ase* mainly occurs in the renal proximal tubule, which is the major site for calcium and phosphate reabsorption. Dietary phosphate limitation causes serum $1,25(OH)_2D_3$ levels to increase (Engstrom *et al.,* 1985; Tanaka *et al.,* 1983), by upregulating *1α(OH)ase* and down regulating *CYP24* activity and mRNA (Yoshida *et al.,* 2001; Engstrom *et al.,* 1985). Despite the large increase in circulating $1,25(OH)_2D_3$ resulting from the increased *1α(OH)ase* during hypophosphatemia, *CYP24* mRNA levels are reduced. The mechanism is not known and probably due to a reduced VDR content (Wu *et al.,* 1996). The increased $1,25(OH)_2D_3$ then acts on intestine and bone to increase the phosphate level to normal. On the contrary, hyperphosphatemia would result in decreased $1,25(OH)_2D_3$ levels to lower the serum phosphate level in the serum (Brown *et al.,* 1999b).

1.8 Molecular Action of $1,25(OH)_2D_3$

In this section, I will describe the molecular actions of $1,25(OH)_2D_3$, the role of VDR and VDR mediated genomic and non-genomic mechanisms.

1.8.1 Nuclear VDR

The nuclear actions of $1,25(OH)_2D_3$ on target genes are mediated through VDR. The molecular mass of this VDR has been estimated between 48KDa (human VDR) and 60KDa (avian VDR) (Christakos *et al.,* 1996). VDR belongs to the superfamily of nuclear receptors including the receptors of retinoic acid, thyroid hormone, and peroxisome proliferator activator (Carlberg and Polly, 1998; Pike, 1997). All the receptors in the family have similarity in sequence and structure. VDR is composed of several functional domains: an A/B domain (at the N-terminus), a DNA-binding domain (C domain), a hinge domain (D domain), and a ligand binding and transactivation domain (E/F domain at the C terminus) (Pike, 1997). The function of the VDR A/B domain is not known. The DNA-binding domain is the most conserved region in the superfamily of nuclear receptors for interacting with the specific DNA sequence in the promoter of target genes, the vitamin D responsive element (VDRE). The ligand binding domain binds $1,25(OH)_2D_3$ and is involved in dimerization with retinoid X receptor (RXR) (Brown *et al*, 1999b). The ligand-dependent activation function domain (AF2 domain), localized near the COOH terminus of the ligand binding domain, interacts with other transcription factors or coactivators (such as TFIIB, SRC-1, SKIP/NcoA-62 and CBP/p300) for both ligand dependent transactivation and transrepression by VDR (Brown *et al.,* 1999b; Jimenez-Lara and Aranda, 1999; Barry *et al.,* 2003).

The crystal structure of the complex of VDR ligand binding domain (LBD) and $1,25(OH)_2D_3$ has been established (Rochel et al 2000). It reveals the three-dimensional arrangement of the ligand-binding pocket around $1,25(OH)_2D_3$ and allows an understanding of the different binding properties of some synthetic analogs (Rochel *et al.,* 2000). Also reported are the crystal structures of the VDR DNA-binding domain (DBD) complexed with VDRE from three different promoters: osteopontin, canonial DR3 and osteocalcin (Shaffer and Gewirth, 2002). These structures reveal the chemical basis for the binding affinities of the different VDREs.

1.8.2 Vitamin D responsive elements (VDRE)

The predominant form of VDRE is a directly repeated arrangement of the hexameric binding site with three spacing nucleotides and designated as DR-3 (Toell *et al.*, 2000). The consensus sequence of the half site is RGKTSA (R=A or G; K=G or T and S=C or G). Besides this type of VDRE, direct repeats with four or six spacing nucleotides (DR4- and DR6- type VDREs) and inverted palindromes with nine intervening nucleotides (IP9-type VDREs) have been reported (Carlberg and Polly, 1998).

1.8.3 VDR and RXR forms a heterodimer

In the absence of ligand, VDR-RXR heterodimer binds to VDRE and recruits a co-repressor complex, such as RIPδ1 (Dwivedi *et al.*, 1998) and Alien (Polly *et al.*, 2000), which repress basal expression most likely through associated histone-deacetylase to facilitate a closed chromatin structure (Omdahl *et al.*, 2002). Once 1,25(OH)$_2$D$_3$ binds to VDR, the conformation change of the VDR-RXR heterodimer results in the release of the corepressor complex, and facilitates the interaction with coactivators. Therefore a "mouse trap" mechanism has been proposed for VDR activation based on the crystal structure of the human retinoic acid receptor γ (Renaud *et al.*, 1995); ligand induced conformation transition in the nuclear receptor LBD results in the modification of AF-2 activating domain whereby the combination of α-helix 12 and α-helices 3 and 5 together creates a docking site for a coactivator of the p160 family (Nolte *et al.*, 1998; Rachez and Freedman, 2000). Such activators with histone acetyltransferase (HAT) activity could modify the chromatin structure. This type of coactivator is then replaced by another coactivator complex lacking HAT activity but interacting with RNA polymerase II (such as Vitamin D Receptor Interacting Protein (DRIP) complex) to initiate transcription (Chinba *et al.*, 2000). Very recently, Barry et al

(2003) have shown that the distinct coregulator Ski-interacting protein (SKIP)/NcoA-62 interacts with VDR through α-helix 10 interface, not the AF-2 domain.

1.8.4   Regulation of VDR

Regulation of VDR abundance plays an important role in modulating the cellular response to $1,25(OH)_2D_3$ (Shao *et al.*, 2001; Krishnan and Feldman, 1997). VDR mRNA and protein expression have been shown to be regulated by $1,25(OH)_2D_3$ and other hormones in a species-, tissue-, and cell type-specific manner, and the physiological mechanism of this regulation affects the synthesis of VDR, not the affinity of VDR for $1,25(OH)_2D_3$ (Krishnan and Feldman, 1997). It has been shown that $1,25(OH)_2D_3$ up regulates the VDR level in renal and intestinal cells (Costa and Feldman, 1986; Krishnan and Feldman, 1997; Issa *et al.*, 1998). This makes sense since $1,25(OH)_2D_3$ will act more efficiently on target genes if the level of $1,25(OH)_2D_3$ is increased. However, the molecular mechanism by which $1,25(OH)_2D_3$ regulates VDR abundance remains unclear (Jehan and DeLuca, 2000; Brown *et al.*, 1999b).

The mouse (Jehan and DeLuca, 1997), chicken (Lu *et al.*, 2000) and human (Miyamoto *et al.*, 1997) VDR promoters and genes have been cloned and thus provide the basis for studying transcriptional regulation. The VDR promoter from different species contains no TATA box. The promoter of mouse VDR is driven mainly though Sp1 binding sites (Jehan and DeLuca, 2000) and in NIH3T3 cells the activity of the promoter increases 3-5 fold in response to forskolin, an activator of the protein kinase A (PKA) pathway (Jehan and DeLuca, 1997). Analysis of the 1.5kb sequence of the mouse VDR promoter failed to find any consensus VDRE (Jehan and DeLuca, 1997), which suggests that a VDRE might be localized farther upstream in the promoter or there might be a different sequence from the classical one. The human VDR (hVDR) has exons 1A, 1B and 1C for the 5'-end noncoding region (Miyamoto *et al.*, 1997). Since upstream exons of the hVDR incorporated into variant transcripts have been identified, it suggests that more than one promoter may direct hVDR gene expression leading to multiple mRNA and protein isoforms (Crofts, 1998; Miyamoto *et al.*, 1997). Crofts *et al.*

(1998) have reported that different VDR isoforms are expressed in different tissues. Sunn *et al.* (2001) discovered a novel N-terminally variant human VDR that has different transactivation capacity and subcellular localization. However, no further investigation has been performed on the molecular regulation of the VDR gene by $1,25(OH)_2D_3$.

1.8.5 Phosphorylation of VDR

In addition to the upregulation of VDR abundance, phosphorylation of VDR can enhance the VDR-mediated transcription by increasing the interaction between VDR and coactivators such as DRIP205 (Barletta *et al*, 2002). VDR can be phosphorylated by PKC (Hsieh *et al.,* 1991), casein kinase II (Jurutka *et al.,* 1996) and PKA (Jurutka and Hsieh, 1993) *in vitro,* however the *in vivo* role of this phosphorylation is not clear. Since VDR contains no consensus sequence for MAP kinases (MAPK) (Solomon *et al.,* 1999), it seems that the MAPK pathway is not involved in VDR phosphorylation. Recent data by Dwivedi *et al.* (2002) in our laboratory also agree with this; ERK1/2 could not phosphorylate VDR in an *in vitro* kinase assay.

1.8.6 VDR knockout animal and related rickets

The lack of functional VDR leads to an insensitivity to $1,25(OH)_2D_3$ in over 30 target tissues, as shown in patients with hereditary 1,25 dihydroxyvitamin D-resistant rickets (VDDR II) and VDR knock out mice (Ylikomi *et al.,* 2002). VDDR II is caused by missense mutations in the VDR DNA binding domain (DBD) (Malloy *et al.,* 1994; Rut *et al.,* 1994) or ligand-binding domain (LBD) (Malloy *et al.,* 1990 and 2001) or truncation of VDR through premature termination codons (Ritchie *et al.,* 1989). In this type of rickets there is a decrease in intestinal calcium and phosphate absorption leading to decreased bone mineralization and rickets with hypocalcemia, hypophosphatemia and elevated serum levels of alkaline phosphatase. After weaning VDR homozygous knockout mice exhibit growth retardation, impaired bone formation, and uterine hypoplasia with impaired folliculogenesis (Yoshizawa *et al.,* 1997). It

21

is surprising that a VDR knock out mouse could be born alive and could be rescued with a high Ca^{2+} diet to appear essentially normal, except for the slow development of alopecia (Amling *et al.*, 1999).

1.8.7 Non-genomic actions of 1,25(OH)$_2$D$_3$

1,25(OH)$_2$D$_3$ mediated induction of target genes requires nuclear VDR and transcriptional activation. This response is referred to as the genomic response. A more rapid response to 1,25(OH)$_2$D$_3$ occurs in minutes and this involves activation of signaling pathways such as PKC and MAP kinases (Nemere *et al.*, 1998; Dwivedi *et al.*, 2002). This is the non-genomic response.

It has been suggested that the non-genomic response may be due to a plasma membrane VDR distinct from the classic nuclear VDR. For example, genomic and non-genomic regulations are achieved by quite different shapes of 1,25(OH)$_2$D$_3$, and especially a 6-s-cis locked analog of 1,25(OH)$_2$D$_3$ which preferentially activates the non-genomic response (Norman *et al.*, 2002 and references therein). Furthermore, for the rapid response of transcaltachia (the rapid hormonal stimulation of intestinal calcium transport), a candidate membrane VDR receptor was reported which had a different ligand binding affinity and molecular weight from nuclear VDR (Nemere *et al.*, 1994). However, there has been no further information on this.

Recently, Capiati et al (2002) showed that 1,25(OH)$_2$D$_3$ induces translocation of the VDR from the nucleus to the plasma membrane in skeletal muscle cells in a short-term (1-10min), and the relocation process involves microtubular transport and tyrosine kinases. The result suggested that nuclear VDR may be the receptor that mediates the non-genomic effect of 1,25(OH)$_2$D$_3$. The rapid nongenomic responses to 1,25(OH)$_2$D$_3$ are abrogated in osteoblasts prepared from VDR null mice in which the first zinc finger for DNA binding was deleted, further supporting the conclusion that the classical VDR mediates the nongenomic actions of 1,25(OH)$_2$D$_3$ (Erben *et al.*, 2002). Erben et al (2002) suggested that the nuclear VDR when

acting at the plasma membrane interacts with proteins so that its ligand specificity is altered, thus explaining the apparent selectivity of $1,25(OH)_2D_3$ analogues for the non-genomic response. The precise molecular mechanisms by which $1,25(OH)_2D_3$ mediates the non-genomic effects is currently of great interest. There is evidence that $1,25(OH)_2D_3$ can in different cell types activate PKA (Vazquez et al., 1997), PKC (Morelli et al., 1996), ERKs (Dwivedi et al., 2002), Src (Buitrago et al, 2002) and PI 3K (Buitrago et al., 2002). The critical issues are whether the nuclear VDR is involved and if so how it couples to the signaling pathways.

1.8.8 Regulation of *1α(OH)ase* and *CYP24* by $1,25(OH)_2D_3$

1.8.8.1 Regulation of *1α(OH)ase* by $1,25(OH)_2D_3$ in kidney and keratinocytes

The inhibitory effect of $1,25(OH)_2D_3$ on *1α(OH)ase* gene expression has been studied in the kidney. A direct inhibitory effect of $1,25(OH)_2D_3$ on *1α(OH)ase* mRNA and activity has been demonstrated in both cultured kidney cells and intact animals (Murayama et al., 1999; Tanaka and DeLuca, 1984; Henry and Luntao, 1989). Expression of *1α(OH)ase* was suppressed by $1,25(OH)_2D_3$ in VDR+/+ mice and VDR+/- mice, but not in VDR -/- mice (Takeyama et al., 1997). These results suggest that the negative feedback regulation of *1α(OH)ase* by $1,25(OH)_2D_3$ requires liganded VDR. Negative vitamin D responsive elements (nVDRE) have been identified in gene promoters for the human PTH and the rat bone sialoprotein gene (Mackey et al., 1996; Li and Sodek, 1993). The nVDRE of the human PTH promoter differs from positive VDREs both in sequence composition and in affinity for VDR/RXR heterodimer. While no nVDRE in the promoter of the *1α(OH)ase* gene has been identified, a region that underlies repression has been reported (Murayama et al., 2000). However, the VDR does not directly bind to this region in the promoter and the mechanism has not been fully elucidated (Murayama et al., 2001).

1,25(OH)$_2$D$_3$ mediates inhibition of *1α(OH)ase* enzyme activity in keratinocytes but does not

affect *1α(OH)ase* mRNA or protein levels (Xie *et al.*, 2002). Xie *et al.* (2002) found that in

keratinocytes inhibition of *1α(OH)ase* enzyme activity by 1,25(OH)$_2$D$_3$ was reversed when

CYP24 activity was blocked with a specific *CYP24* inhibitor VID 400. The data demonstrate

that the apparent low level of *1α(OH)ase* activity in the presence of 1,25(OH)$_2$D$_3$ results from

inactivation of the substrate 25(OH)D$_3$ and the product 1,25(OH)$_2$D$_3$ by induced *CYP24*.

These authors suggest that studies purporting to establish a negative regulation of *1α(OH)ase*

by 1,25(OH)$_2$D$_3$ need to be reconsidered. For example in VDR null mice, there is increased

1α(OH)ase mRNA (Takeyama *et al.*, 1997) an this may reflect increased PTH rather than an

effect of 1,25(OH)$_2$D$_3$ (Xie *et al.*, 2002). As described above preliminary data indicates a

repressive region in the *1α(OH)ase* promoter that responds to 1,25(OH)$_2$D$_3$ (Murayama *et al.*,

2000). Perhaps this inhibitory effect of 1,25(OH)$_2$D$_3$ on *1α(OH)ase* mRNA production is

tissue specific, occurring in kidney but not in keratinocyte cells.

1.8.8.2 Regulation of *CYP24* by 1,25(OH)$_2$D$_3$ in kidney

1,25(OH)$_2$D$_3$ functions to induce *CYP24* gene expression and increase *CYP24* enzyme levels

in different tissues including kidney and bone (Omdahl *et al.*, 2002; Jones *et al.*, 1998).

The molecular mechanism by which 1,25(OH)$_2$D$_3$ increases expression of the *CYP24* gene

has been analyzed in great detail since the gene was cloned from rat (Ohyama and Okuda,

1991, Ohyama *et al.*, 1991), human (Chen *et al.*, 1993) and mouse (Akeno *et al.*, 1997). The

regulation of *CYP24* by 1,25(OH)$_2$D$_3$ requires the VDR as shown by VDR knock out mice

(Takeyama *et al.*, 1997). In promoter studies (Hahn *et al.*, 1994; Kerry *et al.*, 1996; Zierold *et

al.*, 1995), two VDREs were identified in the promoter of the rat *CYP24* gene and were

responsible for vitamin D activation. These two VDREs act in a synergistic manner. An Ets-1

binding site (EBS) was also identified which interacts with VDR/RXR complex bound to the

proximal VDRE and assists in the transactivation process (Dwivedi *et al.*, 2000). $1,25(OH)_2D_3$ apparently activates Ras dependent MAP kinase pathways leading to phosphorylation of transcription factors Ets-1 and RXRα (Dwivedi *et al.*, 2002). The PKC pathway is also involved in $1,25(OH)_2D_3$ mediated induction of *CYP24* promoter activity (Armbrecht *et al.*, 1997; Chen *et al.*, 1993), but the downstream responsive elements have not been identified.

In the next two sections, I would like to introduce two other important hormones for calcium regulation, PTH and calcitonin. I would like to deal with their receptors, the elicited signaling pathways and the regulations of *1α(OH)ase* and *CYP24* expression in kidney and bone cells.

1.9   Molecular action of PTH

1.9.1   PTH

PTH is synthesized and secreted by the parathyroid gland to regulate serum calcium levels. When the serum calcium level is lowered, the production of PTH by the parathyroid is up regulated through CaR (Brown and MacLeod, 2001). The secreted PTH targets chiefly kidney and bone.

PTH stimulates *1α(OH)ase* activity and inhibits *CYP24* activity in the kidney (Murayama *et al.*, 1999; Brenza *et al.*, 1998; Shigematsu *et al.*, 1986; Zierold *et al.*, 2001) as discussed in later sections, and this results in an increase of $1,25(OH)_2D_3$-mediated calcium uptake by the intestine and increased reabsorption of calcium by the kidney (Brown *et al.*, 1999b). Also $1,25(OH)_2D_3$ together PTH increase calcium mobilization from bone to maintain the concentration of the ion (Mannstadt *et al.*, 1999). In this process, PTH acts directly on osteoblasts that possess PTH receptor (PTHR) and indirectly on osteoclasts that lack this receptor (Swarthout *et al.*, 2002). PTH stimulates production of RANKL on osteoblasts/ stromal cells and enhances the interaction of RANKL with RANK, the transmembrane

receptor on osteoclast precursor cells, resulting in the stimulation of the maturation of osteoclasts and consequently of bone resorption (Aubin and Bonnelye, 2000). As mentioned earlier (see section (1.1.2), $1,25(OH)_2D_3$ also participates in the stimulation of osteoclastogenesis.

1.9.2 Signaling pathway and down stream transcription factors mediated by PTHR

PTHR couples to at least two different types of heterotrimeric G proteins, Gs and Gq (Abour-Samra et al., 1992). Therefore it activates at least two downstream signaling systems: the AC/cAMP, and the PLC/inositol phosphate/diacyl glycerol/ intracellular calcium signaling system (Abou-Samra et al., 1992). It was recently shown that PTH at low concentrations stimulates ERK1/2 MAP kinase activity in a PKC-dependent manner in both osteoblastic and kidney cells (Swarthout et al., 2002). MAPK p38 has been shown to be involved in PTH mediated regulation of chondrocyte differentiation (Zhen et al., 2001). However, only a couple of target transcription factors that respond to PTHR signaling are known. PTH induces the c-fos promoter in bone cells through phosphorylation of cAMP response element (CRE)-binding protein that binds to the major CRE (Pearman et al., 1996). The activator protein-1 (AP-1) and the runt domain binding sites were identified as necessary for PTH-induced rat collagenase-3 promoter activity in osteoblastic cells (Selvamurugan et al., 1998).

1.9.3 Regulation of *1α(OH)ase* and *CYP24* by PTH

Abundant evidence has shown that in kidney, PTH plays an important role in $1,25(OH)_2D_3$ metabolism. PTH can up regulate *1α(OH)ase* and down regulate *CYP24* gene expression in the kidney to control the systemic $1,25(OH)_2D_3$ level (Omdahl and May, 1997; Omdahl et al., 2002; Henry,1997). After parathyroidectomy, serum levels of $1,25(OH)_2D_3$ decrease but increase after administration of PTH to the avian and mammalian species (Henry, 1997).

1.9.3.1 Regulation of *1α(OH)ase* by PTH in kidney

PTH is considered as the primary up regulator for *1α(OH)ase* in the kidney. The molecular action of PTH on *1α(OH)ase* has been studied. Brenza *et al.* (1998) demonstrated that PTH could stimulate the mouse *1α(OH)ase* promoter-directed synthesis of luciferase by 17-fold in kidney MCT cells. Kong *et al.* (1999) reported that PTH could stimulate constructs of the *1α(OH)ase* promoter (-1.4kb) about two fold in a kidney AOK-B50 cells. In the human *1α(OH)ase* promoter, the -0.5kb region has been identified as a PTH responsive region (Murayama *et al.*, 2001). Cyclic AMP (cAMP) and forskolin have been shown to mimic the induction of *1α(OH)ase* by PTH in cultured kidney cells (Yoshida T *et al.*, 1999; Kong XF *et al.*, 1999; Korkor *et al.*, 1987). Taken together, these data suggest that the activation of *1α(OH)ase* by PTH is mediated by a cAMP dependent pathway. However no PTH responsive elements in the *1α(OH)ase* promoter have yet been identified. Therefore, one major aim of my current study is to understand the molecular mechanism by which PTH regulates the promoter of the human *1α(OH)ase* gene.

PTHR is also found in osteoblasts, but no information has been reported as to whether PTH regulates *1α(OH)ase* expression in these cells.

1.9.3.2 Regulation of *CYP24* by PTH in kidney and bone

It is well documented that PTH down regulates *CYP24* activity and mRNA in kidney (Armbrecht *et al.*, 1984; Henry *et al.*, 1992). In this event, the level of the circulatory $1,25(OH)_2D_3$ will be increased. The molecular mechanism of the regulation has been studied. PTH is not able to down regulate the *CYP24* promoter activity in transfected kidney cells, but instead modifies *CYP24* mRNA stability (Zierold *et al.*, 2000). In transient transfection assays the PTH action sites for destabilization were not found in either the 5' or 3' untranslated regions and hence must lie in the coding region (Zierold *et al.*, 2001).

PTH also can partially or completely block the $1,25(OH)_2D_3$-mediated induction of *CYP24* mRNA and activity in the kidney (Shinki *et al.*, 1992; Armbrecht *et al.*, 1984; Henry, 1985; Henry *et al.*, 1992). The results suggest that the renal expression of *CYP24* gene is regulated by a balance of PTH and $1,25(OH)_2D_3$ levels in serum.

The regulation of *CYP24* by PTH in bone is different from that in kidney cells. In rats fed a low calcium diet to increase serum PTH and $1,25(OH)_2D_3$, *CYP24* activity is increased in bone but decreased in the kidney (Nishimura *et al.*, 1994). PTH alone has no effect on *CYP24* levels in bone UMR-106 cells, but acts synergistically with $1,25(OH)_2D_3$ to induce the *CYP24* mRNA (Armbrecht *et al.*, 1998), protein (Armbrecht *et al.*, 1998) and activity (Armbrecht and Hodam 1994). The synergistic induction of $1,25(OH)_2D_3$ and PTH on *CYP24* mRNA is through the cAMP signaling pathway (Armbrecht *et al.*, 1998). The molecular mechanism of the synergistic effect is not understood and the biological rationale of this phenomenon is still not clear.

1.10   Molecular action of calcitonin

1.10.1   Calcitonin

Calcitonin is a 34-amino acid peptide hormone produced by the C cells or the parafollicular cells of the thyroid gland, in response to a hypercalcemic signal (Jones *et al.*, 1998). It directly acts on osteoclasts through calcitonin receptor (CTR) inhibiting osteoclastic bone resorption and osteoclast differentiation and reducing the calcium mobilizing activity (Inzerillo *et al.*, 2002). It also acts on kidney enhancing calcium excretion by inhibition of renal tubular calcium resorption (Pondel, 2000). Calcitonin has been used clinically for treatment of a variety of bone disorders, including Paget's disease, hypercalcemia of malignancy, and osteoporosis (Martin, 1999; Pondel, 2000).

1.10.2 Calcitonin receptor (CTR)

Calcitonin binds to its membrane receptor CTR, which like PTHR mentioned above, belongs to the G-protein coupled receptor superfamily with a seven-transmembrane domain (Pondel, 2000). The cloning of calcitonin receptor has disclosed that multiple isoforms are present in a tissue-specific and species-specific manner, which result from alternative splicing of transcripts from a single gene. These isoforms have distinct ligand binding specificity and /or signal transduction pathway utilization and thus could contribute to the modulation of the cellular response to calcitonin. At present at least five isoforms of calcitonin receptor have been identified in different species (Sexton *et al.*, 1999; Shyu *et al.*, 1996; Ho *et al.*, 1999). The most common one, C1a, was widely distributed *in vivo* in different species (eg. rat, pig, mouse and human). This isoform is also referred to as "an insert negative form" in chapter 5. Compared with other isoforms, C1a is the most abundant isoform and occurs at a relatively constant level in all expressing tissues (Ikegame *et al.*, 1995; Kuestner *et al.*, 1994; Gorn *et al.*, 1995). Another isoform, which contains a 16 amino acid insert in the putative first intracellular loop, has been reported in pig and human (Pondel, 2000; Gorn *et al.*, 1995). This isoform appears to be at low levels in most tissues and its expression levels to be much more variable. Compared to C1a, it has a similar or higher binding affinity for calcitonin, but an altered G-protein coupling efficiency leading to a loss of Gq mediated intracellular calcium mobilization and attenuation of Gs-mediated response. It does not signal via PLC/Ca2+-mediated pathways, although it retains some signaling ability via coupling to AC (Kuestner *et al.*, 1994; Moore *et al.*, 1995; Gorn *et al.*, 1995).

1.10.3 Signaling pathway and down stream transcription factors

The calcitonin receptor isoform C1a is well known to couple to multiple heterotrimeric G proteins (G_i, G_q and G_s), leading to downstream cellular responses (Pondel, 2000; Sexton *et al.*, 1999). It is well established that stimulation of CTRs (C1a) results in the activation of the downstream pathways: the AC/cAMP/PKA pathway (Force *et al.*, 1992), the

phosphoinositide-dependent PLCβ/calcium mobilization/PKC pathway (Chabre *et al.*, 1992; Force *et al.*, 1992) and the phosphatidylcholine-dependent phospholipase D pathway/ PKC pathway (Naro *et al.*, 1998). Chen *et al.* (1998) reported that in HEK 293 cells, Shc tyrosine phosphorylation and the activation of the serine/threonine kinases ERK1/2 are elicited by calcitonin.

The downstream transcription factors that respond to calcitonin have not been well investigated. The only report shows that Sp1 binding sites are involved in the calcitonin mediated induction of the human p21[WAF1/CIP1] promoter activity (Evdokou *et al.*, 2000).

1.10.4 Regulation of *1α(OH)ase* and *CYP24* by calcitonin in the kidney

Studies have shown that calcitonin is involved in the regulation of vitamin D metabolism (Galante *et al.*, 1972 and Beckman *et al.*, 1994), but how calcitonin regulates expression of *1α(OH)ase* and *CYP24* is still not well understood.

1.10.4.1   Regulation of *1α(OH)ase* by calcitonin

In early studies, Galante *et al.* (1972) reported that calcitonin enhances production of $1,25(OH)_2D_3$. Calcitonin enhances renal conversion of $25(OH)D_3$ into $1,25(OH)_2D_3$ in vitamin D-deficient rats and calcitonin is the major regulator of *1α(OH)ase* in normocalcemic rats (Shinki *et al.*,1999). Calcitonin induces *1α(OH)ase* mRNA in kidney LLC-PK1 cells, proximal tubule cells (Yoshida N *et al.*, 1999) and also enhances the *1α(OH)ase* activity in human cortical collecting ducts HCD cells (Bland *et al.*, 2001). However, Rasmussen *et al.* (1972) reported that the *1α(OH)ase* activity was actually inhibited by porcine calcitonin *in vitro* using isolated renal tubules from vitamin D-deficient chicks. In addition, calcitonin has no direct effect on *1α(OH)ase* enzyme activity in chick and rat cells (Henry *et al.*, 1997). Bland *et al.* (2001) reported that calcitonin has no effect on *1α(OH)ase* enzyme activity in

proximal convoluted tubules HKC-8 cells. The molecular action of calcitonin on $1\alpha(OH)ase$ expression has also been analyzed using transient transfection assays. In mouse kidney proximal tubule MCT cells, a 0.85 kb region (-889/-30bp) upstream of the human $1\alpha(OH)ase$ gene has been identified as a regulating region responding to calcitonin (Murayama *et al.*, 1998) but the precise calcitonin responsive element has not been identified. Overall, it can be seen that different groups have reported that calcitonin can either up regulate, down regulate or have no effect on $1\alpha(OH)ase$ expression in the kidney. These discrepancies may result from different isolated cells and different experimental condition.

1.10.4.2   Regulation of *CYP24* by calcitonin in kidney

In rat, it has been reported that calcitonin negatively regulates intestinal *CYP24* mRNA expression and activity (Beckman *et al.*, 1994). This implies that calcitonin blocks the degradation of $1,25(OH)_2D_3$ and thus potentiates the toxicity of excess vitamin D_3. However, such a proposal does not fit with the use of calcitonin to treat hypercalcemia. Calcitonin does not affect expression of *CYP24* enzyme activity in either the human cortical collecting duct cell line (HCD) where the hormone induced $1\alpha(OH)ase$ activity or the proximal tubule cell line HKC-8 where the hormone has no effect on $1\alpha(OH)ase$ activity (Bland *et al.*, 2001). At present there is little information on whether or how calcitonin regulates *CYP24* expression in kidney. Therefore one major aim of the thesis was to study how calcitonin affects *CYP24* expression in the kidney and to investigate the relevant molecular mechanism.

1.11   General aim

The general aim of my PhD project is to understand the molecular mechanism by which PTH regulates $1\alpha(OH)ase$ promoter expression, and $1,25(OH)_2D_3$ and calcitonin regulate *CYP24* promoter expression in kidney and/or osteoblast cells, target tissues of the three hormones.

+ +
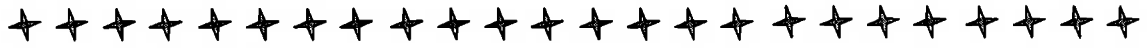
CHAPTER 2
Materials and Methods

+ +
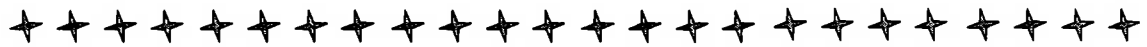
2.1 Materials

2.1.1 Drugs, chemicals and Reagents

Tetrionics, Inc, Madison (WI, USA): 1,25(OH)$_2$D$_3$;

Peninsula Laboratory, INC.: Salmon calcitonin;

Sigma Chemical Co.: Rat PTH (1-34), 40% acrylamide, agarose, ampicillin, bovine serum albumin (BSA), charcoal (activated), deoxyribonucleotide triphosphates (dNTPs), ethidium bromide, ethylenediaminetetra-acetic acid (EDTA), N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (Hepes), salmon sperm DNA, sodium dodecyl sulphate (SDS), phenylmethylsulfonyl fluoride (PMSF), Tris-base, 8-(4-Chlorophenylthio)-Adenosine 3'; 5'- Cyclic Monophosphate (8-CPT-cAMP), 3-isobutyl-1-1-methylxanthine (IBMX), Forskolin and Phorbol 12-myristate 13-acetate (PMA), G418 and Puromycin.

Sources of other important reagents were as follows:

BIOMOL Research Laboratories (INC. USA): H89, calphostin C, SB203580, PD98059, U0126, LY294002.

Promega (Madison, WI): Dual luciferase assay system and luciferase assay system.

Life Tech. BRL: LipofectAMINE 2000.

Boehringer Mannheim Biochemicals (Germany): DOTAP liposomal transfection reagent.

Pharmacia: poly(dI-dC).

Tokyo Kasei: N,N,N',N'-tetramethethylethenediamine (TEMED).

Roche Diagnostics Corp. (Indianapolis, IN, USA): Cesium chloride.

Qiagen Pty. Ltd: DNA gel extraction kit and plasmid preparation kits (Midi and Maxi).

2.1.2 Radiochemicals

[α-^{32}P] dCTP (10μCi/μl) was purchased from Geneworks.

2.1.3 Enzymes

<u>Geneworks</u>: Restriction enzymes, T4 DNA ligase, Pfu polymerase and E.coli DNA polymerase I (Klenow fragment).

<u>Sigma</u>: Ribonuclease A (RNaseA) and Lysozyme.

<u>Boehringer Mannheim</u>: Proteinase K, T4 DNA polymerase and Calf Intestinal Phosphate (CIP).

2.1.4 Buffers and solutions

TBE: 90mM Tris, 90mM boric acid, 2.5mM EDTA, (pH 8.3).

TE: 10mM Tris-HCl (pH 7.5), 1mM EDTA.

GTE buffer: 50mM Glucose, 25mM Tris-HCl (pH8.0), 10mM EDTA (pH 8.0).

Transformation Buffer I (Tfb I): 30mM KAc, 100nM RbCl, 10mM $CaCl_2$ ($2H_2O$), 50mM $MnCl_2$ ($4H_2O$) and 15% Glycerol.

Transformation Buffer II (Tfb II): MOPS (acid), 10mM RbCl, 75mM $CaCl_2$ ($2H_2O$) and 15% Glycerol.

Solution I for plasmid mini-preparation: 50mM Glucose, 25mM Tris·Cl, 10mM EDTA (pH8.0).

Solution II for plasmid mini-preparation: 0.2M NaOH, 1% SDS.

Solution III for plasmid mini-preparation: 5M KAc, 11.5% (v/v) acetic acid.

6×DNA Loading Buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% (w/v) sucrose in water.

Nuclear Extract Lysis Buffer I: 10mM Hepes-KOH, pH7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT and 0.2mM PMSF.

Nuclear Extract Lysis Buffer II: 20mM Hepes-KOH, pH 7.9, 25% Glycerol, 420mM NaCl, 1.5mM $MgCl_2$, 10mM KCl, 0.2mM EDTA, 0.5mM DTT and 0.2mM PMSF.

TM-1 Buffer: 25mM Tris-HCl (pH7.6), 100mM KCl, 0.5mM dithiothreitol, 5mM $MgCl_2$, 0.5mM EDTA and 10% glycerol.

10×Klenow Buffer: 70mM Tris-HCl (pH7.5), 70mM MgCl$_2$.

5×Annealing Buffer: 200mM Tris-HCl (pH7.5), 100mM MgCl$_2$, 250mM NaCl.

5×Ligation Buffer: 250mM Tris-HCl (pH 7.6), 50mM MgCl2, 5mM DTT, 25% (w/v) PTG-6000.

One-Phor-All buffer: 100mM Tris-acetate (pH7.5), 100mM magnesium acetate, 500mM potassium acetate.

All buffers were sterilized by autoclaving or filtration through a 0.2μm filter where necessary.

2.1.5   Cloning vector

pGL3 basic vector and PRL-TK-Luc was purchased from Promega.

Gal (0) and pNL-VIP16 (0) were generously provided by Dr. G Muscat, University of Queensland.

2.1.6   Cloned DNA Sequences

The following cloned DNA sequences were kind gifts from the following:

hCTR and hPTHR were generously provided by Dr. D Findlay (IMVS, Adelaide).

pNF-YAm29 and pNF-YA expression clones were kindly provided by Dr. R Mantovani (University of Milan, Italy).

pPac-NFYA, B and C were generously given by Dr. T Osborne (University of California, USA.) (Liang *et al.*, 2001).

Human Sp1 expression clone was from Dr. M Crossley (Children's Hospital, Boston, USA).

pPac-Sp1 and pPac-Sp3 were provided by Dr. MR Waterman (Vanderbilt University, USA) (Ahlgren *et al.*, 1999).

Overexpression clones of C/EBP α, β and δ were from Dr. RH Costa (University of Illinois, USA).

A-C/EBP was kindly provided by Dr. C Vinson (National Institutes of Health, USA).

NF-κB overexpression clone (pCMVp65+, pCMVp50+) and dominant negative form of IκBα (IκB α S19/23A) were provided by Dr. F Shannon (Australia National University).

pRSV-hVDR, Gal-Sp1, VP16-VDR, VP16-Ets1, VP16-VDR, VP16-RXR, p*CYP24*mEBS-Luc and p*CYP24*mGC-Luc were from Dr. P Dwivedi (University of Adelaide).

pEF-IRES-puro6 vector was generously given by Dr. M Whitelaw (University of Adelaide).

pCMV-PKA (Cα) was a generous gift from Dr. S McKnight (University of Washington, Seattle, WA, USA).

PKC-ζ dominant negative form, PKCζK281M, was from Dr. JW Soh (Columbia University, USA) (Soh *et al.*, 1999).

Ras dominant negative form, Ras17N was from Dr. DA Brenner (University of North California, USA) (Westwick *et al.*, 1994).

ERK1 dominant negative form, ERK1K71R was from Dr. C Hii (Women and Children's Hospital, Adelaide) (Dwivedi *et al.*, 2002).

2.1.7   Synthetic oligonucleotides

Oligonucleotides for sequencing, site-directed mutagenesis and EMSAs were obtained from Sigma or Geneworks. (The primers for HUP8F, HUP6F and HUP9R were sent by Professor John Omdahl, University of New Mexico, USA). The oligonucleotide sequences are as below:

Sequencing oligonucleotides:

CYP24

EA1:   5' GAG CTC CCG GGC CAG CAG CGT GTC GGT 3'

1α(OH)ase

300F: 5' AC GGT ACC TGA CTA GTG TAG CTT GGT C 3'

HUP8F: 5' ACG GTA CCA TGC CTA TTC TGC CAT 3'

HUP6F: 5' TCG GTA CCA ACC CAC CTG CCA TCT 3'

HUP9R: 5' ATC TCG AGG GTC TGG TTC AGG GTG CTC 3'

<u>Mouse NFYA (to confirm the cDNA sequence of NF-Y and NF-YAm29)</u>

mNFYAF   5' GAG ACA AGC ACG GGC TAA GCT AGA G

Site directed mutagenesis (SDM) oligonucleotides with the core sequence underlined in wild type sequence and the mutated oligonucleotides are in bolded letters (**F: Forward primer; R: Reverse primer**).

1α(OH)ase AP-1

(WT   5' GCC TTT CCT AGG A<u>TG AGA CTT</u> TGA GGC 3')

SDMF   5' GCC TTT CCT A**TT** A<u>**CA** AGA CTT</u> TGA GGC 3'

SDMR   5' GCC TCA AG<u>T CT**T** T**GT**</u> AA**T** AGG AAA GGC 3'

1α(OH)ase AP-2

(WT   5' GCA ACA TGA G<u>AC CCA AGG G</u>AG TTG TG 3')

SDMF   5' GCA ACA TGA G<u>**AA** ACA AGG G</u>AG TTG TG 3'

SDMR   5' CAC AAC TCC CT<u>T GT**T** T**CT**</u> CAT GTT GC 3'

1α(OH)ase proximal CRE

(WT   5' CAAGGGAGTTG<u>TGAAGTCA</u>GCCCCAGC 3')

SDMF   5'CAAGGGAGTTG<u>**TATGCT**CA</u>GCCCCAGC 3'

SDMR   5' GCTGGGGCTGAG**CATA**CAACTCCCTTG 3'

1α(OH)ase distal GC box

(WT	5' GAAGTCAG<u>CCCCAGCCCCGCC</u>TACTGTTC 3')
SDMF	5' GAAGTCAG<u>**CCAAAGCTTT**GCC</u>TACTGTTC 3'
SDMR	5' GAACAGTAGGCAAAGCTTTGGCTGACTTC 3'

1α(OH)ase EBS

(WT	5' CCCGCCTA<u>CTGTTCC</u>TGGGTGCTAATCC 3')
SDMF	5' CCCGCCTA<u>CTGTT**TT**T</u>GGGTGCTAATCC 3'
SDMR	5' GGATTAGCACCCAAAAACAGTAGGCGGG 3'

1α(OH)ase proximal GC box

(WT	5' GGAGCTTGGAGA<u>GGGGGCGT</u>CATCACCTC 3')
SDMF	5' GGAGCTTGGAGA<u>AGC **T** TCGT</u>CATCACCTC 3'
SDMR	5' GAGGTGATGACGAAGCTTCTCCAAGCTCC 3'

1α(OH)ase distal CCAAT box

(WT	5' CATGAGGG<u>TCATTGCAACATGAG</u>AC 3')
SDMF	5' CATGAGGG<u>TCA**GA**TCAACATGAGA</u>C 3'
SDMR	5' GTCTCATGTTGATCTGACCCTCATG 3'

1α(OH)ase proximal CCAAT

(WT	5' CAGGAGGAGG<u>GATTGG</u>CTGAGGAGCTTG 3')
SDMF	5' CAGGAGGAGG<u>G**AGCTC**</u>CTGAGGAGCTTG 3'
SDMR	5' CAAGCTCCTCAGGAGCTCCCTCCTCCTG 3'

CYP24 CCAAT box

(WT	5' CAGCGTGCTC<u>ATTGG</u>CCACTCCAGC 3')

| SDMF | 5' CAGCGTGCTC<u>AGCTG</u>CCACTCCAGC 3' |
| SDMR | 5' GCTGGAGTGGCAGCTGAGCACGCTG 3' |

CYP24 GT box

(WT	5' CCCCGCGTCC<u>CTCC</u>CAGCCGGTCCC 3')
SDMF	5' CCCCGCGTCC<u>CTTA</u>CAGCCGGTCCC 3'
SDMR	5' GGGACCGGCTGTAAGGGACGCGGGG3'

Gel shift oligonucleotides (S: sense strand; AS: antisense strand)

Consensus NF-Y binding oligonucleotides (C-NF-Y)

| S: | 5' G TCT GGG CGC TGA TTG GCT GTG GAC TGC G 3' |
| AS: | 5' G CGC AGT CCA CAG CCA ATC AGC GCC CAG A 3' |

Consensus C/EBP binding oligonucleotides (C-C/EBP)

| S: | 5' G TGC AGA TTG CGC AAT CTG CA 3' |
| AS: | 5' G TGC AGA TTG CGC AAT CTG CA 3' |

1α(OH)ase CCAAT box (pCYPα-CCAAT)

| S: | 5' GAG GAG GGA TTG GCT GAG GAG 3' |
| AS: | 5' GCT CCT CAG CCA ATC CCT CCT 3' |

1α(OH)ase mCCAAT oligonucleotide (mCCAAT)

| S: | 5' G AGG AGG GAG CTC CTG AGG AG 3' |
| AS: | 5' G CTC CTC AGG AGC TCC CTC CT 3' |

Consensus Sp1 binding oligonucleotides (Cons-Sp1)

S   5' GAT TCG ATC GGG GCG GGG CGA G 3'

AS: 5' GCT CGC CCC GCC CCG ATC GAA T 3'

1 α(OH)ase distal GC box (pCYPα-GC)

S: 5' GGC CCC AGC CCC GCC TAC 3'

AS: 5' GGT AGG CGG GGC TGG GGC 3'

CYP24 GC box (CYP24-GC)

S: 5' GCC ACA CCC GCC CCC CG 3'

AS: 5' GCG GGG GGC GGG TGT GG 3'

CYP24 CCAAT box (CYP24-CCAAT)

S   5' GTG CTC ATT GGC CAC TCC A 3'

AS   5' GTG GAG TGG CCA ATG AGC A 3'

CYP24-mCCAAT (mut-CCAAT)

S   5' GTG CTC AGC TGC CAC TCC A 3'

AS   5' GTG GAG TGG CAG CTG AGC A 3'

2.1.8   Bacterial strains and culture medium

a) E.coli DH5α: supE44 ΔlacU169 (p80 lac ZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1. The host bacteria strain for recombinant plasmid DNA was received from the E.coli Genetic Stock Center, Yale University, New Haven.

Long-term storage of these strains and plasmid-transformed bacteria was performed by adding equal volume of 80% glycerol to an overnight culture and storing at -80°C until use. The glycerol stock was streaked onto agar plates to obtain the single colony, which then was

inoculated into liquid growth medium. The bacteria grew at 37°C with continuous shaking to provide adequate aeration. Both the agar plates and the liquid growth medium were supplemented with appropriate antibody (Ampicillin if not mentioned) where required.

b) Bacterial growth media

All the growth media were prepared with miliQ water and sterilised by antoclaving. Antibiotics were added after the solution had cooled to 50°C.

Luria broth (LB): 1% (w/v) Bacto-tryptone (Difco), 0.5% (w/v) yeast extract (Difco), 1% (w/v) NaCl, pH7.0.

Agar plates were prepared by adding 1.5 (w/v) Bacto-agar (Difco) to the L broth. 100 µg/ml Ampicillin were added where required.

Psi (ψ) Broth: 2% (w/v) Bacto-tryptone (Difco), 0.5% (w/v) yeast extract (Difco), 0.5% MaSO$_4$, pH 7.6.

SOC: 1% (w/v) Bacto-trypton (Difco), 0.5% (w/v) yeast extract (Difco), 1% (w/v) NaCl, 10mM MgCl$_2$, 10mM MgSO$_4$, 20mM glucose, pH 7.0.

2.1.9   Tissue culture cell lines and media

a)   Cell lines

UMR106-06: Rat osteosarcoma cell line was received from Dr. TJ Martin, St. Vincent's Institute of Medical Research, University of Melbourne.

LLC-PK1: Pig kidney epithelial cell line was received from CSL.

AOK-B50: Proximal tubule cell line derived from porcine LLC-PK1 cells, with stably expression of opossum PTH receptors, was a kind gift from Professor J Omdahl, University of New Mexico, USA.

HEK-293T: Human primary embryonic kidney cell line was from J Health, Oxford University, UK.

<u>HR-12</u>: A modified kidney cell line derived from HEK-293T cells with stably expressed human calcitonin receptors was received from Dr. D Findlay, IMVS, Australia.

<u>JEG-3</u>: Human choriocarcinoma cells were purchased from ATCC (HTB36).

<u>SL3</u>: *Drosophila* epithelial cell line from Ovegon-Rembryos was a kind gift from P Scotti, Aukland, New Zealand.

b)   Solutions

<u>Phosphate Buffered Saline</u> *(PBS)*: 136mM NaCl, 2.6mM KCl, 1.5mM KH_2PO_4 and 8mM Na_2HPO_4 (pH7.4). The solution was sterilized by autoclaving in 20psi at 140°C for 25 min.

c)   Media

<u>Dulbecco's Modified Eagle medium</u> (DMEM), <u>low calcium DMEM</u>, <u>RPMI 1640</u> and <u>Schneider's Drosophila medium</u> were purchased from Gibco BRL.

<u>Fetal Calf Serum</u> (FCS) was from CSL.

<u>Trypan Blue</u> was from Sigma.

<u>Trypsin, puromycin and geneticin</u> (G418) were from Gibco BRL.

d)   Flasks and plates for culture

<u>Cell culture flasks</u>: 25 cm^2, 75 cm^2 and 125 cm^2 flasks were purchased from BD Biosciences, France.

<u>Culture plates</u> (24-well, 6-well and 96-well plates) and *Petridishes* (10mm, 60mm and 100mm) from Becton Dicklnson Labware, NJ, USA.

2.1.10 Miscellaneous

<u>X-ray film</u>: Kodak, USA

<u>3MM paper</u>: Whatman Ltd.

<u>Puc19/EcoRI and Spp1/EcoRI DNA markers</u>: Geneworks.

2.2 Recombinant DNA methods

2.2.1 General methods for DNA manipulation

The following methods were performed essentially as described in "Molecular Cloning: A laboratory manual" (Sambrook *et.al.*,1989): Growth, maintenance and preservation of bacterial; preparations and quantifications of DNA and RNA; restriction enzyme digestion and ligation; agarose and polyacrylamide gel electrophoresis; end-filled labelling of DNA fragments using the Klenow fragment of DNA polymerase and autoradiography.

All manipulations involving in viable organisms that contained recombinant DNA were carried out with accordance to the regulations and approval of the Australian Academy of Science Committee on recombinant DNA and the University Council of the University of Adelaide.

2.2.2 Plasmid DNA preparation

For analytical restriction digestion and sequencing, preparation of plasmid DNA was performed using mini-preparation procedure (section a). Preparation of plasmid DNA for transfection of tissue culture cell lines, was generally obtained from midi-plasmid DNA kit from Qiagen (section b) or by cesium-chloride (CsCl) density gradient centrifugation in a Beckman TL-100 bend top ultracentrifuge and TLA-100.2 rotor (section c).

a)   Mini-preparation

The rapid alkaline hydrolysis procedure of Birnboim and Doly (1979) was used for mini-preparation of plasmid DNA from 1.5ml overnight culture. Briefly, the bacteria pellet was lysed by 100 µl of Solution I, 200 µl of Solution II and 150 µl of Solution III subsequently. After centrifugation, the plasmid was precipitated using 400 µl of isopropanol and further cleaned up by 70% ethanol.

b)   Midi-preparation using midi-plasmid DNA preparation kit (Qiagen)

Isolation of up to 100 μg plasmid DNA was performed using the midi-preparation kit according to the manufacturer's instructions.

c)   Large scale preparation by cesium-chloride (CsCl) density gradient procedure

Large amount of plasmid DNA used for the transfection of tissue culture cell lines was routinely obtained using a modified version of Sambrook *et al.* (1989). 20 ml of desired clone culture was inoculated into 250ml of LB medium with appropriate antibiotics (Ampicillin) and incubated at 37°C with shaking. Then the bacterial cells were pelleted at 4°C in a SS-34 rotor at 6000 rpm for 5min. Plasmid DNA was extracted using the alkaline lysis procedure: 10ml of fresh prepared GTE buffer with lysozyme (2mg/ml) was used to lyse the cells on ice for 20min; 20ml of fresh prepared Solution II (0.2M NaOH, 1% SDS) was added and mixed with the cell lysate by inverting the tubes and then 15ml of NaAc (3M, pH 4.6) was added, mixed and left on ice for 10min. The supernatant was collected and passed through a funnel containing cloth band-aid and then the volume recorded in a 50ml measuring cylinder. Plasmid DNA was precipitated by the addition of 0.6 volumes of isopropanol and incubated on ice for 10min. The plasmid DNA was pelleted at 9000 rpm for 20 min and the supernatant discarded. The pelleted plasmid DNA was allowed to air dry and resuspended in 4 ml of 1×TE. To separate plasmid DNA from RNA and genomic DNA, the plasmid was further purified by the CsCl/ethidium bromide density gradient procedure. 4.2 grams of CsCl was mixed with the dissolved plasmid DNA by vortexing thoroughly. 100 μl of 10mg/ml ethidium bromide was added as the marker of plasmid DNA. The mixture was then centrifuged in a Beckman TL-100 benchtop ultracentrifuge and TLA-100.2 rotor at 80,000rpm at 20°C for overnight. Plasmid DNA with ethidium bromide (the lower most central red band) was obtained using syringe and needles. The ethidium bromide was removed by butanol

extraction. The plasmid DNA was then precipitated with 2.5 volumes of 100% ethanol, rinsed with 70% ethanol, resuspended in 1xTE buffer, and quantified by spectrophotometry and analysed by agarose gel electrophoresis to confirm concentration and supercoiling.

2.2.3 Restriction enzyme digestions of DNA

For analytical restriction digests, 1μg of DNA was incubated with 1 unit each of the restriction enzyme(s) in the appropriate buffer suggested by the manufacturer for minimum 1 hr or overnight. The digestion was terminated by the addition of 1/6 volumes of the urea DNA loading buffer and electrophoresed.

2.2.4 Preparation of DNA restriction fragments

Plasmid DNA was digested with the appropriate restriction enzymes as above. The digested fragment(s) was isolated from 0.8-2.0% agarose gel up to the fragment's size using DNA gel extraction kit (Qiagen) according to the manufacture's instruction.

2.2.5 Preparation of cloning vectors

Vector DNA was linearized with the appropriate restriction enzyme(s) and if required then digested with calf intestinal phosphates (CIP) at 37°C for 1 hour. Further incubation at 65°C for 15min was followed to remove 5' terminal phosphate groups. The vector DNA was isolated by electrophoresis on a 1.0% agarose gel and purified by DNA gel extraction kit (Qiagen) according to the manufactures suggestions. The DNA was resuspended at a concentration of 20-50ng for ligation.

2.2.6 Ligation of DNA

A 10μl ligation reaction was set up with 20ng of vector DNA, a 3 molar excess of the insert DNA, 50mM Tris-HCl (pH7.4), 10mM $MgCl_2$, 1mM ATP and 1unit of T_4 DNA ligase. The

reaction was incubated at 14°C for 16 hours. To determine the background level of uncut and recircularized vector DNA, a control ligation reaction without insert DNA was set up contemporarily. Both sample and control reactions were followed by transformation.

2.2.7 Transformation into E.coli with recombinant plasmid

a)   Preparation of competent E.coli

E.coli cells were made competent by the calcium chloride method describing on "Molecular Cloning" (Sambrook *et al.*, 1989). A single colony, obtained by streaking on the LB plate, was inoculated in 2ml of Psi (ψ) broth and kept growing at 37°C. The overnight culture was then subcultured into 200ml of ψ broth for further growing with continuous shaking until the OD_{600} reached 0.4-0.6. The cells were placed on ice for 5min and centrifuged in a HB-4 rotor at 4000rpm for 5 min at 4°C. The cells was resuspended in 80ml of Tfb1 and kept on ice for 5min. After centrifugation, the cells were resuspended in 8ml of Tfb2 and placed on ice for 15min. Aliquots of the competent cells were stored -80°C until use.

b)   Transformation of plasmid in competent cells

Aliquot of competent cells was taken out from the freezer and thawed on ice. 100ng of plasmid DNA was added to 50μl of competent cells and incubated on ice for 30min. After heat shock at 42°C for 2min, the cells were chilled on ice for 5 min, diluted with 900μl of LB medium and then kept growing at 37°C for 30min. 200μl of the transformed cells was then plated onto the LB agar plate with Ampicillin and incubated at 37°C for overnight. Individual colony was now ready for mini-preparation of plasmid DNA.

2.2.8   Polymerase chain reaction (PCR)

To amplify the fragment from plasmid, 10ng of plasmid DNA was used in a standard 50μl of reaction (5μl of 10 × Pfu buffer, 100μM of each forward and reverse primers and 5mM of

dNTP) using the PCR programme (94°C for 4min and then 30 cycles of 95°C for 1min, 50°C for 1min and 72°C for 20min) in a PTC-100™ programmable thermal controller. Before running the programme, 20 μl of mineral oil was overlaid onto the top of the mixture to prevent evaporation.

2.2.9   Automatic sequencing of the recombinant DNA

To sequence the recombinant DNA, 20μl of cocktail mixture was set up with 0.5μg of plasmid DNA, 100ng of primer and 8μl of Big Dye reaction buffer. Then 20μl of mineral oil was overlaid onto the top to prevent evaporation. The reaction mixture then undergoes the sequencing cycle using the sequencing program (25 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 min) in a PTC-100™ programmable thermal controller.

To dry the samples, 2μl of 3M NaAc pH4.6 and 50μl of 95% ethanol were added to the reaction mixture, which were then allowed to precipitate by 80μl of 75% isopropanol at – 80°C for 15 min and pelleted by centrifuging at maximum speed for 20 min. The pellet was washed by 250μl of 75% isopropanol once. The samples were dried and sent to the IMVS for Big Dye sequencing analysis using an ABI computer program.

2.2.10   Quikchange site-directed mutagenesis

Site-directed mutagenesis of the 0.3kb, or 1.5kb *1α(OH)ase* or 298bp *CYP24* promoter constructs was carried out using the method by QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA, USA). According to the manufacturer's instruction, the oligonucleotide primers were designed and temperature cycling was performed. The site-directed mutagenesis primers designed were shown as 2.1.7. Two complementary oligonucleotide primers containing the desired mutation were used and the oligonucleotides were extended upon by the action of Pfu polymerase (2.5u/μl) in a cycling program (95°C for 30sec, 55°C for 1 min and 68°C for 11 min for 18 times). Incorporation of the

oligonucleotides results in a nicked vector free from methylation. The end product was then digested with DpnI, which is sensitive to the methylated and semi-methylated DNA to remove the parental (non-mutated) DNA. Following digestion, the *1α(OH)ase* promoter or *CYP24* promoter constructs contained desired mutation were then transformed into competent DH5α cells. The recovered transformants were mini-prepared and sequenced to confirm incorporation of the correct mutation.

2.3 Synthesis of plasmid

2.3.1 Generate the deletion constructs of *1α(OH)ase*

Human genomic DNA was amplified by PCR with primers designed from the human *1α(OH)ase* cDNA to generate DNA with 1501 bp and 531 bp of 5' flanking region of the *1α(OH)ase* gene that terminated at +42 bp in the 5' untranslated region. The primers (HUP9F and HUP8R for 1495bp constuct; HUP6F and HUP8R for 531bp constructs) shown as section 2.1.7 were flanked by a 5' KpnI site or a 3' XhoI site. DNA fragments with 1501 bp of 5' flanking region and 531 bp of flanking region that terminated at + 42 bp in the 5' untranslated region, were cloned into the pGL3 basic vector upstream of the firefly luciferase reporter gene (Promega). The sequence of the PCR products was confirmed by automated sequencing.

Deletions of the *1α(OH)ase* gene 5' flanking region with a common termination site at +42 bp were generated using appropriate restriction enzymes. The pGL3 construct with 1501 bp of flanking region was digested with KpnI and SacI at natural sites in the flanking region. The remaining pGL3 construct was purified, blunt ended and religated to generate 997 bp of flanking region. To obtain 884 bp of flanking region, the pGL3 construct with 1501 bp of flanking region was digested with Hind III (at the natural site at 884 bp in the promoter and one in the linker) and the fragment was ligated into the Hind III site of pGL3-Basic. To generate 305 bp of flanking region, the pGL3 construct with 1501 bp flanking region was digested with Kpn I and Spe I, blunt ended and religated and then the putative CRE-d site

destroyed by Spe I, was recovered by insertion of 5 bp using site directed mutagenesis and the primer 5' CTATCGATA GGTACTGACTAGTGTAGCTTGG 3'.

2.4   Methods for transient expression of promoter constructs in tissue culture cell lines.

2.4.1   Maintenance, subculture and storage of mammalian cells

The following cell lines were maintained in DMEM supplemented with 10% FCS: UMR106, HEK-293T cells and LLC-PK1 cells.

JEG-3 cells were cultured in DMEM with 5% FCS.

HR-12 cells were cultured in DMEM with 10% FCS and 200μg/ml G418.

AOK-B50 cells were maintained DMEM with 7% FCS.

Drosophila SL3 cells were maintained with Revised Schneider's medium containing10% FCS.

Cells were subcultured when they reached 70-80% confluence: the medium was gently poured off and the cells were washed once with PBS. For $75cm^2$ flask, 1 ml of trypsin was added in the cells and left for 1min with gently mixing at room temperature (LLC-PK1 and AOK-B50 cells were required for 5 min at 37°C). Cells were detached by tapping the flask, resuspended in 9 ml of culture medium and pelleted by centrifuging at 1500 rpm for 2 min. The cells were then resuspended with 1ml PBS, 20μl of which was used for counting cell number by a Haemocytometer. The required numbers of the cells were seeded into new flask for maintenance or into 24-well plates for transient transfection and dual-luciferase assay. *Drosophila* SL3 cells grew at 25°C incubator without CO_2 provided, all other cells grew in 37°C incubator with 10% CO_2.

Cells were trypsinized as above when they reached 70-80% confluence. After centrifugation, the cells were resuspended with 1 ml of freezing medium (20% DMSO: 80% FCS),

transferred into 2 ml freezing vials, kept at -80°C overnight and then stored in liquid nitrogen until further use.

2.4.2 Preparation of stripped foetal calf serum

To strip the foetal calf serum (FCS), 160ml of FCS were mixed with 50µl stripping buffer containing 125mg Dextran and 1.2g of activated charcoal at 50-60°C for 30min with constant stirring. The cocktail was chilled on ice and then kept at 4°C overnight. The pellet was discarded after centrifugation in HB-4 rotor at 10000rpm for 10min. The spinning step was repeated a few times more until the left charcoal was removed. The stripped serum was sterilized by filtration through a 0.2µm filter and the aliquot of the stripped serum was kept at –20°C until further use.

2.4.3 Transient transfection of mammalian cells with recombinant DNA

All transfections for mammalian cells were performed with the liposomal transfection reagent DOTAP following optimisation of conditions. In preparation for transfection, approximately 2×10^5 cells were seeded into 24 well trays in 400 µl of DMEM containing 10% foetal calf serum to achieve 60-70% confluence and the cells were allowed to attach for 6-7 h. Transfections were performed in triplicate and each transfection was performed using 300 ng of *1α(OH)ase* promoter luciferase construct or 200ng of *CYP24* promoter luciferase construct and also 50 ng of the thymidine kinase (TK) promoter-*Renilla* luciferase (Promega) as a control for transfection efficiency. Over expression plasmids (eg. C/EBPα, β and δ) and dominant negative mutants (eg. NFYAm29 and Ras17N) were also cotransfected wherever indicated. For all the transient transfection performed in chapter 5, 200ng of *CYP24* promoter luciferase construct was only transfected. All plasmid DNA was diluted to a final volume of 5µl and mixed with 1.5µg of DOTAP diluted in 5µl of Hepes buffer (20mM, pH7.4). DNA-DOTAP complex formation was achieved by incubating the mixture for 15-20 min at room

temperature and transfected into the cells. After transfection overnight, the media was replaced with either RPMI 1640 containing 10% charcoal-stripped foetal calf serum (for AOK-B50 cells only) or serum free medium RPMI 1640. Cells were treated with PMA, H89, calphostin C, PD98509, SB203580, U0126 or LY294002 if required, and then further treated with PTH (1-34), calcitonin, $1,25(OH)_2D_3$ or vehicles (2% cysteine hydrochloride, water or ethanol respectively) for 24 h before harvesting. For the condition of low calcium medium, the same number of AOK-B50 cells as above was seeded into 24 well plates with DMEM containing 10% of foetal calf serum. After overnight, the medium was replaced by low calcium medium with 7% foetal calf serum and the transfection was performed. The next day, the medium was replaced again by low calcium medium with no foetal calf serum and PTH was added for further 24 hours treatment.

2.4.4 Dual luciferase assay (DLR assay) system

After the incubation period, the media was aspirated and the wells were washed once with PBS. Then the cells were lysed using $100\mu l$ of 1×passive lysis buffer for 20min at 25°C. Cell lysates were transferred into marked eppendorf tubes, centrifuged for 30 sec and stored at − 20°C until further use. Dual luciferase activity was measured using a TD-20/20 luminometer. The first luminescence of reporter firefly luciferase was recorded by adding $25\mu l$ of LAR II substrate into $10\mu l$ of cell lysate. The second luminescence of control *Renilla* luciferase was quantified by the addition of 25 μl of Stop and Glo substrate to quench the first reaction and simultaneously initiate the *Renilla* luciferase reaction. Data are presented as relative luciferase activity, the ratio of the first luminescence with the second luminescence.

2.4.5 Luciferase assay (LR assay) system

For all the transient transfection assays in chapter 5, the cells were lysed using $100\mu l$ of 1x reporter assay buffer for 20min at 25°C. Cell lysates were subjected to luciferase assay using

a TD-20/20 luminometer. The luminescence of reporter firefly luciferase was recorded by adding 25µl of luciferase assay substrate into 10µl of cell lysate. The data are presented as the ratio of firefly luciferase activity to total cell protein content by Bradford assay.

2.4.6 Bradford assay to determine the concentration of protein

The protein content from the cell lysate was determined using 2µl of supernatant by the protein microassay kit (Bio-Rad) according to the manufacturer's instructions. Bovine serum albumin (BSA) was used as the protein standard.

2.4.7 Transient transfection of *Drosophila* SL3 cells with recombinant DNA

Transfections for *Drosophila* SL3 cells were performed using LipofectAMINE 2000 (Life Tech. BRL). 2×10^5 cells were seeded into 24 well plates in 400µl of Revised Schneider's medium containing 10% FCS. The cells were allowed to grow for 24 hours before transfection. Transfections were performed in triplicate and each transfection was performed using 200ng of *CYP24* or *1α(OH)ase* promoter luciferase construct and also 10-200ng of pPac-derived expression vectors. Total amount of DNA was adjusted by an addition of vector. All plasmid DNA was diluted to a final volume of 50µl and mixed with 2µl of LipofectAMINE 2000 diluted in 50 µl of RPMI medium. DNA-LipofectAMINE complex formation was achieved by incubating the mixture for 20 min at room temperature and transfected into the cells. Cells were harvest after 48 h for luciferase assays using the luciferase assay system (section 2.4.5). The data are presented as fold stimulation where the value of luciferase activity normalized to total cell protein for the reporter alone set to 1.

2.4.8 Mammalian two-hybrid assay

JEG-3 cells were cultured in 24-well plates in DMEM with 5% charcoal-stripped foetal calf serum for 24 hours before transfection. Cells of each well were cotransfected with 200ng

reporter plasmid pG5E1b and 50ng of pRL-TK-Luc (transfection efficiency control) with 500ng GAL4-Sp1 and each of VP16-Ets1, VP16-VDR or VP16-RXR expression plasmid using DOTAP transfection protocol as mentioned in section 2.4.3. After 24 hours, cells were cultured with fresh replaced serum free medium, treated with $1,25(OH)_2D_3$ for 24 hours and harvested for DLR assays.

2.5 Methods for making stable cells from kidney HR-12 cells and AOK-B50 cells

2.5.1 Testing the resistant ability of HR-12 cells and AOK-B50 cells to Puromycin

Cells were seeded into five 10mm-petridishes with 2 ml culture medium (HR-12 cells at 2×10^5 cells/petridish; AOK-B50 cells at 1×10^5 cells/ petridish). After the cells were attached, different concentration of puromycin were added into each plate: 0 μg/ml, 1μg/ml, 10μg/ml, 20μg/ml and 30μg/ml. The cell survival rates after 48 hours were checked and then the right concentration of puromycin was selected for making the stably transfected cells. For current studies, the stable cells created from HR-12 cells and AOK-B50 cells were selected by 1μg/ml puromycin.

2.5.2. Creating stable cell lines

Cells were subcultured into one 60mm-petridish with 5 ml culture medium (HR-12 cells at 1×10^6 cells/petridish; AOK-B50 cells at 0.5×10^6 cells /petridish). Once attached, the cells were transfected with the cocktail of 50μl DNA mixture (10μg promoter construct, 100ng pEF-IRES-Puro vector) and 50μl DOTAP mixture (30μg DOTAP). The DNA/DOTAP cocktail was left on room temperature for 20min before being transfected into the cells. The transfected cells grew at 37°C for about 24 hours until the confluence was almost reached and then were subcultured into two 100mm-petridish with 8 ml culture medium for further 24 hours. The media was replaced with the fresh medium and 1μg/ml puromycin was added into

the medium for 4-7 days or the medium colour change into yellow. The step for replacing medium was repeated once and the cells were left for 4-7 days. The cells were pooled into one $25cm^2$ flask with the selection medium and grew until the confluence was reached. Then small amount of cells was counted and subjected into luciferase assay (section 2.4.5) and Bradford assays for the efficiency of the stable cell lines.

2.6   Gel mobility shift assays

2.6.1   Preparation of nuclear extracts from mammalian tissue culture cells

Nuclear extract was prepared from AOK-B50 cells, HEK-293T as described previously (Dwivedi *et al.*, 2000). Briefly, mammalian cells in a 75-cm^2 flask were counted when 70-80% confluence and resuspended with 400µl of cold Lysis Buffer I by gently flicking. Cells were left on ice for 15 min to swell for hypotonic lysis and then pelleted by centrifuge. The cells was resuspended in 100µl of Lysis Buffer II and incubated on ice for 30 min for high salt extraction. After centrifugation, the protein concentration of the supernatant was quantified by performing Bradford assay. The nuclear extract was kept at –80°C until use.

2.6.2   Preparation of end-filled labeling DNA probe with $[\alpha-^{32}P]$ dCTP

2.5µg of sense and antisense oligomers (the relevant sequence as shown in section 2.1.7) was annealed in a 10µl volume using 2µl of 5x annealing buffer. The oligomers were heated at 95°C for 2 min and transferred to a 65°C heating block. The block was allowed gradually to cool to room temperature for 30min. The annealed oligomers (0.5µg) were end filling labeled by $[\alpha-^{32}P]$ dCTP in 12µl volume containing 1.5µl of 10x Klenow buffer, 2 units of Klenow fragments and 25µCi $[\alpha-^{32}P]$ dCTP. The reaction buffer was incubated at room temperature for 20-30min. For each labeling sample, 1µg of poly (dI-dC), 2µl of 3M NaAc and 40µl of 100% ethanol were used to precipitate the probe on dry ice for 15min. The probes were pelleted by spinning at maximum speed for 20min and rinsed once with 50µl of 70% ethanol.

Once air-dried, the probes were dissolved in 10μl of 1x TE buffer and radioactivity were counted using scilination solution.

2.6.3 Gel mobility shift assays

Double stranded oligonucleotides were designed to contain the specific sequence in the *1α(OH)ase* or *CYP24* promoter. Oligonucleotides including a known C/EBP binding site (Con-C/EBP, Santa Cruz), a known Sp1 binding site (Con-Sp1 box from Santa Cruz) or a known NF-Y binding site (Con-NF-Y from rat Hexokinase II promoter, Osawa *et al.,* 1996) were designed as a control.

Each double stranded oligonucleotide was labelled by end filling with [α-^{32}P] dCTP using Klenow fragment. DNA binding reactions were carried out using 6μg of nuclear extracts and 1μg poly(dI-dC) in a final volume of 12μl in TM-1 binding buffer on ice for 15-30min. Radiolabeled probes (200,000cpm) were added and the samples were incubated on ice for further 20-30min. For supershift assays, polyclonal or monoclonal antibodies were included in the binding reaction and incubated on ice for 30min prior to addition of probe. Monoclonal anti-Sp1, polyclonal anti-C/EBPα and C/EBPβ were purchased from Santa Cruz Biotechnology Inc. and polyclonal anti-NF-YB was generous provided by Dr. R Mantovani (University of Milan) (Mantovani *et al.,* 1992). Monoclonal antibody for VDR was purchased from Affinity BioReagents (NJ, USA). Gel shift competition assays were carried out using the unlabelled competitor oligonucleotides at molar excess concentration (10-50 fold) by incubation in the binding reaction. The retarded complex was resolved on a 4% non-denaturing polyacrylamide gel in a low ionic strength running buffer (0.5× TBE) at 4°C. The gel was dried and exposed to X-ray film for 2-3 days at −70°C.

✦ ✦

CHAPTER 3

Basal and parathyroid hormone induced expression of the human 25-hydroxyvitamin D 1α-hydroxylase gene promoter in kidney AOK-B50 cells

✦ ✦
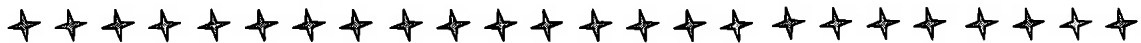
3.1 Introduction

1α(OH)ase, the key rate controlling enzyme for the synthesis of circulatory 1,25(OH)$_2$D$_3$, is mainly expressed in the kidney. A cDNA clone for *1α(OH)ase* from the kidney of mice lacking VDR was first reported by Takeyama *et al* (1997). Later cDNA and genomic sequences of *1α(OH)ase* were cloned from rat (Shinki *et al.*, 1997) and human (Kong XF *et al.*, 1999) thus facilitating studies at the molecular level. Renal expression of *1α(OH)ase* is controlled by physiological regulators including PTH, CT, calcium, phosphate and 1,25(OH)$_2$D$_3$ itself, but the chief regulators are PTH and 1,25(OH)$_2$D$_3$.

Infusion of a pharmacological dose of PTH (1-34) induces expression of *1α(OH)ase* mRNA in mice by 2-fold (St-Arnaud *et al.*, 1997) and similar studies in rats have resulted in a 3-4 fold induction (Murayama *et al.*, 1999). *In vitro* experiments with cultured kidney cells have established that PTH increases expression of the *1α(OH)ase* mRNA (Murayama *et al.*, 1999). However, the molecular mechanism by which PTH upregulates *1α(OH)ase* gene expression remains unclear.

PTH receptor belongs to the superfamily of G-protein coupled receptors and is known to couple to adenylyl cyclase (AC) /cAMP and phospholipase C (PLC) /inositol phosphate/diacylglycerol (DAG) signaling systems (Abou-Samra *et al.*, 1992). PTH can also activate MAP kinase pathways via cAMP (Verheijen and Defize, 1997). The evidence available to date indicates that protein kinase A (PKA) is involved in PTH action on *1α(OH)ase* expression (Murayama *et al.*, 1999; Kong XF *et al.*, 1999; Henry, 1985).

The aim of this chapter is to investigate the molecular mechanism as to how PTH regulates *1α(OH)ase* gene expression in kidney AOK-B50 cells and to specifically identify the trans-acting elements in the human *1α(OH)ase* promoter which are involved in basal and PTH induced expression, and to identify the PTH mediated signaling pathways.

Part of this chapter was published as " Gao *et al.*, (2002): Basal and parathyroid hormone induced expression of the human 25-hydroxylase gene promoter in kidney AOK-B50 cells:

55

role of Sp1, Ets1 and CCAAT box protein binding sites. *Int J Biochem Cell Biol.* 34: 921-930".

3.2 Results

3.2.1 PTH mediated induction of *1α(OH)ase* promoter in LLC-PK1 cells

To determine the effect of PTH on *1α(OH)ase* in kidney cells, transient transfection assays were initially undertaken in porcine proximal tubule LLC-PK1 cells, where *1α(OH)ase* and PTH receptor (PTHR) are highly expressed (Mannstadt *et al.*, 1999; Hewison *et al.*, 2000). Using a DOTAP transfection protocol, LLC-PK1 cells were transiently transfected with a construct containing 305bp of rat *1α(OH)ase* promoter fused to the firefly luciferase gene as reporter and a construct with PRL-TK directed *Renilla* luciferase as an internal control for transfection efficiency. The cells were treated with PTH (1-34) at 10^{-7} M for 24h and luciferase activity was determined in cell lysates. This concentration of PTH (1-34) has been employed by others in similar cell culture studies (Brenza *et al.*, 1998; Kong XF *et al.*, 1999). The data showed that PTH is not able to induce the *1α(OH)ase* promoter directed luciferase reporter activity in LLC-PK1 cells (Figure 3.1). It was possible that this cell line might have insufficient endogenous PTHR. To test this, an expression plasmid for human PTHR was cotransfected with the *1α(OH)ase* (305bp) construct into LLC-PK1 cells. The data showed that in the presence of exogenous PTHR, PTH could induce the *1α(OH)ase* promoter about 2-fold (Figure 3.1). AOK-B50 cells are LLC-PK1 cells stably transfected with opossum PTH receptor (Bringhust *et al.*, 1993). Therefore in this chapter, AOK-B50 cells have been employed to study the molecular mechanism by which PTH mediates regulation of the *1α(OH)ase* gene.
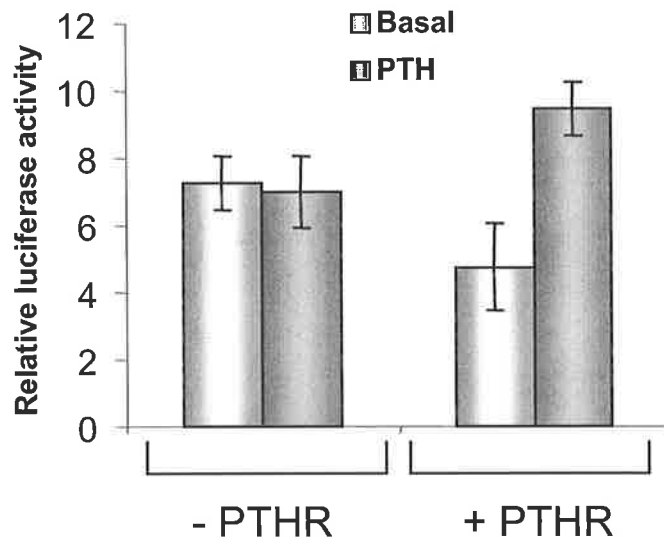
Figure 3. 1 Transient expression of wild-type *1α(OH)ase* construct in LLC-PK1 cells. The cells were cotransfected with either 200ng of human PTH receptor (hPTHR) or vectors with wild-type pCYPα-305-Luc in either presence or absence of PTH (10^{-7}M). The relative luciferase activity shown represent the mean ± S.D firefly luciferase /*Renilla* luciferase ratios of triplicate samples. The experiment was repeated at least twice and similar results were observed.

3.2.2 Deletion analysis of the *1α(OH)ase* 5' flanking region

Human genomic DNA from a chromosome 12 clone was employed as template in PCR reactions with appropriate primers, to generate fragments that encompassed 1501 bp and 531 bp of 5' flanking sequence of the *1α(OH)ase* gene, and terminated at +42 bp in the 5' untranslated region. These fragments were inserted upstream of the firefly luciferase cDNA coding sequence. Using appropriate restriction enzymes, other 5' deletions of the flanking region were generated of length 997 bp, 884 bp and 305 bp. The above five deletion constructs were obtained in conjunction with Prof. J. Omdahl at University of Mexico, USA. Since a methyltransferase-like gene is located about 1.5kb upstream from the *1α(OH)ase* gene (Omdahl *et al.*, 2001), the luciferase construct with 1501 bp of sequence is assumed to encompass the entire 5' flanking region of the gene.

These deletion constructs were transiently transfected individually into AOK-B50 kidney cells. The results in Figure 3.2 show that the 305 bp-promoter construct designated pGL3-305bp (or pCYPα-305-Luc), exhibited substantial basal expression (compared with the promoter-less pGL3 vector) and that this expression increased as the promoter length was extended to -531 bp. Expression remained constant until -997 bp but decreased somewhat when further extended to −1501 bp. In the presence of PTH peptide at 10^{-7} M for 24h, expression of the 305 bp promoter construct was increased 2.03 ± 0.06 fold relative to the basal and similar levels of fold induction were observed with all deletion constructs but not with the vector control. This pattern of PTH induction was observed in three separate experiments performed in triplicate (Figure 3.2). In other experiments, luciferase activity was measured at 3, 6 and 12 h after PTH treatment and the level of induction was also found to be about 2-fold (Figure 3.3). It is noteworthy that PTH mediated induction occurs as early as 3h and stays the same level until 24h. This finding argues against the possibility that endogenous *1α(OH)ase* induced at earlier times leads to $1,25(OH)_2D_3$ production and repression of the promoter construct measured after 24h since it has been reported that renal *1α(OH)ase*
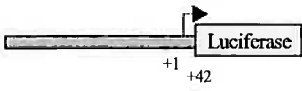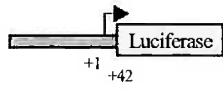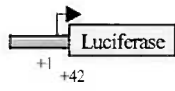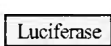
		Basal (RLA)	PTH (RLA)	Fold induction
pCYPα-1501-Luc	[Luciferase] +1 +42	3.59 ± 0.35	7.70 ± 0.86	2.15 ± 0.24
pCYPα-997-Luc	[Luciferase] +1 +42	4.49 ± 0.05	8.60 ± 0.57	1.93 ± 0.15
pCYPα-884-Luc	[Luciferase] +1 +42	4.88 ± 0.32	8.89 ± 0.38	1.82 ± 0.08
pCYPα-531-Luc	[Luciferase] +1 +42	4.50 ± 0.31	8.02 ± 0.54	1.78 ± 0.12
pCYPα-305-Luc	[Luciferase] +1 +42	2.73 ± 0.05	5.55 ± 0.16	2.03 ± 0.06
pGL3-Basic	[Luciferase]	0.05 ± 0.01	0.05 ± 0.02	1.0 ± 0.01

Figure 3.2   Localization of basal and PTH responsive regions in the $1\alpha(OH)ase$ promoter in AOK-B50 cells. Five deletion constructs were generated by PCR and restriction enzyme digestion as described in the Chapter 2. The relative luciferase activity (RLA) shown represents the mean ± S.D firefly luciferase ratios of triplicate samples. The fold induction is the ratio of relative luciferase activity from PTH (10^{-7}M) treated cells to that from untreated cells (Basal). The experiment was repeated at least twice and similar results were observed.
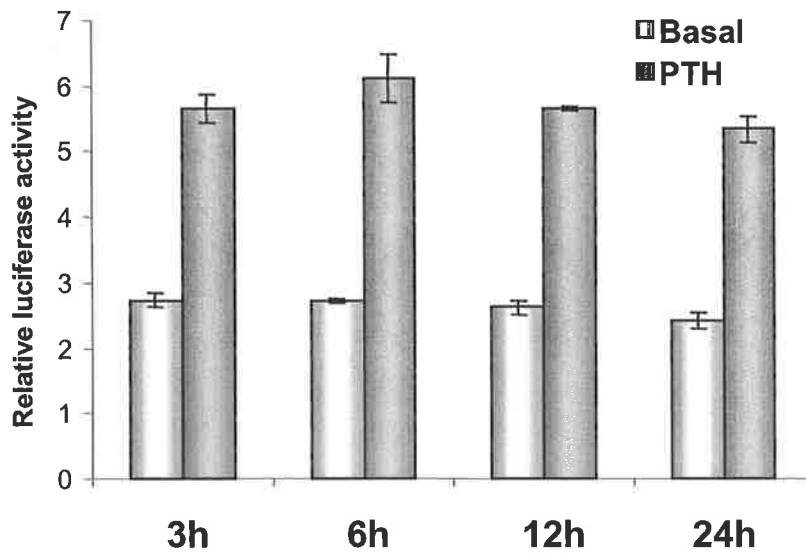
Figure 3.3 Effect of expression of wild-type pCYPα-305-Luc by PTH for different exposure time in AOK-B50 cells. AOK-B50 cells were transiently transfected with pCYPα-305-Luc and treated with PTH at 10^{-7}M for 3, 6, 12 or 24h. The RLA shown represents the mean ± S.D firefly luciferase /*Renilla* luciferase ratios of triplicate samples. The experiment was repeated at least twice and similar results were observed.

expression can be inhibited by $1,25(OH)_2D_3$ (Omdahl et al., 2002). A series concentration of PTH was also employed and it was found that $10^{-7}M$ was required for the 2-fold increase of promoter induction (Figure 3.4). In addition, all transfection experiments were conducted in media containing charcoal stripped foetal calf serum and hence deficient in $1,25(OH)_2D_3$ and PTH.

The results establish that the first 305 bp of promoter contains a regulatory element(s) that responds to PTH and studies here focused on this region. It has been reported that endogenous $1\alpha(OH)ase$ enzyme activity levels in human proximal tubule HKC-8 kidney cells can be directly influenced by calcium in the media through the calcium sensing receptor (CaR) with lowered calcium resulting in enhanced expression (Bland et al., 1999). However, during our studies it was found that PTH induced expression of pCYPα-305-Luc was not further increased when cells were cultured in modified DMEM that lacked calcium (Gibco). The cells were seeded into 24-well trays in 400µl of DMEM with 7% FCS and were left overnight. The normal medium was replaced with modified DMEM lacking calcium the next day and the cells were allowed to grow for 24h prior to transfection. After overnight transfection, the cells were treated with $10^{-7}M$ PTH in the fresh replaced medium without calcium for further 24h and the dual luciferase activity was determined. The basal expression of pCYPα-305-Luc reduced to 0.52 ± 0.10 from 3.27 ± 0.09 when the medium lacks calcium, however PTH mediated induction still remained around 2-fold.

We next investigated the response to PTH (1-34) at $10^{-7}M$ of pCYPα-305-Luc when this construct was stably transfected into AOK-B50 cells. Under these conditions, the assembly of nucleosomes on the plasmid may more closely resemble that in vivo compared with transiently introduced plasmid. We found that the pooled stably transfected cells gave levels of PTH induction after 24h of about 2-fold (from 1415.5 ± 321.7 to 2944.5 ± 125.6), a result similar to that seen in the transient expression analysis. This level of induction is comparable to that determined in vivo in response to PTH (1-34) treatment (St-Arnaud et al., 1997;
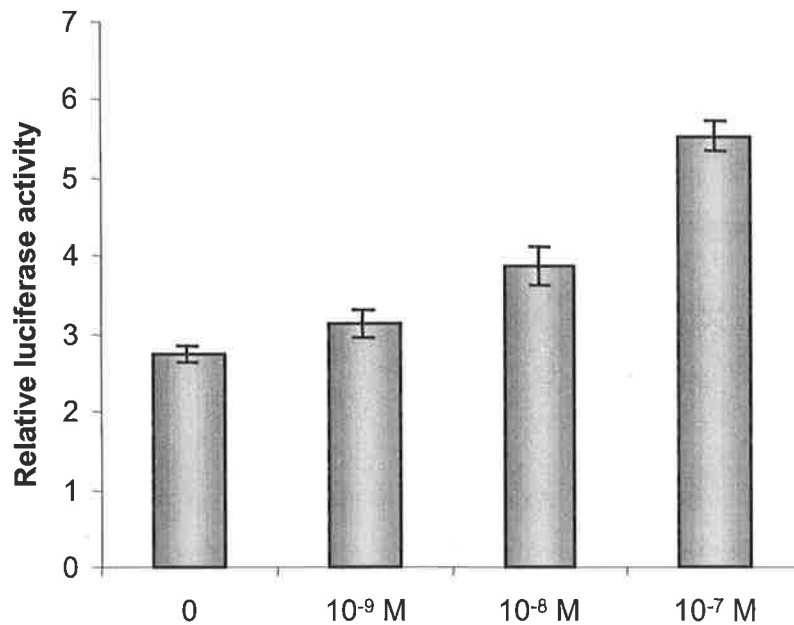
Figure 3.4   Effect of a range of PTH concentrations on expression of wild-type pCYPα-305-Luc in AOK-B50 cells. AOK- B50 cells were transiently transfected with pCYPα-305-Luc and treated with 10^{-9} to 10^{-7}M PTH for 24h. The relative luciferase activity shown represents the mean ± SD firefly luciferase /*Renilla* luciferase ratios of triplicate samples. The experiment was repeated at least twice and similar results were observed.

Murayama *et al.,* 1999). Hence, the lack of chromatin structure likely to be associated with the transiently transfected promoter plasmid does not appear to alter PTH responsiveness.

3.2.3   Identification of regulatory elements in the promoter

A number of possible transcriptional activator binding sites have been identified by DNA sequence analysis in the first 305 bp of the human *1α(OH)ase* promoter (Kong XF *et al.,* 1999; Brenza *et al.,* 1998) and several likely PTH responsive sites located including two cAMP-responsive elements (proximal and distal CREs designated as CRE-p and CRE-d respectively), two GC boxes (proximal and distal GC boxes designated as GC-p and GC-d respectively), an AP-1 site and an AP-2 site (Figure 3.5). The relative contribution of each putative site to basal activity and PTH induction of pCYPα-305-Luc was determined. Each site was selectively mutated within pCYPα-305-Luc as mentioned in chapter 2. The constructs were introduced into AOK-B50 cells and luciferase activities were measured. The data in Figure 3.5 show that the distal putative GC box site (GC-d, 5'CCAGCCCCG 3') located at -133/–125 and the inverted proximal CCAAT box site (CCAAT-p, 5'ATTGGCT 3') located at –74/-68 are major contributors to basal expression. Mutagenesis of the GC-d site (5'AAAGCTTTG3') and the CCAAT-p site (5'AGCTCCT3'), lowered wild type promoter basal expression by 93% and 88% respectively. The sequence 5'CTGTTCCTGG 3' located between GC-d and the CCAAT-p box resembles a consensus Ets protein binding site located on the antisense strand and containing the Ets core 5'GGAA3' sequence. This site contributed to basal expression although to a lesser extent and mutagenesis (5'CTGTTTTTGG3') lowered expression to 45% of the wild type. Surprisingly, mutagenesis of each of the other putative transcription factor binding sites resulted in an increase in basal expression particularly for the putative AP-2 and CRE-proximal sites. The results shown in Figure 3.5 are representative of similar data obtained in five independent experiments performed in triplicate.

	Basal (RLA)	PTH (RLA)	Fold Induction
pCYPα-305-Luc	2.73 ± 0.05	5.55 ± 0.16	2.03 ± 0.06
pmCRE-d-Luc	4.16± 0.19	10.15 ± 0.55	2.44 ± 0.33
pmAP1-Luc	4.16 ± 0.21	7.94 ± 0.76	1.91 ± 0.44
pmCCAAT-d-Luc	4.65 ± 0.13	9.95 ± 0.23	2.14 ± 0.13
pmAP2-Luc	5.49 ± 0.15	14.04 ± 0.98	2.56 ± 0.44
pmCRE-p-Luc	7.13 ± 0.31	13.76 ± 0.66	1.93 ± 0.23
pmGC-d-Luc	0.20 ± 0.01	0.56 ± 0.02	2.83 ± 0.22
pmEBS-Luc	1.18 ± 0.15	2.44 ± 0.13	2.07 ± 0.27
pmCCAAT-p-Luc	0.32 ± 0.02	0.40 ± 0.01	1.28 ± 0.10
pmGC-p-Luc	4.01 ± 0.32	11.22 ± 1.28	2.80 ± 0.79
pGL3 basic-Luc	0.05 ± 0.01	0.05 ± 0.02	1.0 ± 0.01

Figure 3.5 Effect of site-directed mutagenesis of the putative transcription factor binding sites in the human *1α (OH)ase* 305 promoter on basal and the PTH responses. The site-directed mutagenesis was performed as described in Chapter 2. The mutant promoter constructs were transiently transfected into AOK-B50 cells and treated with PTH (10^{-7}M) for 24h. The relative luciferase activity (RLA) represents the mean ± S.D firefly luciferase/*Renilla* luciferase ratios of triplicate samples. The experiment was repeated at least five times and similar results were observed. The fold induction shown is the ratio of relative luciferase activity from PTH treated cells to that from untreated cells (basal). The distal putative CRE (pmCRE-d-Luc) was not mutated but was destroyed by digestion with SpeI to generate a promoter length of 300bp.

The promoter therefore appears to contain both positive and negative regulatory elements that contribute to overall basal expression. While the putative NF-κB site at −219 bp/-209 bp was not mutated in these studies, other experiments in which the NF-κB subunits p50 and p65 and a dominant negative mutant of the inhibitor IκB α subunit, IκBαS32/36A, were over-expressed in AOK-B50 cells failed to reveal evidence for NF-κB involvement in basal expression of the transfected pCYPα-305-Luc construct (Figure 3.6). In these experiments, exogenous p50 and p65 did not increase basal expression while the dominant negative mutant, IκB α S19/23A, did not repress expression even when tested at 500ng.

In response to PTH (1-34), all mutant promoter constructs, except the construct containing the mutated CCAAT-p box, continued to display levels of induction of approximately 2-fold (Figure 3.5). With the mutated CCAAT-p box, induction was consistently reduced to a low level (1.28 ± 0.10 fold in this experiment). By comparison, mutagenesis of the distal GC box (GC-d) that resulted in marked basal repression, continued to respond to PTH with a 2.83 ± 0.22 fold level of induction. Similar data to that shown in Figure 3.5 were obtained in 5 separate experiments performed in triplicate following PTH treatment. I interpret these data to demonstrate that the CCAAT box site, but not the nearby Ets or GC-d site, is a contributor to PTH responsiveness of the 305-bp promoter.

3.2.4 Characterization of the distal putative GC box

To determine the identity of proteins that bind the putative distal GC box site, we performed electromobility shift assays (EMSAs) with an oligonucleotide encompassing the putative GC box from the native *1α(OH)ase* promoter (or *pCYPα-GC*) and nuclear extracts from AOK-B50 cells. Double stranded oligonucleotides encompassing the distal putative GC box, or the consensus Sp1 binding site (from Santa Cruz) were radiolabeled by end- filling using Klenow fragment I. Addition of nuclear extracts from AOK-B50 cells to an aliquot of the ^{32}P labelled distal putative GC box oligonucleotide results in the formation of one major and three minor
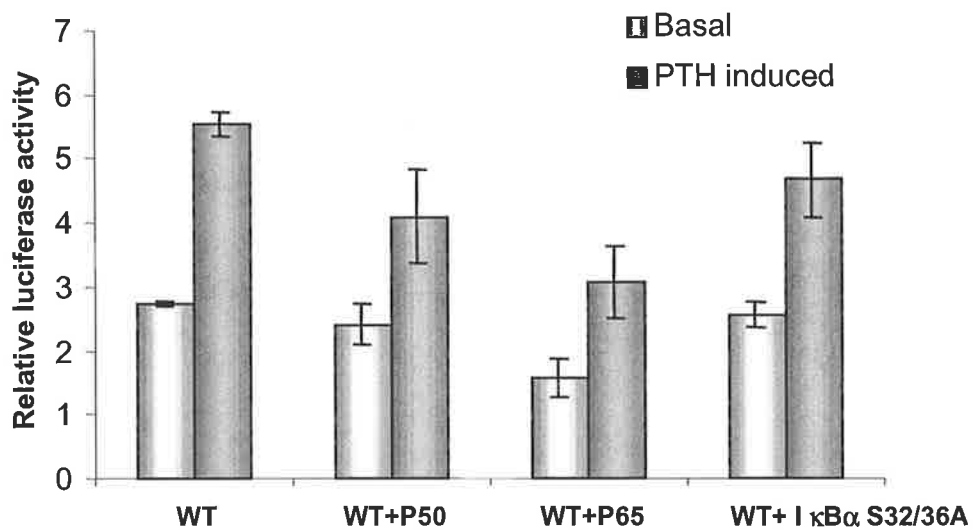
Figure 3.6 Cotransfection of NF-κB subunits or a dominant negative of NF-κB inhibitor IκBα, IκBαS32/36A together with pCYPα-305-Luc in AOK-B50 cells. The relative luciferase activity represents the mean ± S.D firefly luciferase/*Renilla* luciferase ratios of triplicate samples. The experiment was repeated at least twice and similar results were observed.

protein-DNA complexes, which were designated as complexes I, II, III and IV with the major band being complex III (Figure 3.7A). The four protein-DNA complexes were also observed using the radiolabelled consensus Sp1 oligonucleotide (Figure 3.7A, Lane 1), which suggests that the distal putative pCYPα-GC box binds Sp1. To determine the specificity of the four protein-DNA complexes, self-competition experiments were performed with 10 and 50-fold molar excess of the unlabelled pCYPα-GC. The formation of all four protein-DNA complexes was totally inhibited. However, 50-fold molar excess of an unlabelled oligonucleotide containing a VDRE (5' TCGACAGCCACATCCTCTGGAAC 3') was not able to compete with any the binding proteins. The results suggest that the four protein-DNA complexes are specifically binding to the distal putative GC box. To establish the identity of the trans-acting factors binding to the putative GC box, we carried out supershift assays with monoclonal antibody specific for Sp1 (Figure 3.7B). The addition of anti-Sp1 antibody results in a very weak supershift of complex I (Fig 3.7B, Lane2, Band SS) and has no effect on the other protein-DNA complexes. As a negative control, addition of an antibody specific for VDR had no effect on the formation of any complex. The data suggest that complex I contains Sp1 and that other proteins also can bind to the putative regulatory site. The identity of the major protein complex III is unknown.

To determine whether Sp1 activates basal expression of the *1α(OH)ase* gene promoter through the distal GC box site, we coexpressed plasmids containing the Sp1, or Sp3 cDNA under the control of the *Drosophila* melanogaster actin 5C promoter (designated as pPac-Sp1 or pPac-Sp3) together with pCYPα-305 in *Drosophila* SL3 cells. In these cells, Sp family members are absent. Cotransfection of pPac-Sp1 (1ng-10ng) leads to activation of pCYPα-305-Luc in a dose dependent manner (Figure 3.8). However, cotransfection of 10ng of pPac-Sp3 has no effect on the pCYPα-305-Luc activity. The data suggest that Sp1 but not Sp3 specifically acts on the *1α(OH)ase* promoter. To test if the Sp1 functions through the distal GC box site, I cotransfected the pPac-Sp1 expression cDNA together pCYPα-305-Luc or
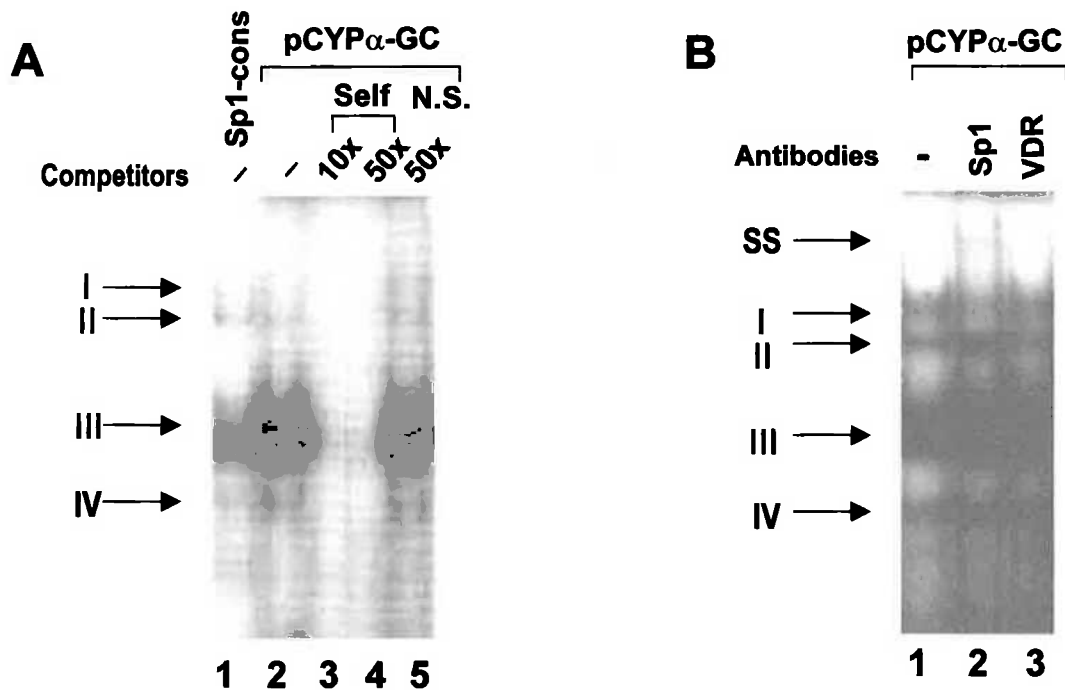
Figure 3.7 Binding of nuclear proteins to the GC box. A double stranded oligonucleotide to GC (pCYPα-GC) and a consensus Sp1 binding oligonucleotide (Sp1-cons) were radio labeled by end-filling and incubated with nuclear extracts from AOK-B50 cells. The major retarded complexes are arrowed and numbered as I to IV. **A,** the formation of complexes with labeled pCYPα-GC was competed with two unlabeled probes (i.e. self or non-specific probe CYP24-VDRE). **B,** for supershift assays, nuclear extracts were incubated with or without monoclonal antibody to Sp1 or VDR prior to addition of the labeled probe pCYPα-GC. A very weak supershifted band is observed (SS) in Lane 2.
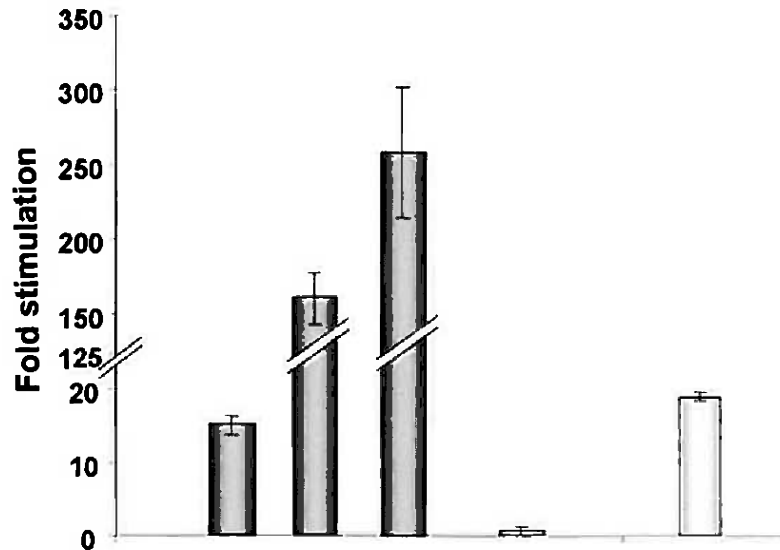
Figure 3.8 Cotransfection of pCYPα-305-Luc construct with expression plasmids for Sp1 and Sp3 into *Drosophila* SL3 cells. SL3 cells were transiently transfected by LipofectAmine 2000 with the indicated amounts of Sp1, Sp3 together with pCYPα-305-Luc or pmGC-305-Luc. Total DNA was adjusted by an addition of mock DNA plasmid. Cells were harvest 48 h after transfection and luciferase activity determined. The results were shown as fold induction, the ratio of relative luciferase activity from Sp factor transfected cells to that from mock DNA-transfected cells. Data represent one of three independent experiments performed in triplicate.

distal Sp1 mutant construct (designated as pmGC–305-Luc) into SL3 cells. Overexpression of

10ng pPac-Sp1 results in a fold activation of pmGC-305-Luc around 19.58 ± 0.80 comparing

fold activation of pCYPα-305-Luc at 254.63 ± 47.09 (Figure 3.8). The data indicated that

while Sp1 acts mostly through the distal GC box, some other weak Sp1 binding site may exist

in the 305 region of the *1α(OH)ase* promoter

3.2.5 Characterization of the CCAAT box

To characterize the inverted CCAAT site in the *1α(OH)ase* promoter, I performed EMSAs

using a radiolabelled double stranded oligonucleotide encompassing the putative proximal

CCAAT site from *1α(OH)ase* promoter (pCYPα-CCAAT) and nuclear extracts from AOK-

B50 cells (Figure 3.9). Two retarded bands were observed designated as complex I and II. To

determine the binding specificity of the DNA-protein complexes, competition assays using

unlabelled pCYPα-CCAAT and *1α(OH)ase* mutated CCAAT (mCCAAT) oligonucleotides

were undertaken. The addition of 50-fold molar excess of unlabelled pCYPα-CCAAT

inhibited the formation of complex I, but did not affect complex II (Figure 3.9A, Lane 3). On

this basis, complex II is considered non-specific. The addition of 50-fold molar excess of

unlabelled mutated CCAAT oligonucleotide had little effect on complex I formation (Figure

3.9A, Lane 5).

A number of transcription factors have been identified that can bind to a CCAAT motif (eg.

C/EBP and NF-Y) and one or more could participate in basal and /or PTH induction of the

1α(OH)ase promoter. The C/EBP family has at least six isoforms (C/EBP α, β, γ, δ, ε and ζ)

and C/EBP dimerisation is a prerequisite to DNA binding (Lekstrom-Himes and

Xanthopoulos, 1998). NF-Y constitutes a trimer of NF-YA, B and C subunits and either of the

subunits was required for DNA binding (Mantovani, 1999). I therefore tested oligonucleotides

containing the consensus NF-Y binding sequence (5' GTCTGGGCGCTG**ATTGG**CTGTG

GACTGCG 3', Osawa *et al.*, 1996) or the consensus C/EBP binding sequence (5'
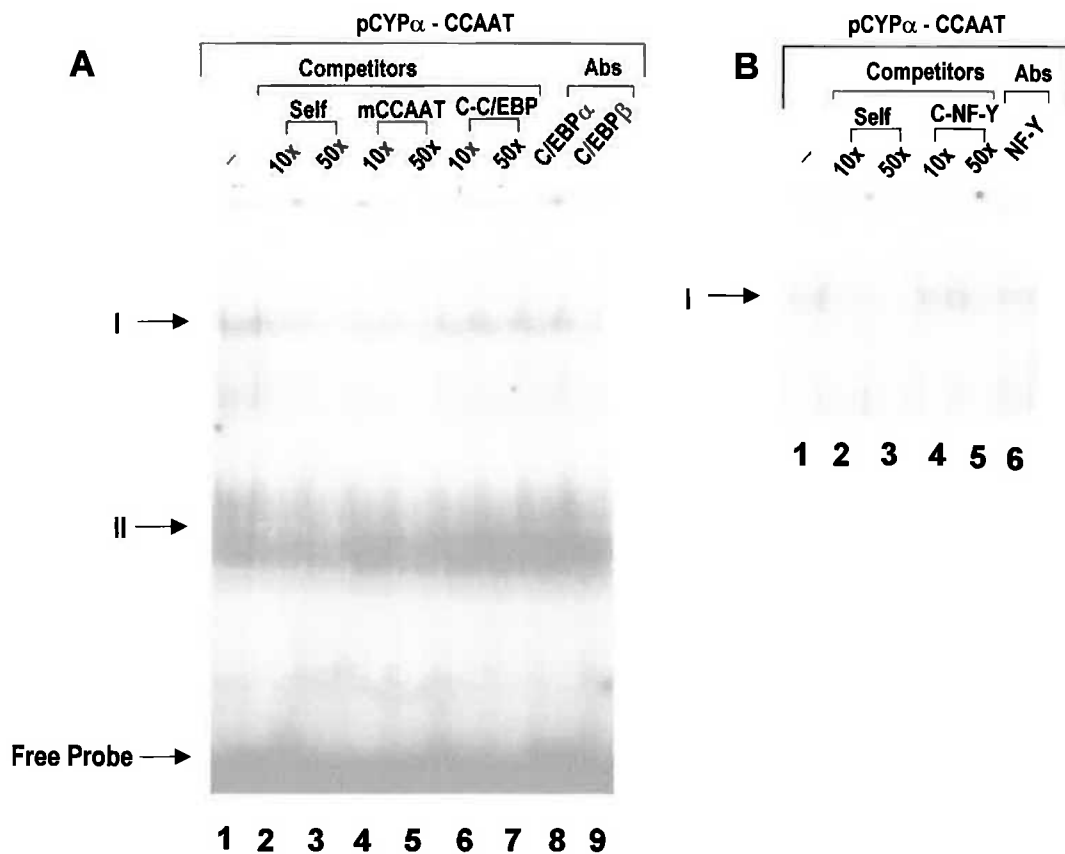
Figure 3.9 Binding of nuclear proteins to the CCAAT box. A double stranded oligonucleotide to CCAAT (pCYPα-CCAAT) was radio labeled by end-filling and incubated with nuclear extracts from AOK-B50 cells. The major retarded complexes is arrowed. Free probe is arrowed at the bottom of the gel. **A**, the formation of complexes with labeled pCYPα-CCAAT was competed with three unlabeled probes (i.e. self, mutant pCYPα-CCAAT or consensus C/EBP binding oligonucleotide (C-C/EBP)). For supershift assays, nuclear extracts were incubated with polyclonal antibody to C/EBPα (Lane 8) or C/EBPβ (Lane9) prior to addition of the labeled probe pCYPα-CCAAT. **B**, the formation of complexes with labeled pCYPα-CCAAT was competed with two unlabeled probes (i.e. self or consensus NF-Y) (lane 6). For supershift assays, nuclear extracts were incubated with polyclonal antibody to NF-YB.

GTGCAGA**TTGCG**CAATCTGCA 3', Santa Cruz). Addition of 50-fold molar excess of either unlabelled consensus C/EBP oligonucleotide (C-C/EBP) or unlabelled consensus NF-Y oligonucleotide (C-NF-Y) did not lead to inhibition of formation of complex I (Figure 3.9A and 3.9B). The results indicate that the protein binding to the CCAAT box of the *1α(OH)ase* promoter is not C/EBP or NF-Y. To further confirm this, gel mobility shift experiments employing various antibodies were carried out. The data show that the specific band could not be affected after addition of either polyclonal antibody to C/EBPα, C/EBPβ or NF-YB (Figure 3.9A and 3.9B).

To confirm that the ubiquitous transcription factor NF-Y is not involved in the CCAAT box activity, I investigated the role of NF-Y in PTH mediated induction of the *1α(OH)ase* gene promoter using transient transfection assays. Of the heterotrimeric complex, NF-YA appears to play the critical role in activation of the binding activity of NF-Y during cell differentiation and proliferation (Chang *et al.,* 1994; Marziali *et al.,* 1999; Bolognese *et al.,* 1999; Inoue *et al.,* 1999). A dominant negative mutant form of NF-YA (pNF-YAm29) with three amino acids mutated in the DNA binding domain is functionally inactive both *in vitro* and *in vivo* (Jackson *et al.,* 1995; Mantovani *et al.,* 1994). Overexpression of pNF-YAm29 (at concentrations of 200ng-1000ng) resulted in a dose dependent reduction of pCYPα-305-Luc basal expression (30-60%) (Figure 3.10A). However, in the presence of PTH peptide the lowered levels of basal expression, produced by the dominant negative mutant, remained sensitive to PTH and were increased about 2-fold comparable with wild type induction (Figure 3.10A). These results suggest that NF-Y is not involved in the PTH induction mechanism. The basal inhibition possibly represents a non- specific titration of an unknown transcription factor by pNF-YAm29 and this has not been pursued further.

To determine if the C/EBP family members participate in basal or PTH induction of pCYPα-305-Luc, I examined the role of the dominant negative mutant of C/EBP, A-C/EBP, that is known to heterodimerise with all C/EBPs (Krylov *et al.,* 1995). Overexpression of A-C/EBP

in AOK-B50 cells resulted in a concentration dependent inhibition of both basal expression and PTH induced expression, with marked inhibition observed at 1μg of expression vector (Basal from 2.8 to 0.8; PTH mediated induction from 5.7 to1.2) (Figure 3.10A). This result indicates that a C/EBP isoform is required for basal expression and also for the PTH mediated induction process.

There is evidence that C/EBP β is phosphorylated in response to cAMP raising the possibility that this isoform may participate in the PTH response. However, overexpression in AOK-B50 cells of wild type C/EBP β at concentrations of 200ng, did not affect increase basal or PTH responsiveness of the transfected *1α(OH)ase* promoter (Figure 3.10B). Hence, we conclude that C/EBP β is most unlikely to be involved in *1α(OH)ase* promoter activity in kidney cells. Cotransfection of another C/EBP member, C/EBPδ together with *1α(OH)ase* actually reduced basal and PTH mediated induction (Figure 3.10B). The mechanism underlying this is not understood at this stage. However, the data indicate that C/EBPδ is not involved in PTH induced expression of *1α(OH)ase*.

In parallel experiments C/EBP α isoform was also overexpressed. In contrast to C/EBP β, 200ng of C/EBP α lowered basal expression of pCYPα–305-Luc wild type construct by about 82% (Figure 3.10B). The reason for this is not known and experiments with overexpressed Sp1 partially reversed the inhibition perhaps suggesting titration of Sp1 (Data not shown). Importantly, in the presence of overexpressed C/EBP α, the observed lowered basal expression of the wild-type promoter remained sensitive to PTH peptide, and indeed was consistently elevated to levels of 3-4 fold that were higher than the 2-fold level obtained with PTH peptide alone (Figure 3.10B). The 3 or 4-fold level of induction was lowered to 2-fold when the CCAAT box was inactivated suggesting that the additional 2-fold level of induction was mediated by another CCAAT box site in the promoter (Data not shown). One other site (TCATTGCAACATGAG) that resembled a CCAAT box at (-73/-59) in the 305bp promoter was mutated individually and the constructs were transiently transfected into AOK-B50 cells.
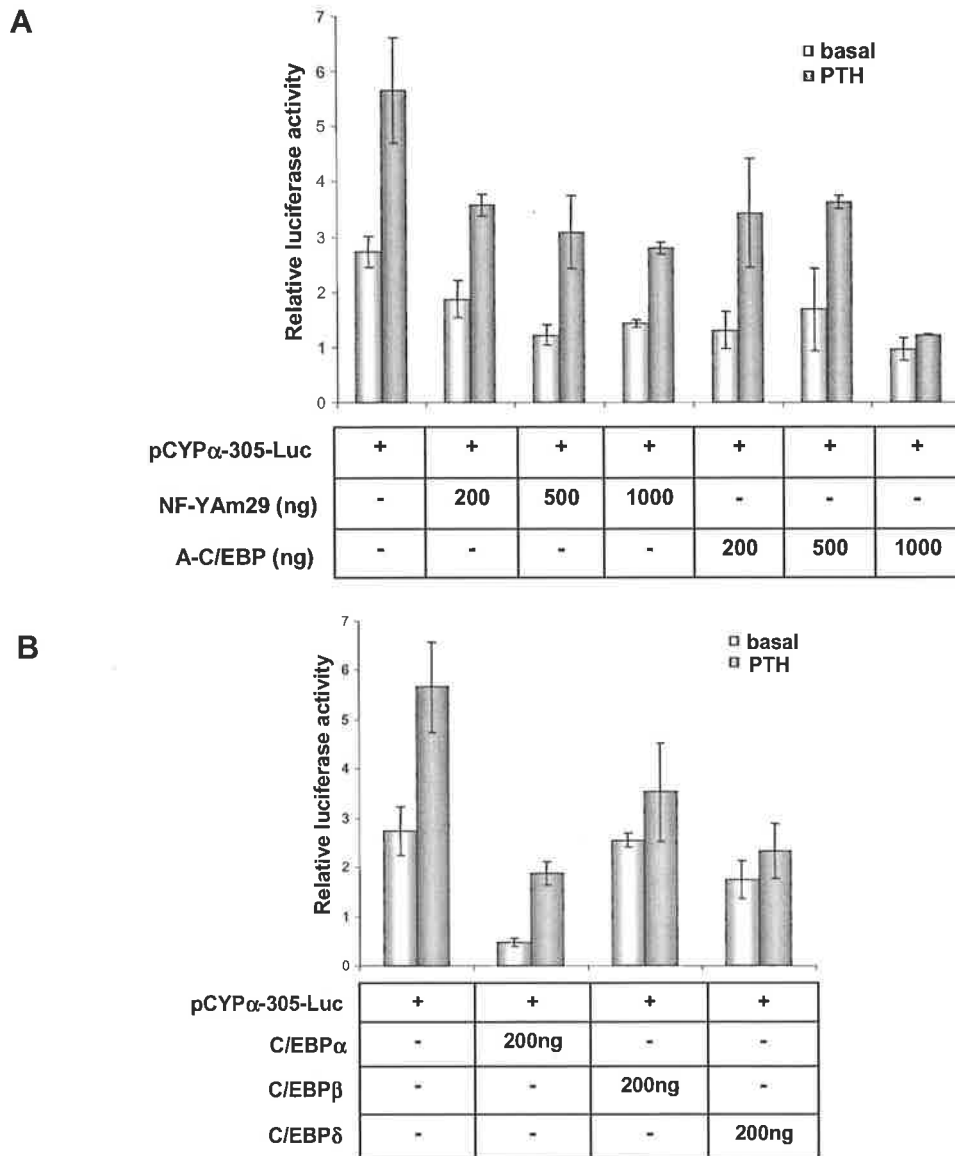
Figure 3.10 Involvement of C/EBP member or NF-Y in basal or PTH mediated induction in AOK-B50 cells. A, Effect of dominant negative C/EBPs, A-C/EBP or a dominant negative NF-Y, NF-YAm29. AOK-B50 cells were cotransfected with increasing amounts of each above construct together with pCYPα-305-Luc and treated with 10^{-7} M PTH for 24h. **B,** Cotransfection of C/EBP isoforms, C/EBPα, β and δ, with pCYPα-305-Luc into AOK-B50 cells. The relative luciferase activity represents the mean ± S.D firefly luciferase/*Renilla* luciferase ratios of triplicate samples. Data represent one of three independent experiments performed in triplicate.

However there was no effect on the high level of induction observed in the presence of PTH and exogenous C/EBP α (Data not shown). The enhanced PTH induction seen in the presence of exogenous C/EBP α continued to be seen with each of the mutant promoter constructs examined in Figure 3.5, establishing that the action of overexpressed C/EBP α is not dependent on any of these promoter sites (Data not shown).

In conclusion, data from overexpression of A-C/EBP suggested that a C/EBP family member may be involved in PTH mediated induction. However the known CCAAT binding proteins C/EBPα, β and δ do not appear to be involved. Therefore the identity of the CCAAT binding protein remains unknown and whether it is in fact of member of the C/EBP family is not clear.

3.2.6 Effect of inhibitors on basal and PTH induced expression

To evaluate whether protein kinase A (PKA), protein kinase C (PKC), ERK or p38 mitogen activated protein (MAP) kinase pathways contribute to basal or PTH-induced expression of the pCYPα-305-Luc construct in transiently transfected AOK-B50 cells, the effects of known specific pharmacological kinase inhibitors (H89, calphostin C, PD098059 or SB 203580 respectively) on promoter activity were determined. The data in Figure 3.11 show that basal expression of the promoter construct was not affected by the PKA inhibitor H89. However, PTH-induced levels were completely inhibited, which implicated PKA in the induction mechanism. Neither the PKC inhibitor calphostin C nor the MAP kinase pathway inhibitors PD98059 and SB203580 affected basal or PTH induced expression.

3.3 Discussion

The up-regulation of renal *1α(OH)ase* expression by PTH under hypocalcemic conditions is a key physiological response in vitamin D metabolism resulting in increased $1,25(OH)_2D_3$ synthesis and enhanced systemic calcium levels. While this action of PTH has been known for
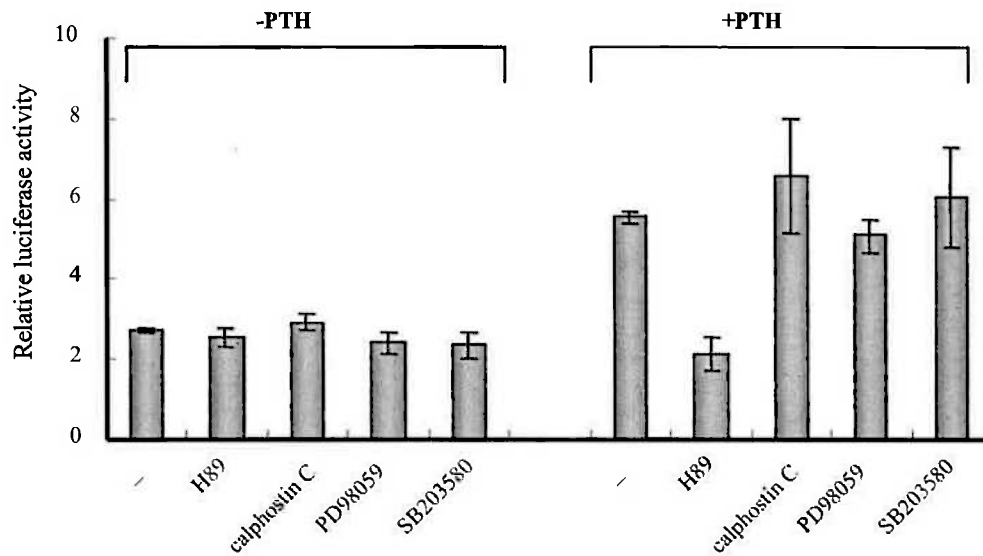
Figure 3.11 Effect of PKA, PKC and MAP kinase inhibitors on pCYPα-305-Luc. AOK-B50 cells were transiently transfected with plasmid pCYPα-305-Luc and pre-incubated with H89, calphostin C, PD98059 and SB203580 for 1.5h before 24h incubation with PTH or vehicle. The relative luciferase activity shown represent the mean ± S.D firefly luciferase/*Renilla* luciferase ratios of triplicate samples from one of three independent experiments.

many years, the molecular mechanism underlying the response has not been elucidated. Transient expression analysis with mouse and human *1α(OH)ase* promoter constructs has identified regions of PTH responsiveness located within the first 1.4kb of the human promoter (Kong XF *et al.,* 1999) and in other studies, further upstream at –4.4kb/-3.2kb (Murayama *et al.,* 1999). The physiological significance of the latter finding is unclear since this region lies in an upstream gene. However, no specific transcription factor-binding site that responds to PTH has been reported in the 5' flanking region of the *1α(OH)ase* gene.

AOK-B50 cells with stably expressed oppossum PTH receptor (Bringhust *et al.,* 1993) were used in the current study and PTH responsiveness was located in the first 305 bp of *1α(OH)ase* promoter sequence. Within this sequence, I have identified the regulatory control elements that drive basal expression of the *1α(OH)ase* gene and importantly have identified a transcription site that contributes to PTH up-regulation. For basal expression, mutagenesis and expression analysis has identified three functional clustered sites. These sites comprise a GC box site, a CCAAT box site and a putative site for an Ets family member.

Using *Drosophila* SL3 cells, I have shown over-expression of Sp1 but not Sp3 markedly stimulates basal expression of pCYPα-305-Luc through the GC-d site and on this basis it seems likely that Sp1 contributes to basal expression in the AOK-B50 cells. EMSAs employing an oligonucleotide encompassing the distal GC box site and nuclear extracts from AOK-B50 cells reveal four specific protein-DNA complexes and the one with the slowest mobility can be partially supershifted with Sp1 antibody. The result suggests the involvement of Sp1 in the basal transactivation of the *1α(OH)ase* promoter.

Multiple proteins are known to bind to CCAAT box sites. In EMSA experiments with nuclear extracts from AOK-B50 cells, I have identified one major protein complex band that specifically interacts with the CCAAT box oligonucleotide. Cross-competition assays demonstrated that neither an excess molar of consensus NF-Y nor C/EBP binding oligonucleotides affects the formation of the specific protein-DNA complex. In addition,

supershifting antibody for NF-YB has no significant effect on this specific band. Other experiments with supershifting antibodies to C/EBP α and β also failed to affect this band. Hence, further work is required to identify the protein that binds to the functional CCAAT box. We have not as yet investigated the identity of the protein(s) that bind to the putative Ets protein-binding site.

Importantly, the level of PTH induction was only affected when the proximal CCAAT box site was mutated in the promoter. When this site was mutated, the level of PTH induction was not maintained and this finding was seen in repeated experiments. This represents the first report for the identification of a promoter site in the 1α-hydroxylase gene promoter that is involved in PTH up-regulation. Over expression of NF-YAm29, a dominant negative form of NF-Y has no effect on the level of PTH mediated induction, however over expression of A/CEBP at 1μg significantly inhibited PTH activation. The data suggests that a C/EBP member is critical for PTH mediated induction, however the significance is difficult to access particularly since C/EBP members investigated did not interact with the CCAAT site directly as shown by supershift EMSAs.

Over expression of C/EBP α further enhances PTH mediated induction of *1α(OH)ase* promoter activity but how this is enhanced remains unclear since it apparently is not dependent on a CCAAT box in the promoter. In this regard, the data presented here resembles that reported for the over expression of C/EBP α on the promoter of the p8 gene (Vasseur *et al.*, 1999), where transcriptional activation was apparently independent of any CCAAT box binding site in the promoter.

In marked contrast to the mutagenesis of the proximal CCAAT box, mutagenesis of the nearby GC box site resulted in a low level of basal expression similar to that observed with the mutated CCAAT box but remained sensitive to PTH. A putative EBS (-133/-120) also contribute to basal expression but not PTH mediated induction. It is not clear whether the three proteins driving basal expression, Sp1, CCAAT binding proteins and Ets that bind in close

proximity are functionally cooperative. There is evidence from other studies that Sp1 can interact with CCAAT box proteins or with Ets proteins (Yamada *et al.,* 2000; Ge *et al.,* 2001; Xia and Zhang, 2001). Whether there is cooperation between the three proteins on the *1α(OH)ase* promoter is not clear. Since mutagenesis of either the GC-d or CCAAT-p sites results in markedly lower levels of basal expression, it can be reasoned that a physical interaction occurs between these sites. However, the presence of an Ets protein between these sites makes this unlikely. Moreover, mutagenesis of the Ets site does not affect basal expression as greatly as mutagenesis of either the flanking GC or CCAAT box sites, which does not support the proposal that there is significant interaction between these proteins. On this basis, we propose that the CCAAT binding protein interacts with the promoter and responds to PTH independently of the nearby functional Sp1 and Ets proteins, but this proposal require further investigation.

Experiments with pharmacological inhibitors indicated that MAP kinase pathways and PKC activities are not involved in either basal or PTH induced expression of *1α(OH)ase* in transiently transfected AOK-B50 cells. However, the protein kinase inhibitor H89 completely prevented PTH induction but did not alter basal expression of the *1α(OH)ase* promoter. While H89 shows selective inhibition for PKA it has recently been shown to inhibit a histone kinase that acts downstream of MAP kinases. Since MAP kinase inhibitors of the ERK1/2 and p38 MAP kinase pathways, PD98059 and SB203580 respectively, have no effect on PTH induction in our experiments (Figure 3.11), it is most unlikely that the H89 inhibition of induction reflects inhibition of histone phosphorylation. We suggest the possibility that PTH may alter the phosphorylation status of the CCAAT box binding protein through a PKA-dependent mechanism. Future work will therefore focus on characterization of the CCAAT protein and its phosphorylation status in the presence and absence of PTH.

A model to explain PTH induced expression of the *1α(OH)ase* gene promoter can be proposed (Figure 3.12). The basal expression of the promoter is predominantly driven by the
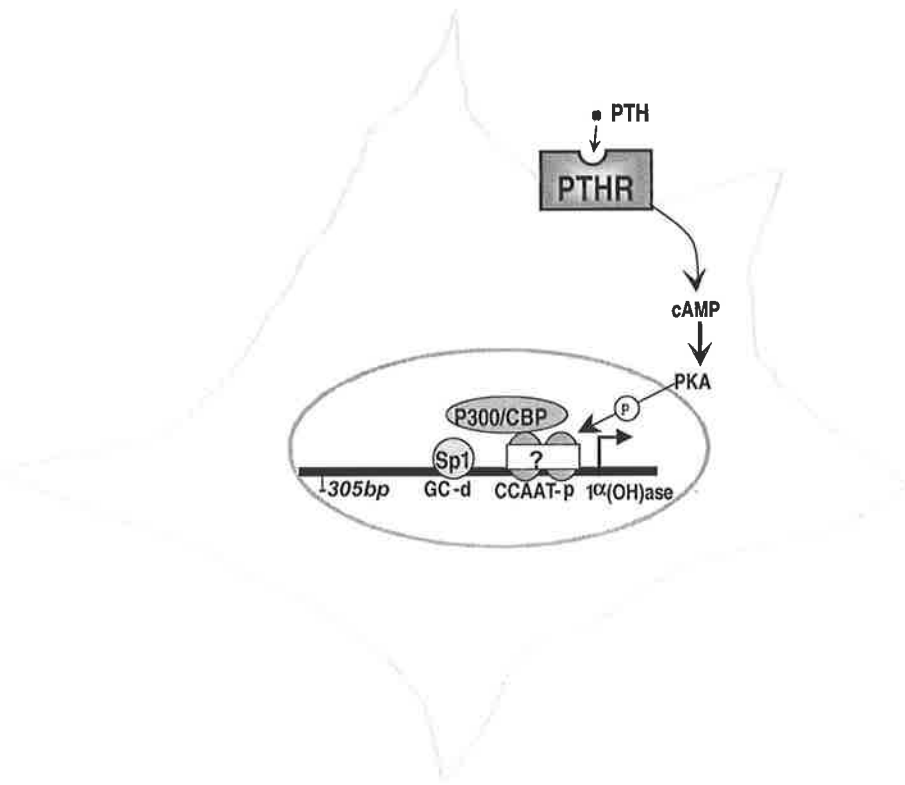
Figure 3.12 A model for transcription regulation of *1α(OH)ase* gene by PTH.

distal GC box and the proximal CCAAT box. Sp1 binds the distal GC box site but the protein binding to the CCAAT box is unknown. PTHR belongs to the superfamily of G-protein coupled seven-transmembrane receptors. Liganded PTHR results in an activation of a G protein that in turns activates AC, increasing the intracellular concentration of cAMP. cAMP then activates PKA. We propose that PKA phosphorylates the CCAAT binding protein and that this affects transcription events through increasing the binding of the CCAAT binding protein to DNA or by promoting interaction with a coactivator such as p300/CBP.

✦ ✦

CHAPTER 4

Regulation of the rat CYP24 gene expression in kidney and osteoblast cells: Role of Sp1 and CCAAT binding proteins in basal and $1,25(OH)_2D_3$ mediated induction of the promoter.

✦ ✦

4.1 Introduction

$1,25(OH)_2D_3$ is a key physiological regulator of *CYP24* expression in the kidney and other tissues (see Introduction). In a positive feed back response, expression of *CYP24* is markedly up-regulated in the presence of excessive $1,25(OH)_2D_3$. In mice null for *CYP24*, there is elevated serum $1,25(OH)_2D_3$ and the mice display symptoms of $1,25(OH)_2D_3$ toxicity (Yoshizawa *et al.*, 1997). Hence the physiological role of *CYP24* is to modulate the cellular and circulating levels of $1,25(OH)_2D_3$ through catabolism of the hormone.

The molecular mechanism by which $1,25(OH)_2D_3$ stimulates *CYP24* gene expression in kidney has been extensively studied by us and other research groups. In the rat *CYP24* promoter, two vitamin D responsive elements (VDREs) were identified (Hahn *et al.*, 1994) and shown to synergise with each other in the response to $1,25(OH)_2D_3$ (Kerry *et al.*, 1996). The two VDREs were designated as VDRE1 at –150/-136 and VDRE2 at –258/-244. VDRE1 shows lower binding affinity of VDR/RXR complex in an electromobility shift assay (EMSA) but contributes to transactivation of the *CYP24* gene promoter in response to $1,25(OH)_2D_3$ to a greater extent compared with VDRE2. To explain this it was suggested that nearby transcription factors around VDRE1 form an enhanceosome to interact with VDR/RXR or coactivators that would facilitate the transactivation through VDRE1. One such site is a binding site for Ets-1 (EBS) (-128/-119) located downstream of VDRE-1; mutagenesis of this site markedly lowers induction of the promoter through VDRE-1 with Ets-1 probably interacting with VDR (Dwivedi *et al.*, 2000).

Further studies in our laboratory have shown that the genomic (ie transcriptional) activation of the *CYP24* promoter is critically dependent on contributions from signaling pathways including ERK1/2/5 and PKC (Dwivedi *et al.*, 2002). Dwivedi *et al.* (2002) has shown that $1,25(OH)_2D_3$ activates ERK1/2/5 MAPK activity and this leads to phosphorylation of Ets-1 and RXR α. If the activation of these ERKs is inhibited, then $1,25(OH)_2D_3$ induction is severely repressed. A search was undertaken in our laboratory to determine whether there

were other transcription factor binding sites near VDRE1, in addition to an Ets-1 site (EBS). A computer programme MatInspector (V2.2) (http://transfac.gbf.de/) revealed a putative GC box (-114/-101) downstream from the EBS and a putative CCAAT box (-62/-51) located further down stream on the non-coding strand. The aim of this chapter was to investigate the functionality of the GC and CCAAT boxes in transiently transfected kidney and osteoblast cell lines expressing the first -298bp of rat promoter fused to the firefly luciferase reporter vectors and to determine their contributions to basal and $1,25(OH)_2D_3$ induced expression. The EMSAs and some transient transfection assays related to characterization of the GC box were performed in conjunction with Dr. Prem Dwivedi.

4.2 Results

4.2.1 Identification of functional GC and CCAAT boxes in the *CYP24* promoter

As mentioned, analysis of the rat *CYP24* promoter sequence between -298bp and +1 bp using a MatInspector (V2.2) program revealed a GT box (-100 CGTCC<u>CTCC</u>CAGC -88) (Bouwman and Philipsen, 2002), a GC box (-114 ACAC<u>CCGCCC</u>CCCG-101) immediately downstream of the EBS and an inverted CCAAT box at (-62 GCTC<u>ATTGG</u>CCA -51) (Figure 4.1). A TATA-like box at -30/-24 (ATAAATA) has previously been identified (Hahn *et al.*, 1994).

Inoue *et al.* (1999) have reported that the molecular mechanism of vitamin D_3-induced transcription of the P27kip1 gene apparently does not involve VDR, but involves a CCAAT box and GC box in the promoter of the gene. However, the CCAAT box and GC boxes identified here are not able to respond to $1,25(OH)_2D_3$ directly since we have demonstrated that $1,25(OH)_2D_3$ induction of the -298bp rat *CYP24* promoter is completely inhibited when both VDREs are inactivated (Kerry *et al.*, 1996). To evaluate whether the putative GC, GT and CCAAT boxes can contribute to basal and/or $1,25(OH)_2D_3$-dependent *CYP24* promoter activity, the core sequence of each site was individually mutated in a vector containing -

-298

A G G C C A G C T G C A G G A G G A G G G C G A G G A G G C G T G C

      -258   **VDRE-2**   **-244**

T C G C A G C G C A C C C G C T G A A C C C T G G G C T C G A C C C G

C C T T T C T C A G G T T A T C T C C G G G G T G G A G T C C A C C G

G T G C G T C T G C C G G G C C A G C A G C G T G T C G G T C A C C G

      -150   **VDRE-1**   **-136**   **-128**

A G G C C C C G G C G C C C T C A C T C A C C T C G C T G A C T C C A

EBS -119   **-114**   **GC**   **-101 -100**   **GT**

T C C T C T T C C C A C A C C C G C C C C C C G C G T C C C T C C C A G

-88   **-62**   **CCAAT**

C C G G T C C C C C T T G C C C T G C T C A G C G T G C T C A T T G G C

-51   **-30 TATA -24**

C A C T C C A G C A T G C C C T G C T T C C A T A A A T A C G A G G T

                         +1   **EXON1**

C C C T A T G C C T G G A A T C T T G G G G A G G T A C A A C G A

Figure 4.1 Nucleotide sequence of the 5' flanking region (-298) of the rat _CYP24_ gene . The transcription factor binding sites are boxed and numbered for the location. The transcription starting site is also arrowed.

298bp of *CYP24* promoter sequence fused to the firefly luciferase reporter gene (Figure 4.2). The wild type and the mutants of GT, GC and CCAAT boxes were designated as p*CYP24*WT-Luc, p*CYP24*mGT-Luc, p*CYP24*mGC-Luc and p*CYP24*mCCAAT-Luc respectively. These mutant constructs and the wild type construct were individually introduced into either kidney HEK-293T cells or for comparison, rat osteosarcoma UMR106 cells, using the DOTAP transfection protocol. A recombinant vector pRL-TK expressing *Renilla* luciferase was also transfected as an internal control for transfection efficiency. Due to the limited amounts of VDR in kidney HEK-293T cells, 200ng of pRSV-hVDR was over expressed in all transient transfection assays in this cell line. The transfected cells were treated with 10^{-7}M $1,25(OH)_2D_3$ for 24 hours, lysed and subjected to the dual luciferase assay.

As seen in Figure 4.3 and 4.4, mutagenesis of the putative GT box does not significantly alter either the basal or the $1,25(OH)_2D_3$ mediated induction of the *CYP24* promoter activity in either cell line. However, the putative GC box contributed to basal expression in both HEK-293T and UMR106 cells with levels decreasing about 50% from 0.15 to 0.07 (Figure 4.3) and from 0.25 to 0.14 (Figure 4.4) respectively. In response to $1,25(OH)_2D_3$, the p*CYP24*WT-Luc gave about a 23-fold level of induction, but this was reduced to about 11-fold when the mutated GC box was tested in HEK-293T cells. Similar data was obtained in UMR106 cells, where the inactivated GC box construct showed 34-fold induction compared with 60-fold by the wild type construct.

The CCAAT box sequence also contributed substantially to both basal and $1,25(OH)_2D_3$-induced expression. When this site was inactivated, basal activity of the promoter decreased in HEK-293T cells (from 0.15 to 0.05) and in UMR106 cells (from 0.25 to 0.09). The CCAAT box was found to be more important for $1,25(OH)_2D_3$ induction in HEK-293T cells with inactivation of this site resulting in a loss of 75% of induced activity (from 23-fold to 5-fold) while in UMR106 cells inhibition was about 40% (from 60-fold to 38-fold).

To further confirm that each of the GC and CCAAT box sites contributed substantially to basal expression and $1,25(OH)_2D_3$ responsiveness, the two sites were mutated simultaneously
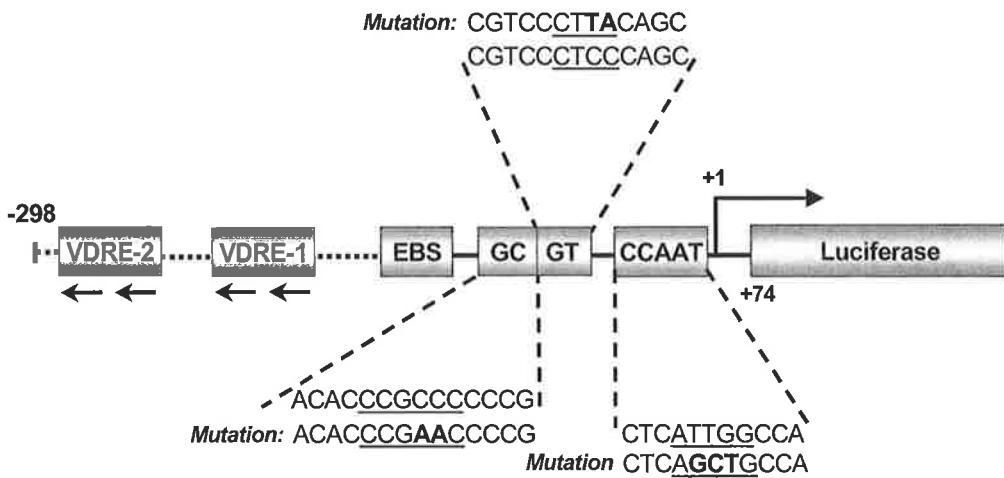
Figure 4.2 **Diagrammatic representation of the proximal region of the rat** *CYP24* **promoter.** Illustrated are the transcriptional initiation site, VDRE1, VDRE2, EBS, putative GC, GT and CCAAT sites. Regions of the promoter sequence are expanded to illustrate the three putative sites and their corresponding mutated sequences (mutated nucleotides as bold type).

	Basal (RLA)	$1,25(OH)_2D_3$ (RLA)	Fold ($\pm\ 1,25(OH)_2D_3$)
pCYP24 WT-Luc	0.15 ± 0.02	3.47 ± 0.07	23.43 ± 0.47
pCYP24 mGC-Luc	0.07 ± 0.02	0.69 ± 0.09	10.73 ± 0.03
pCYP24 mGT-Luc	0.13 ± 0.04	3.03 ± 0.20	23.33 ± 0.83
pCYP24 mCCAAT -Luc	0.05 ± 0.01	0.24 ± 0.03	4.79 ± 0.60
pCYP24 mGC+CCAAT -Luc	0.03 ± 0.01	0.08 ± 0.01	2.56 ± 0.33
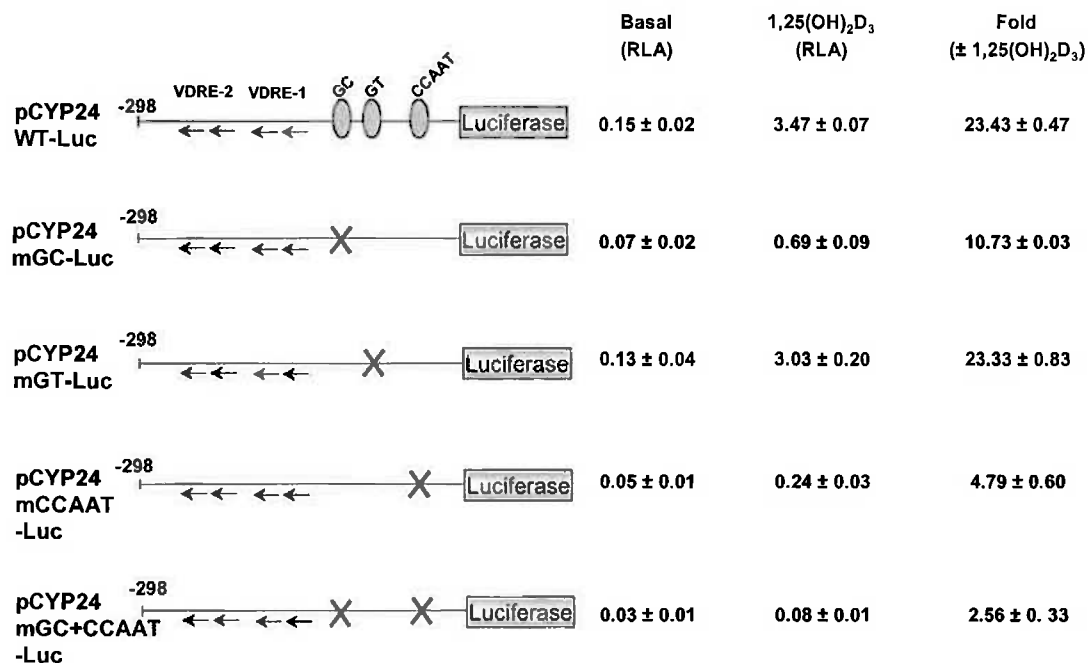
Figure 4.3   Effect of site-directed mutagenesis of the putative GC, GT and CCAAT sites in the rat *CYP24* 298bp promoter on basal and the $1,25(OH)_2D_3$ response in HEK-293T cells. HEK-293T cells were transiently transfected with wild-type, p*CYP24*WT-Luc, p*CYP24*mGC-Luc, p*CYP24*mGT-Luc, p*CYP24*mCCAAT-Luc or p*CYP24*mGC+CCAAT-Luc and treated with $1,25(OH)_2D_3$ for 24h. The relative luciferase activity (RLA) represents the mean ± S.D firefly luciferase/*Renilla* luciferase ratios of triplicate samples. The fold induction shown is the ratio of relative luciferase activity from $1,25(OH)_2D_3$ treated cells to that from untreated cells (ethanol). Data are the representative of three independent experiments performed in triplicate.

	Basal (RLA)	1,25(OH)$_2$D$_3$ (RLA)	Fold (± 1,25(OH)$_2$D$_3$)
pCYP24 WT-Luc	0.25 ± 0.05	14.92 ± 3.42	60.43 ± 13.70
pCYP24 mGC-Luc	0.14 ± 0.02	4.61 ± 0.68	33.66 ± 4.29
pCYP24 mGT-Luc	0.18 ± 0.03	12.33 ± 0.35	68.52 ± 1.96
pCYP24 mCCAAT -Luc	0.09 ± 0.02	4.98 ± 0.85	38.34 ± 6.56
pCYP24 mGC+CCAAT -Luc	0.06 ± 0.02	3.62 ± 0.64	24.42 ± 6.42

Figure 4.4   Effect of site-directed mutagenesis of the putative GC, GT and CCAAT sites in the rat *CYP24* 298bp promoter on basal and the 1,25(OH)$_2$D$_3$ response in UMR106 cells. UMR106 cells were transiently transfected with wild-type, p*CYP24*WT-Luc, p*CYP24*mGC-Luc, p*CYP24*mGT-Luc, p*CYP24*mCCAAT-Luc or p*CYP24*mGC+CCAAT-Luc and treated with 1,25(OH)$_2$D$_3$ for 24h. The relative luciferase activity (RLA) represents the mean ± S.D firefly luciferase/*Renilla* luciferase ratios of triplicate samples. The fold induction shown is the ratio of relative luciferase activity from 1,25(OH)$_2$D$_3$ treated cells to that from untreated cells (ethanol). Data are the representative of three independent experiments performed in triplicate.

and transfected into either HEK-293T or UMR106 cells. In HEK-293T cells, basal expression was further decreased (0.03) and the $1,25(OH)_2D_3$ mediated induction of *CYP24* promoter activity substantially reduced to about 3-fold (Figure 4.3). In UMR106 cells, mutagenesis of the two sites resulted in basal expression being further decreased to 0.06 and $1,25(OH)_2D_3$ mediated induction of *CYP24* activity reduced to 24-fold (Figure 4.4).

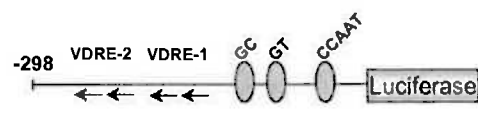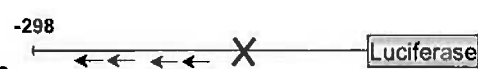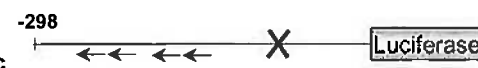Hence the GC and CCAAT box sequences together contribute substantially to basal expression with expression inhibited about 75% in both cell lines when both sites are inactivated. As well, these sequences are important for $1,25(OH)_2D_3$ induction particularly in HEK-293T cells where inactivation of both the GC and CCAAT boxes lowering induction by nearly 90%. In view of this, it was important to characterize the GC and CCAAT boxes by identifying the binding transcription factors, pursue their relationship with other nearby binding proteins, and finally to investigate the signaling pathways possibly impinging on the two sites. Firstly however, the expression of the wild type and mutant constructs were examined in stably transfected cells.

4.2.2 Functional role of GC and CCAAT boxes in stable cell lines

The roles of GC and CCAAT boxes were next analyzed in stable cell lines created from HEK-293T cells. Each construct (p*CYP24*WT-Luc, p*CYP24*mGC-Luc and p*CYP24*mCCAAT-Luc) was cotransfected using a DOTAP transfection protocol with a puromycin resistant vector (pEF-IRES-puro6) into HEK-293T cells (See 2.5.2). Transfected cells were then cultured in selection medium with puromycin and the resistant colonies were pooled. These three pooled stable cell lines were designated as H298, HmGC and HmCCAAT respectively.

The three lines were treated with 10^{-7}M $1,25(OH)_2D_3$ for 24 hours and firefly luciferase activity was determined in cell lysates (Figure 4.5). The luciferase activity was normalized by the protein content in each sample. The basal *CYP24* directed luciferase activity was 1.64 in HmGC cells, only half of that in wild type H298 cells which was 3.38. In response to $1,25(OH)_2D_3$ the luciferase activity in HmGC cells was induced 26-fold and that in H298

	Basal (RLA)	1,25(OH)$_2$D$_3$ R(LA)	Fold (± 1,25(OH)$_2$D$_3$)
H298	3.38 ± 0.67	143.33 ± 19.08	42.4 ± 5.65
HmGC	1.64 ± 0.41	42.23 ±3.91	25.66 ± 6.03
HmCCAAT	0.33 ± 0.07	3.03 ± 0.35	9.21 ± 0.05

Figure 4.5 Stably transfected HEK-293T cells expressing constructs containing wild-type, mutated putative GC and CCAAT box in the rat *CYP24* promoter. The relative luciferase activity (RLA) represents the mean ± S.D firefly luciferase/protein content ratios of triplicate samples. The fold induction shown is the ratio of relative luciferase activity from 1,25(OH)$_2$D$_3$ treated cells to that from untreated cells (ethanol). Data represent one of three independent experiments performed in triplicate.

cells about 42-fold. These results were comparable with that from transiently transfected cells (Figure 4.3). When the CCAAT box was mutated, the basal activity of *CYP24* promoter was reduced by 10-fold (from 3.38 to 0.33). With this mutant, the 1,25(OH)$_2$D$_3$ mediated induction of 42-fold was lowered to 9-fold, a reduction of 80%. The dependence on the CCAAT box for 1,25(OH)$_2$D$_3$ induction in the stable cell line is comparable with that in transiently transfected cells where 1,25(OH)$_2$D$_3$ induction was reduced by 75% in HEK-293T cells.

4.2.3 Characterization of the GC box binding proteins

It is well known that Sp1 and Sp3, two ubiquitous transcription factors, can bind to GC boxes (Suske, 1999). To determine if Sp1 can bind to the GC box in the *CYP24* promoter, EMSAs were performed with an oligonucleotide (*CYP24*-GC) encompassing the *CYP24* GC box site (5' GCCACACCCGCCCCCCG 3') and nuclear extracts prepared from both HEK-293T cells and UMR106 cells (Figure 4.6 and Figure 4.7). Retarded DNA nuclear protein complexes were analyzed on a 4% non-denaturing polyacrymide gel. Three retarded bands (I, II and III) were detected using radio labeled *CYP24*-GC oligonucleotide and the nuclear extracts from HEK-293T cells (Figure 4.6A, Lane 2). Competition experiments showed that 50-fold molar excess of unlabeled *CYP24*-GC probe inhibited formation of complexes I-III (Lane 3) and competition experiments with a consensus Sp1 binding sequence (5' GATTCGATCGGGGCGGGGCGAG 3') also significantly inhibited formation of complexes I-III (Lane 4). Formation of complex I however was particularly affected by each competitor in lanes 3 and 4. A nonspecific inhibitor (an oligonucleotide encompassing the *CYP24* VDRE1, 5' TCGACAGCCACATCCTCTGGAAC 3') had no effect on complexes I-III (Lane 5). In the profile of DNA binding with the consensus Sp1 binding sequence, only complex I was observed with a similar mobility (Figure 4.6A, compare Lane 1 and Lane 2). The results suggest that complex I contained Sp1.
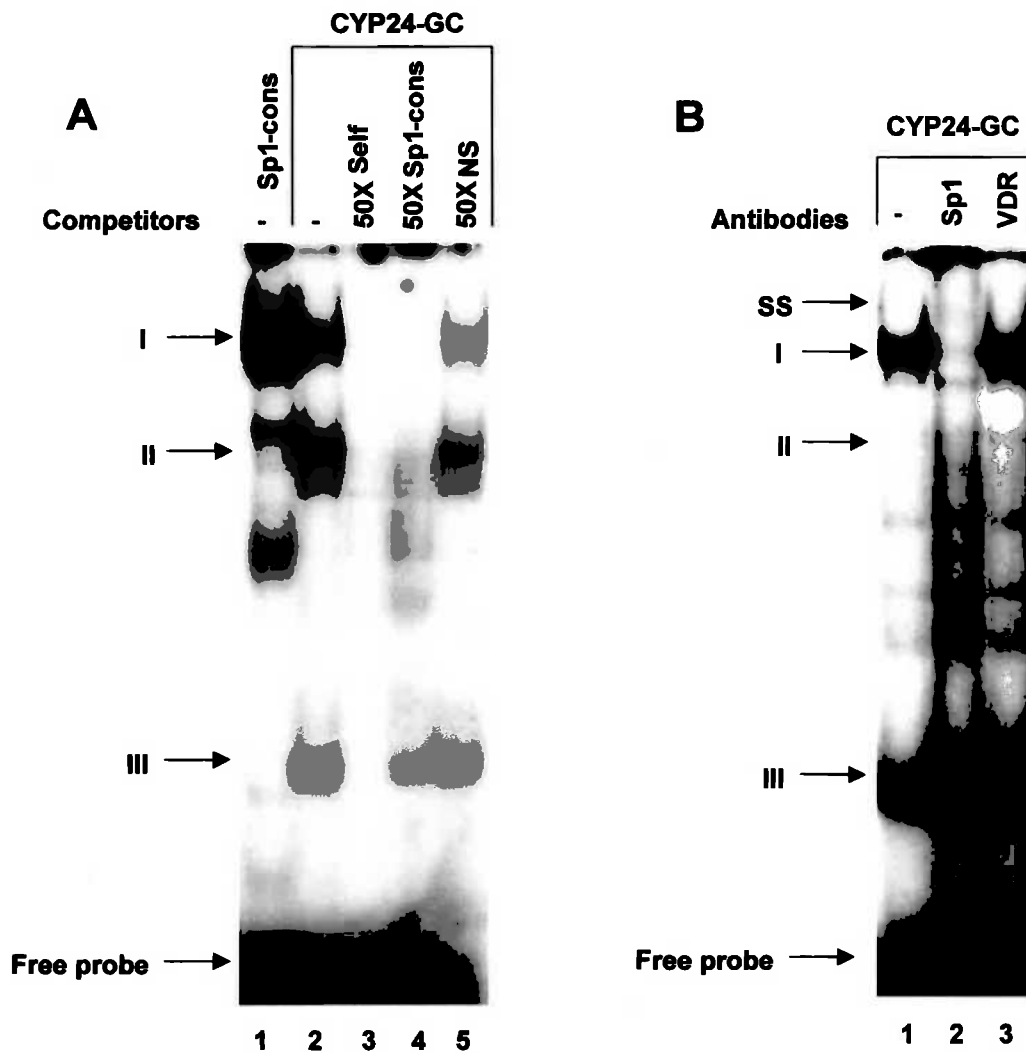
Figure 4.6 Binding to the GC box in the *CYP24* promoter using the nuclear proteins from HEK-293T cells. A double stranded nucleotide encompassing the GC box (*CYP24*-GC) was labeled by end-filling with [α-^{32}P]dCTP and incubated with nuclear extracts from HEK-293T cells. The major retarded complexes are arrowed and numbered as I to III. **A**, the formation of complexes with labeled *CYP24*-GC was competed with three unlabeled probes (i.e. self, consensus-GC or nonspecific probe *CYP24*-VDRE). **B**, for supershift assays, nuclear extracts were incubated with monoclonal antibody to Sp1 or VDR prior to addition of the labeled probe *CYP24*-GC.

Figure 4.7 Binding to the GC box in the *CYP24* promoter using the nuclear proteins from UMR106 cells. A double stranded nucleotide encompassing the GC box (*CYP24*-GC) was labeled by end-filling with [α-^{32}P]dCTP and incubated with nuclear extracts from UMR106 cells. The major retarded complexes are arrowed and numbered as I to IV. **A**, the formation of complexes with labeled *CYP24*-GC was competed with three unlabeled probes (i.e. self, consensus-GC or nonspecific probe *CYP24*-VDRE). **B**, for supershift assays, nuclear extracts were incubated with monoclonal antibody to Sp1 or VDR prior to addition of the labeled probe *CYP24*-GC.

To further confirm that Sp1 interacts with the GC site of the *CYP24* promoter, I performed a supershift assay using monoclonal anti-Sp1 antibody with nuclear extracts from HEK-293T cells (Figure 4.6B, Lane 2). The antibody against Sp1 led to the formation of a very weak supershifted band (Band SS) at the expense of complex 1 (Figure 4.6B, compare Lanes 1 and 2). However, a control antibody against VDR had no effect on the formation of any complex (Figure 4.6B, Lane 3). The result therefore establishes that Sp1 present in HEK-293T cells interacts with the GC box sequence and corresponds to band I.

Similar results were also observed using the nuclear extracts from UMR106 cells (Figure 4.7). Four major nuclear-protein complexes (I-IV) were consistently detected with a weaker variable band appearing between III and IV (Figure 4.7A, Lane 2). Competition experiments with 50-fold molar excess unlabeled *CYP24*-GC probe resulted in marked inhibition of formation of complexes I-IV (Figure 4.7A, Lane 3) and competition experiments with the consensus Sp1 binding oligonucleotide significantly inhibited formation of complexes I and II (Figure 4.7A, Lane 4). However, a nonspecific probe containing the *CYP24* VDRE had no effect on complexes I-IV (Figure 4.7A, Lane 5). In the profile of DNA binding with the consensus Sp1 binding sequence, complexes I, II and III apparently ran with same mobility as those detected with the *CYP24*-GC box oligonucleotide (Figure 4.7A, compare lanes 1 and 2). Addition of the monoclonal antibody against Sp1 resulted in the almost complete loss of complex I but only a very weak supershifted band (SS) was seen (Figure 4.7B, compare Lanes 1 and 2), with the control antibody against VDR having no effect on the formation of any of the four complexes (Figure 4.7B, Lane 3). The results using nuclear extracts from both cell lines therefore demonstrate that Sp1 can bind with the GC box sequence present in the *CYP24* promoter.

4.2.4 Characterization of the CCAAT binding proteins

Many proteins can bind to CCAAT sequences or inverted CCAAT sequences, in particular C/EBP isoforms (CCAAT enhancer binding protein) (Lekstrom-Himes and Xanthopoulos,

1998) and NF-Y (Nuclear factor Y) (Mantovani, 1999). To characterize the inverted CCAAT box in the promoter of the *CYP24* gene, EMSAs were carried out using a double stranded oligonucleotide encompassing the CCAAT box site (5' GTGCTCATTGGCCACTCCA 3') and nuclear extracts from HEK-293T cells. Eight major nuclear protein-DNA complexes (Complexes I to VIII) were detected (Figure 4.8). Only complexes I, II and IV appeared specific since a 50-fold molar excess of unlabeled *CYP24*-CCAAT oligonucleotide resulted in the strong inhibition of their formation (Figure 4.8A, Lane3) while a 50-fold molar excess of unlabeled *CYP24*-mCCAAT oligonucleotide (5 GTGCTCAGCTGCCACTCCA 3') had no effect on their formation (Figure 4.8A, Lane 9). Complex III appears non-specific since its formation could also be totally inhibited by addition of 50-fold molar excess of *CYP24*-mCCAAT oliognucleotide (Figure 4.8A, Lane 9).

Competition experiments showed that formation of complex II was substantially inhibited with a 10-fold molar excess of a consensus NF-Y binding oligonucleotide (5' GTCTGGGCGCTGATTGGCTGTGGACTG CG 3') compared with complexes I, III and IV. (Figure 4.8A, Lane 4). At 50-fold molar excess of the consensus NF-Y oligonucleotide, formation of I to IV was noticeably inhibited. The data tentatively suggest that complex II contains NF-Y (Figure 4.8A, Lane 5).

Cross competition experiments with 50-fold molar excess of a consensus C/EBP binding oligonucleotide (5' GTGCAGATTGCGCAATCTGCA 3') resulted in strong inhibition of specific bindng complexes IV and I (Figure 4.8A, Lane 7). However, it is to be noted that formation of complex II was not affected at all (Figure 4.8A, Lane 7). NF-Y is a ubiquitous heterotrimer consisting of three subunits (NF-YA, NF-YB and NF-YC) and all the three are necessary for DNA binding (Mantovani, 1999). To determine if complex II is NF-Y, EMSA experiments were undertaken with polyclonal antibodies specific for NF-YB subunit, C/EBPα and C/EBPβ. As shown in Figure 4.8B, anti-NF-YB antibody significantly supershifted complex II (compare Lanes 1 and 4, SS), while anti-C/EBPα and β antibodies had no effect
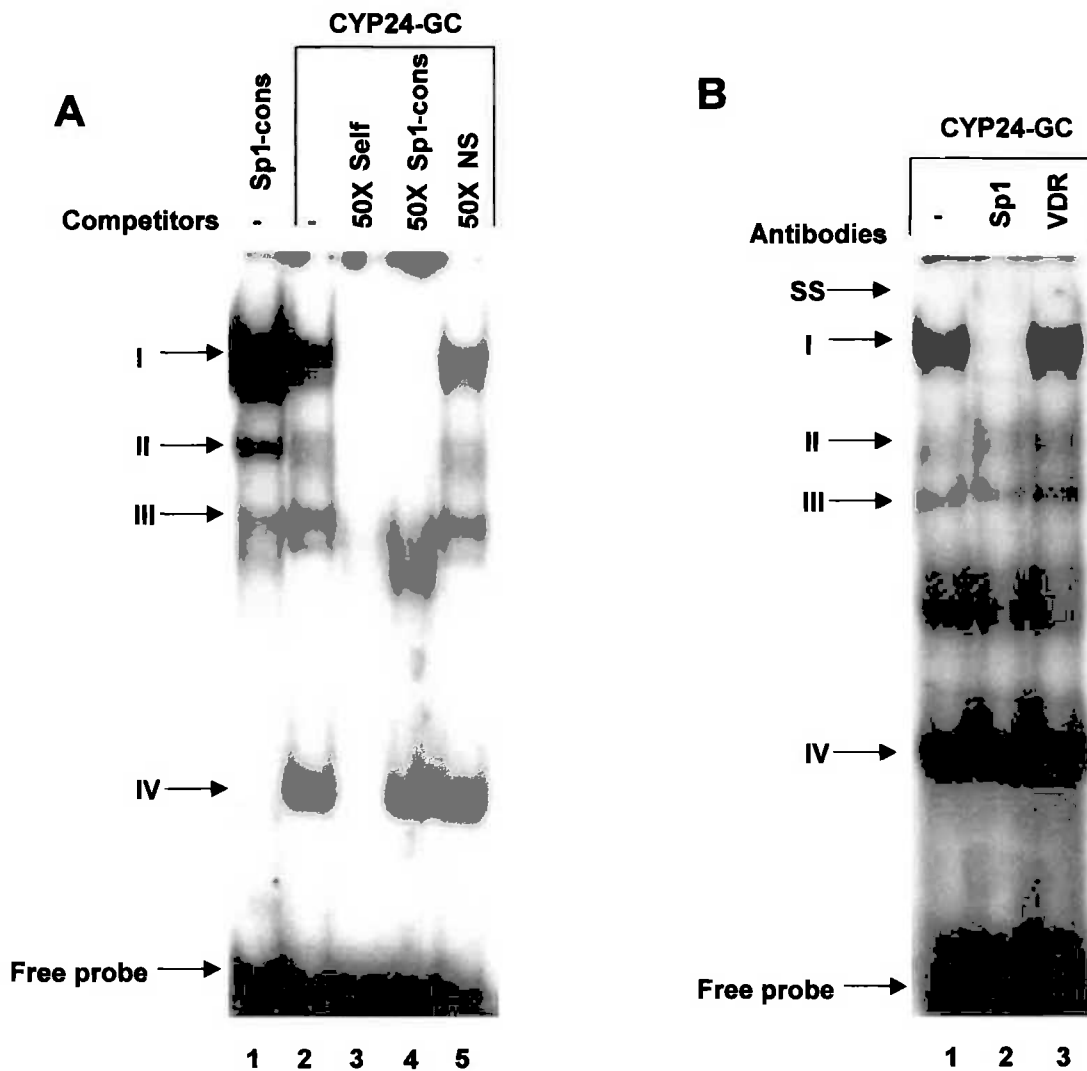
Figure 4.8 Binding to the CCAAT box in the *CYP24* promoter using the nuclear proteins from HEK-293T cells. A double stranded nucleotide encompassing the CCAAT box (*CYP24*-CCAAT) was labeled by end-filling with [α-^{32}P] dCTP and incubated with nuclear extracts from HEK-293T cells. The major retarded complexes are arrowed and numbered as I to VIII. **A**, the formation of complexes with labeled *CYP24*-CCAAT was competed with four unlabeled probes (i.e. self, consensus NF-Y binding sequence, consensus C/EBP binding sequence or *CYP24*-mCCAAT). **B**, for supershift assays, nuclear extracts were incubated with polyclonal antibody to C/EBP α , C/EBPβ and NF-YB prior to addition of the labeled probe *CYP24*-CCAAT.

on any of the complexes (Figure 4.8B, Lanes 2 and 3). The results demonstrate that NF-YB can bind to the CCAAT box of the *CYP24* gene promoter but as well, additional factors present in HEK-293T cells also recognize the element possibly including a C/EBP protein. Electromobility shift assays (EMSA) using the double stranded oligonucleotide encompassing the CCAAT box site were also performed with nuclear extracts from UMR106 cells (Figure 4.9). Three major nuclear protein-DNA complexes were detected. Binding complexes II and I were specific and were competed with a 50-fold molar excess of unlabeled *CYP24*-CCAAT (Figure 4.9A, Lane 3) and were also significantly inhibited with a 50-fold molar excess of unlabeled consensus NF-Y binding oligonucleotide (Figure 4.9A, Lane 5). Complex III appears non-specific since its formation could not be inhibited with a 50-fold molar excess of unlabeled *CYP24*-CCAAT. The addition of a 50-fold molar excess of unlabeled consensus C/EBP binding oligonucleotide (Figure 4.9A, Lane 7) or a 50-fold molar excess of unlabeled *CYP24*-mCCAAT oligonucleotides (Figure 4.9A, Lane 9) led to the disappearance of the binding complex II, but had no effect on complex I. It is concluded from this data, that complex I not complex II may contain NF-Y. This was confirmed using the polyclonal antibodies. Incubation with the anti-NF-YB antibody resulted in loss of complex I and appearance of a supershifted band shown as SS (Figure 4.9B, Lane 2). In contrast, the polyclonal antibodies to C/EBPα (Figure 4.9B, Lane 3) and β (Figure 4.9B, Lane 4) had no effect. Overall, the data in Figure 4.8 and Figure 4.9 demonstrate that the CCAAT box sequence binds NF-Y but clearly other protein complexes present in nuclear extracts can also bind.

4.2.5   Functionality of Sp1 and NF-Y in basal expression of *CYP24* promoter activity

To study the functionality of Sp1 and NF-Y in vivo, we initially cotransfected expression clones for Sp1 or NF-YA with p*CYP24*WT-Luc (-298) into kidney and bone cells using a DOTAP transfection protocol. However, basal activity of the transfected promoter was not altered most likely due to high endogenous levels of these proteins (Data not shown).
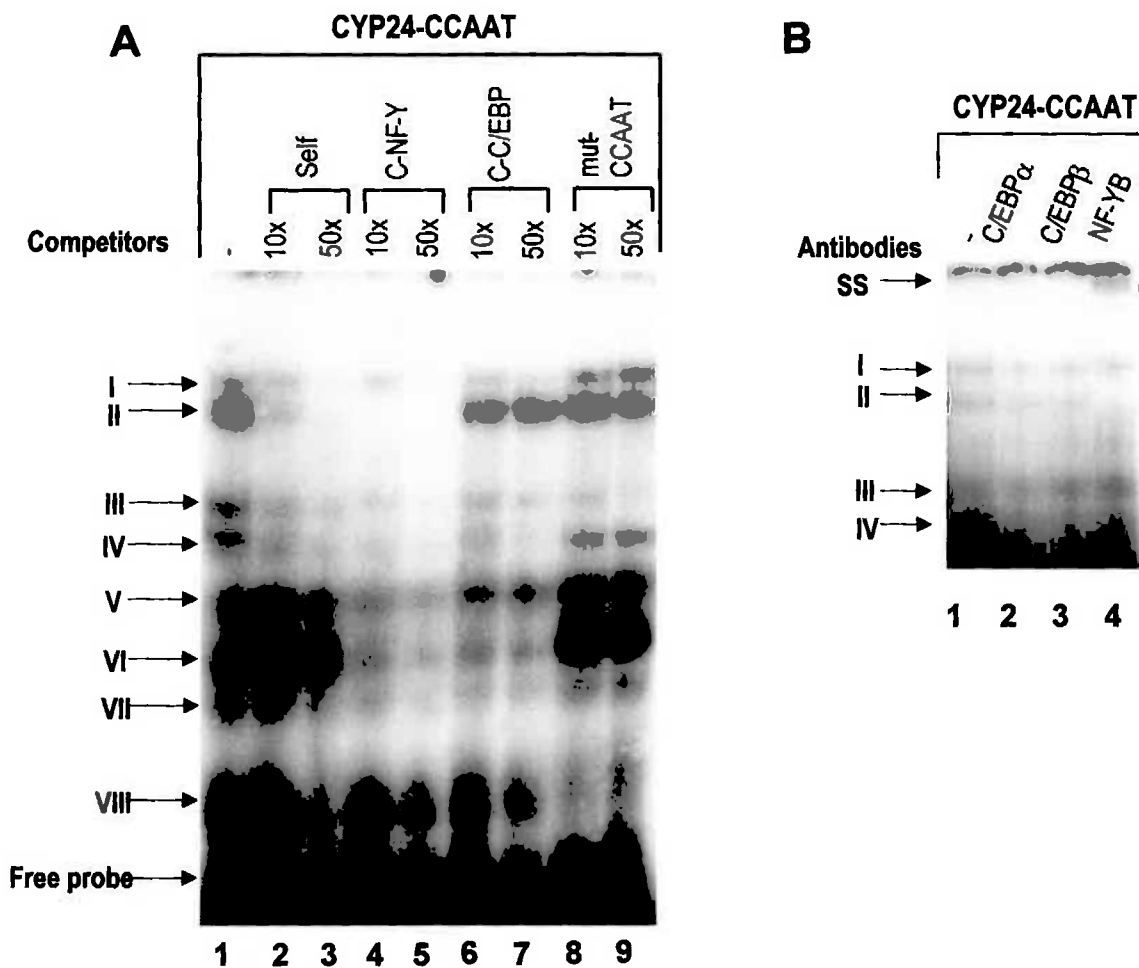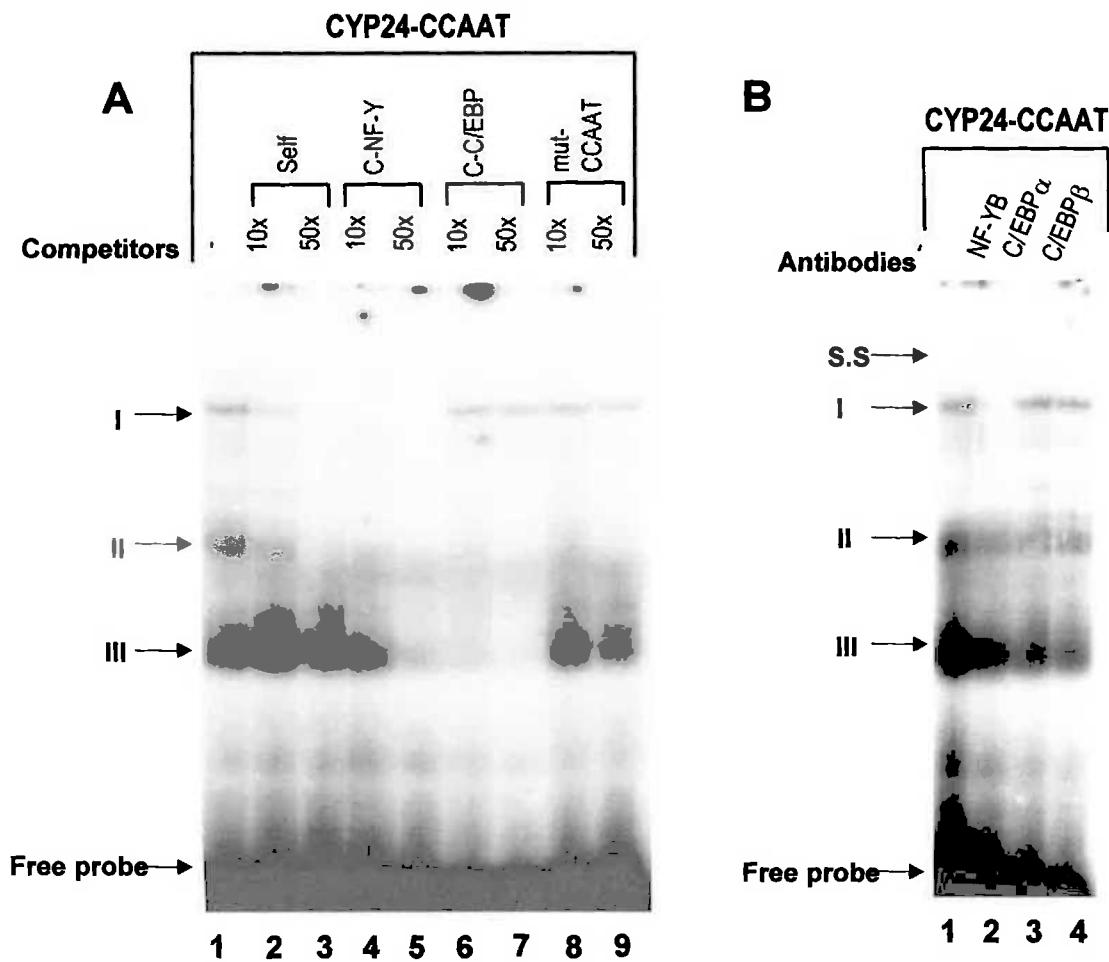
Figure 4.9 Binding to the CCAAT box in the *CYP24* promoter using the nuclear proteins from UMR106 cells. A double stranded nucleotide encopassing CCAAT (*CYP24*-CCAAT) was labeled by end-filling with [α-^{32}P] dCTP and incubated with nuclear extracts from UMR 106 cells. The major retarded complexes are arrowed and numbered as I, II and III. **A,** the formation of complexes with labeled *CYP24*-CCAAT was competed with four unlabeled probes (i.e. self, consensus NF-Y binding sequence, consensus C/EBP binding sequence or *CYP24*-mCCAAT). **B,** for supershift assays, nuclear extracts were incubated with polyclonal antibody to C/EBP α , C/EBPβ and NF-YB prior to addition of the labeled probe *CYP24*-CCAAT.

Drosophila SL3 cells were employed that lack endogenous Sp members (Ahlgren *et al.*, 1999) and NF-Y proteins (Liang *et al.*, 2001). Expression plasmids for Sp1, Sp3 and NF-Y subunits (A, B, C) driven by the *Drosophila* melanogaster actin 5C promoter [designated pPac-Sp1, pPac-Sp3 and pPac-NF-Y (A, B, C)] were introduced into *Drosophila* SL3 cells together with the -298bp *CYP24* promoter construct. The data were presented as fold induction, the ratio of relative luciferase activity from Sp1 factor transfected cells to that from appropriate vector transfected cells (Figure 4.10 and 4.11).

Figure 4.10 shows that the *CYP24* promoter was strongly activated in a dose-dependent manner by cotransfection of pPac-Sp1 (1ng to 10ng) from 42-fold to 387-fold comparing with the activity in the absence of pPac-Sp1. In contrast expression of Sp3, another member of the Sp family, from 1-10ng (Figure 4.10) had no effect on expression. Hence Sp1, not Sp3, is likely to function specifically on the *CYP24* promoter. To confirm Sp1 functions through the GC box, 10ng of Sp1 was cotransfected with pCYP24mGC-Luc as well as pCYP24WT-Luc. The results showed that Sp1 induced-*CYP24* promoter activity was decreased from 387-fold to 105-fold when the GC box was mutated (Figure 4.10). It seems that Sp1 activation is at least partially dependent on the GC box.

When the three NF-Y subunits were expressed together in different amounts (from 0.1 to 10ng), an increase in expression of luciferase of about 8-fold was consistently observed (Figure 4.11). However, exogenous NF-Y did not activate the pCYP24mCCAAT-Luc construct (Figure 4.11) demonstrating that NF-Y mediated activation is entirely through the CCAAT box. When both Sp1 and NF-Y subunits (at 10ng) were over expressed together no cooperation was observed but instead, the level of expression was actually decreased compared with Sp1 alone (data not shown). This result indicates that there is no synergy between these sites in the *CYP24* promoter and the reduced expression observed could reflect titration of exogenous regulatory proteins.
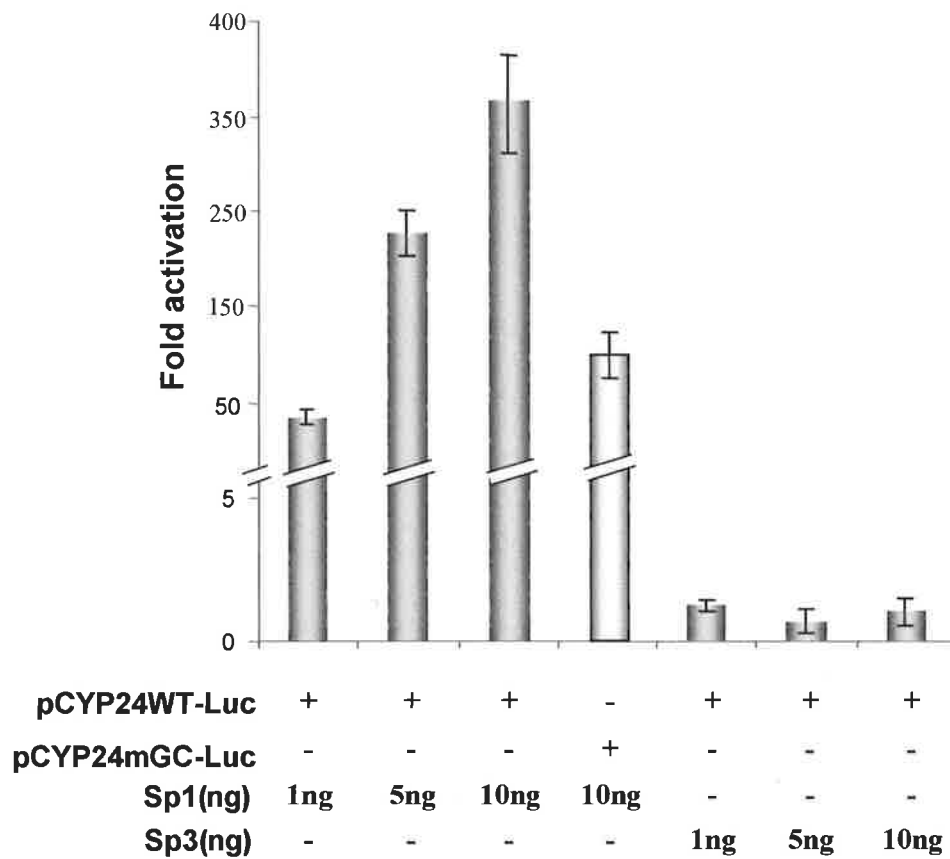
Figure 4.10 Cotransfection of p*CYP24*WT (-298)-Luc construct or p*CYP24*mGC (-298)-Luc with expression plasmid for Sp in *Drosophila* SL3 cells. *Drosophila* SL3 cells were transiently transfected by LipofectAmine 2000 with indicated amount of Sp1 or Sp3 together with p*CYP24*WT-Luc or p*CYP24*mGC-Luc. Total DNA was adjusted by an addition of mock DNA plasmid. Cells were harvest 48 h after transfection and luciferase activity determined. The results are shown as fold induction, the ratio of relative luciferase activity from Sp factor transfected cells to that from mock DNA-transfected cells. Data represent one of three independent experiments performed in triplicate.
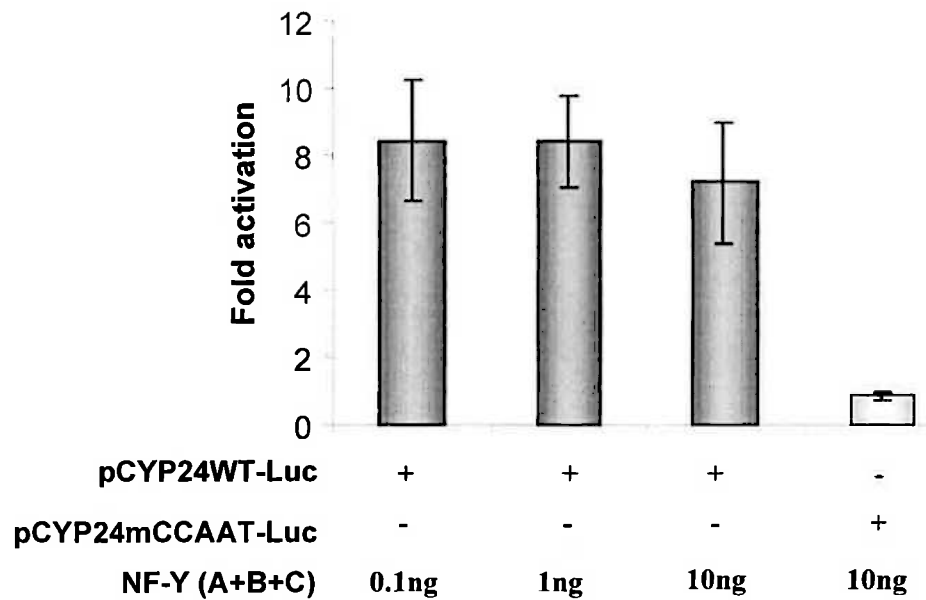
Figure 4.11 Cotransfection of p*CYP24*WT (-298)-Luc construct with expression plasmid for NF-Y into *Drosophila* SL3 cells. *Drosophila* SL3 cells were transiently transfected with equal amount of each NF-Y subunit (total amount as indicated in the figure) together with p*CYP24*WT-Luc or p*CYP24*mCCAAT-Luc. Total DNA was adjusted by an addition of mock DNA plasmid. Cells were harvest 48 h after transfection and luciferase activity were determined. The results were shown as fold induction, the ratio of relative luciferase activity from NF-Y factor transfected cells to that from mock DNA-transfected cells. Data represent one of three independent experiments performed in triplicate.

4.2.6   Effects of a dominant negative mutant of NF-YA (NF-YAm29) in HEK-293T cells

As mentioned in chapter 1, of the heterotrimeric complex, NF-YA seems to play the critical role in activation of the binding activity of NF-Y during cell differentiation and proliferation (Chang *et al.*, 1994; Marziali *et al.*, 1999; Bolognese *et al.*, 1999; Inoue *et al.*, 1999b). A dominant negative mutant form of NF-YA (NF-YAm29) with three amino acids mutated in the DNA binding domain is functionally inactive both *in vitro* and *in vivo* (Jackson *et al.*, 1995; Mantovani *et al.*, 1994). The NF-YAm29 construct (100ng, 200ng and 500ng) together with 200ng of p*CYP24*WT-Luc or p*CYP24*mCCAAT-Luc was introduced into HEK-293T cells and the relative luciferase activity determined. In the presence of increasing amounts of NF-YAm29, the basal p*CYP24*WT-Luc luciferase activity was consistently reduced, however the expression by p*CYP24*mCCAAT-Luc construct was not significantly altered (Figure 4.12A). The data further confirmed that NF-Y functions through the CCAAT box in the proximal *CYP24* promoter and drives basal expression.

However the effect on $1,25(OH)_2D_3$ induction of cotransfecting NF-YAm29 gave a surprising result. While basal expression was again reduced (by about 40% at 500ng), the level of wild type $1,25(OH)_2D_3$ induced activity was not reduced in the presence of the dominant negative (Figure 4.12B). In fact, $1,25(OH)_2D_3$ fold induction levels were slightly increased in the presence of the dominant negative form construct (Figure 4.12B). The interpretation of this data is not easy. Firstly, we consider that NF-Y contributes in a positive fashion to basal expression. However, we suggest that NF-Y is replaced by another CCAAT box binding protein in response to $1,25 (OH)_2D_3$. NF-Y may therefore act negatively in the $1,25(OH)_2D_3$ induction mechanism so that its removal by the dominant negative mutant will enhance induction.

4.2.7   Double mutation in GC box and EBS

It can be seen in Figure 4.1 that the known binding site (EBS) for the transcription Ets-1 lies two turns of the helix immediately upstream from the GC box. There was a strong possibility
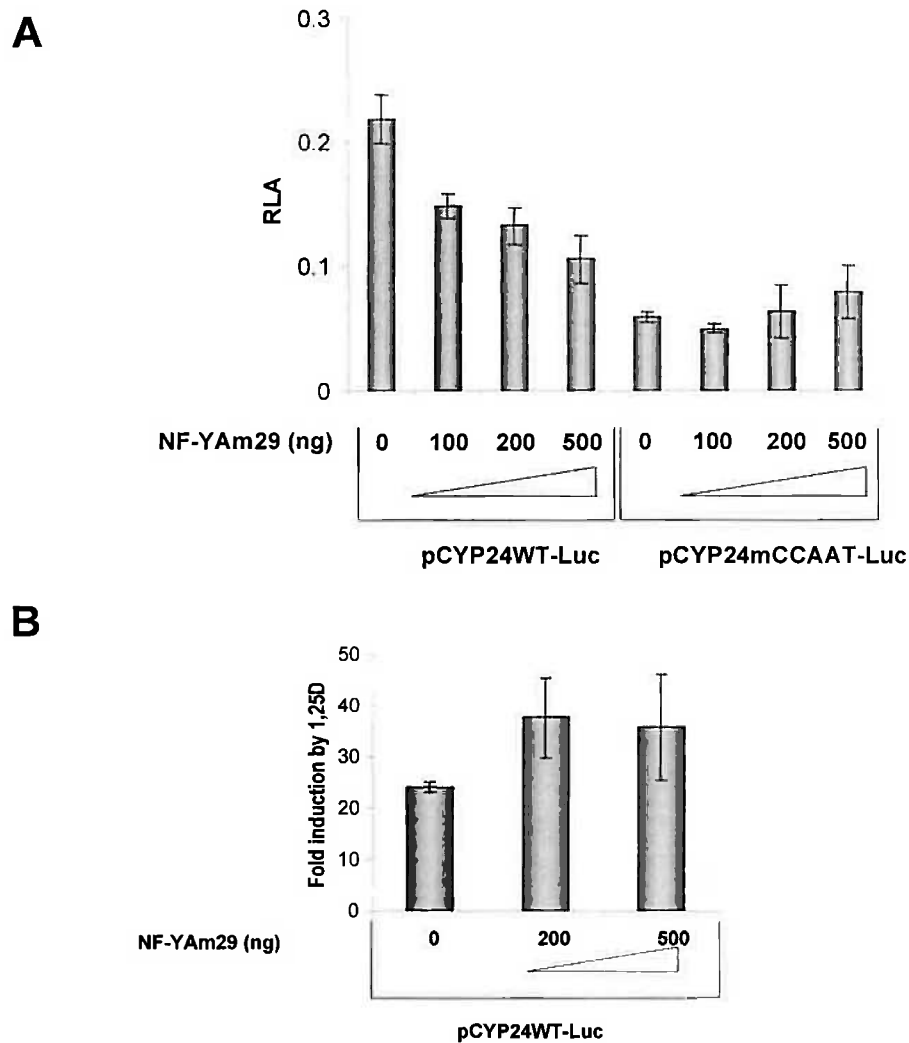
Figure 4.12 Effect of a dominant negative NF-YA mutant on the basal expression and 1,25(OH)$_2$D$_3$ mediated induction of _CYP24_ promoter activity. **A,** A dominant negative mutant from of NF-YA (NF-YAm29) expression plasmid was cotransfected at increasing amount as indicated into HEK-293T cells with either the p_CYP24_WT-Luc and p_CYP24_mCCAAT-Luc reporter plasmid. The relative luciferase activity (RLA) represents the mean ± S.D firefly luciferase/_Renilla_ luciferase ratios of triplicate samples. **B,** the cells transfected with NF-YAm29 expression construct and p_CYP24_WT-Luc also treated with 1,25(OH)$_2$D$_3$. Results were expressed as fold induction, the ratio of relative luciferase activity from 1,25(OH)$_2$D$_3$ treated cells to that from untreated cells. Data represent one of three independent experiments performed in triplicate.

that these two sites may cooperate in basal and 1,25(OH)$_2$D$_3$ induced expression. The two sites within the 298bp construct were therefore mutated simultaneously (p*CYP24*mEBS+mGC-Luc) and introduced into HEK-293T cells and UMR106 cells. As well the p*CYP24*WT-Luc, p*CYP24*mEBS-Luc and p*CYP24*mGC-Luc constructs were also examined.

In HEK-293T cells mutagenesis of EBS alone has no effect on basal expression, but reduces fold induction by 1,25(OH)$_2$D$_3$ from 23 to 13-fold (Figure 4.13). When the GC box was mutated, the basal expression was reduced from 0.15 to 0.07 and 1,25(OH)$_2$D$_3$ mediated induction of *CYP24* promoter activity was reduced from 23-fold to 10-fold as mentioned in section 4.2.1. Double mutagenesis of the two sites has no further effect on the basal expression (0.06) compared to p*CYP24*mGC-Luc construct (0.07), but 1,25(OH)$_2$D$_3$ induced activity of the *CYP24* promoter was further reduced to 8-fold.

Similar results were seen in UMR106 cells (Fig 4.14). Mutagenesis of the EBS results in reduced fold activation by 1,25(OH)$_2$D$_3$ (from 60-fold to 36-fold) but has no effect on basal expression. When the GC box was mutated, the basal expression was reduced from 0.25 to 0.14 and 1,25(OH)$_2$D$_3$ mediated induction of *CYP24* promoter activity was reduced from 60-fold to 33-fold as mentioned in section 4.2.1. Compared to the p*CYP24*mGC-Luc construct, double mutagenesis of EBS and GC box results in 1,25(OH)$_2$D$_3$ induced *CYP24* promoter activity being further decreased to 6-fold although there was no additional effect on basal activity of *CYP24* promoter (0.13) (Figure 4.14).

While evidence for synergy between two binding sites has been proposed on the basis that the value from the double mutation is greater than the sum of each individual mutations (Valor *et al.*, 2002) that is not the situation here. Over expression studies employing Ets-1 and Sp1 could provide evidence for synergy, but these studies were not undertaken here. Another approach was employed to determine whether these proteins could interact.

	Basal (RLA)	1,25(OH)$_2$D$_3$ (RLA)	Fold (± 1,25(OH)$_2$D$_3$)
pCYP24WT-Luc	0.15 ± 0.02	3.47 ± 0.07	23.13 ± 0.47
pCYP24mEBS-Luc	0.15 ± 0.02	1.85 ± 0.06	12.76 ± 0.43
pCYP24mGC-Luc	0.07 ± 0.02	0.69 ± 0.09	10.73 ± 0.03
pCYP24mEBS +mGC-Luc	0.06 ± 0.01	0.45 ± 0.05	7.62 ± 0.81
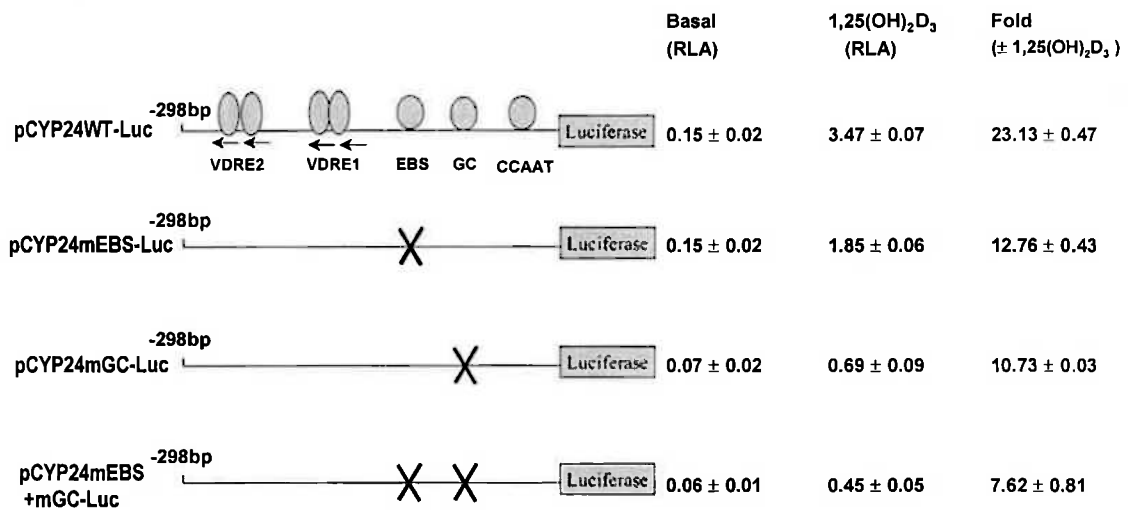
Figure 4.13 Effect of site-directed mutagenesis of the EBS and GC sites in the rat *CYP24* 298bp promoter on basal and the 1,25(OH)$_2$D$_3$ response in HEK-293T cells. HEK-293T cells were transiently transfected with p*CYP24*WT-Luc, p*CYP24*mEBS-Luc, p*CYP24*mGC-Luc or p*CYP24*mEBS+mGC-Luc and treated with 1,25(OH)$_2$D$_3$ for 24h. The relative luciferase activity (RLA) represents the mean ± S.D firefly luciferase/*Renilla* luciferase ratios of triplicate samples. The fold induction shown is the ratio of relative luciferase activity from1,25(OH)$_2$D$_3$ treated cells to that from untreated cells (ethanol). Data are the representative of three independent experiments performed in triplicate.

	Basal (RLA)	1,25(OH)$_2$D$_3$ (RLA)	Fold (±1,25(OH)$_2$D$_3$)
pCYP24WT-Luc	0.25 ± 0.05	14.92 ± 3.42	60.43 ± 13.70
pCYP24mEBS-Luc	0.24 ± 0.05	8.62 ± 0.78	36.23 ± 3.27
pCYP24mGC-Luc	0.14 ± 0.02	4.61 ± 0.68	33.66 ± 4.29
pCYP24mEBS +mGC-Luc	0.13 ± 0.01	0.79 ± 0.11	6.14 ± 0.81

-298bp VDRE2 VDRE1 EBS GC CCAAT Luciferase

Figure 4.14 Effect of site-directed mutagenesis of the EBS and GC sites in the rat *CYP24* 298bp promoter on basal and the 1,25(OH)$_2$D$_3$ responses in UMR106 cells. UMR106 cells were transiently transfected with p*CYP24*WT-Luc, p*CYP24*mEBS-Luc, p*CYP24*mGC-Luc or p*CYP24*mEBS+mGC-Luc and treated with 1,25(OH)$_2$D$_3$ for 24h. The relative luciferase activity (RLA) represents the mean ± S.D firefly luciferase/*Renilla* luciferase ratios of triplicate samples. The fold induction shown is the ratio of relative luciferase activity from 1,25(OH)$_2$D$_3$ treated cells to that from untreated cells (ethanol). Data are the representative of three independent experiments performed in triplicate.

4.2.8 Protein–protein interactions between Sp1, Ets1, VDR and RXR using the mammalian two-hybrid assay

In work from this laboratory, Dwivedi *et al.* (2002) have demonstrated using a mammalian two-hybrid assay that Ets-1 can physically interact with either VDR or RXR. For this reaction, the ligand 1,25(OH)$_2$D$_3$ was not important. It was proposed on this basis, that VDR/RXR interacts with Ets-1 bound two turns of the helix downstream from VDRE1 (Figure 4.1 and Dwivedi *et al.*, 2002). Appreciating that the GC and EBS sites are also in close proximity (Figure 4.1), a study was undertaken to determine whether Sp1 could interact with Ets-1 in the mammalian two-hybrid assay. In these experiments, an interaction between Sp1 with VDR or RXR was also investigated. The mammalian two-hybrid system (Dwivedi et al, 1998) utilizes a plasmid with a firefly luciferase gene downstream of five yeast GAL4 binding sites fused to the E1b promoter (pG5E1b–Luc). In these experiments, the yeast GAL4 DNA binding domain was fused to the full length human Sp1 (GAL4-Sp1) and cotransfected into JEG-3 cells together with the full leng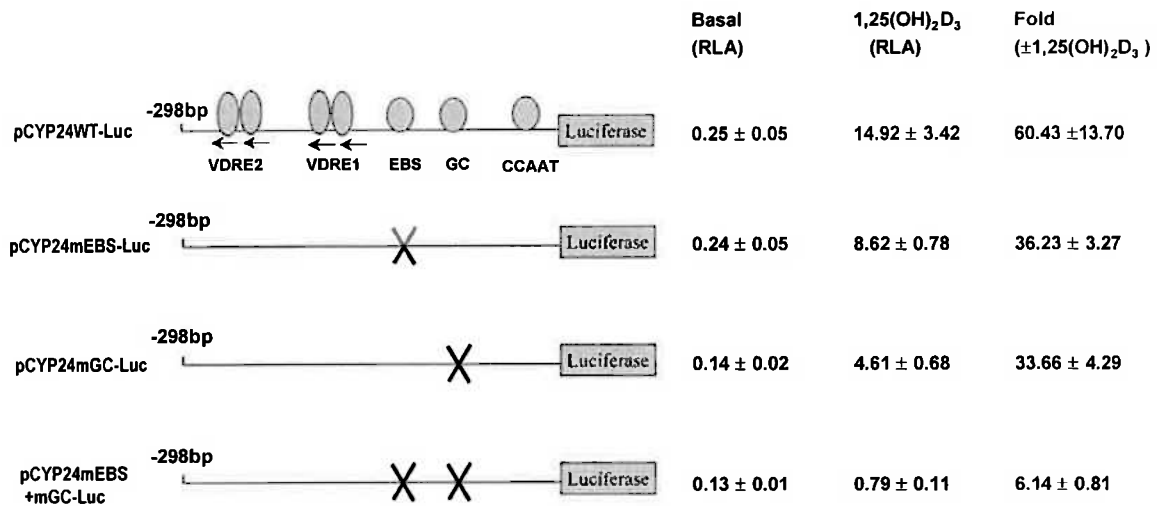th human VDR, RXR or Ets 1 protein linked to the transactivation domain of herpes simplex virus VP16 (VP16-VDR, VP16-RXR or VP16-Ets1). Transactivation of pG5E1b-Luc is only achieved when the GAL4 fusion protein interacts with the VP16 fusion protein physically.

The control luciferase activity was regarded as 100% when GAL4-Sp1 was cotransfected together with VP16 empty vector (Figure 4.15). A substantial increase in luciferase activity to 580% was observed when VP16-Ets1 was cotransfected with GAL4-Sp1. The results demonstrated that Sp1 interacts with Ets1 efficiently. It was observed that VP16-VDR did not significantly interact with GAL4-Sp1 since the pG5E1b luciferase reporter remains 100% (Figure 4.15). When VP16-RXR was cotransfected with the fusion protein GAL4-Sp1, the reporter luciferase activity was increased to 350% demonstrating that GAL4-Sp1 interacts with VP16-RXR (Figure 4.15). In addition, the interaction between GAL4-Sp1 and VP16-RXR was not as strong as the interaction between GAL4-Sp1 and VP16-Ets1. These experiments indicate that on the *CYP24* promoter an interaction between Sp1 and Ets-1 seems
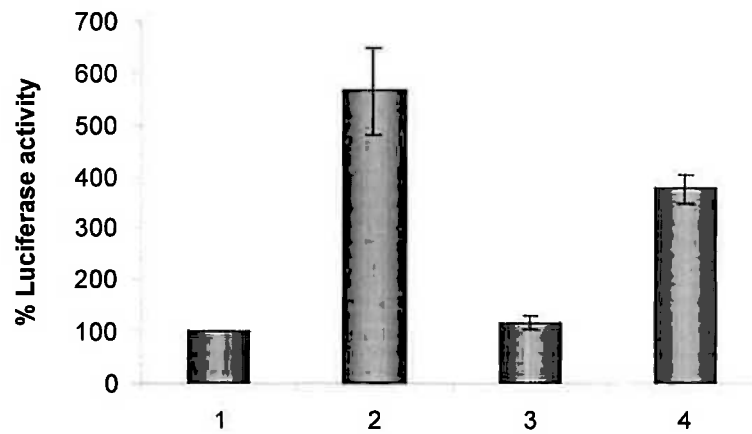
	1	2	3	4
GAL4-Sp1	+	+	+	+
VP16 (0)	+	-	-	-
VP16-Ets1	-	+	-	-
VP16-VDR	-	-	+	-
VP16-RXR	-	-	-	+

Figure 4.15 An interaction between Sp1 and Ets1 and an interaction between Sp1 and RXR in a mammalian two-hybrid assay. JEG-3 cells were cotransfected with 200ng of each GAL4 chimera and 200ng of either VP16 or VP16 chimera as indicated, together with 200ng of reporter plasmid, pG5E1b-Luc. Results shown are the mean ± S.D. from at least three independent experiments.

likely suggestive of transcriptional cooperation. The significance of the interaction between Sp1 and RXR is not clear.

4.2.9 Investigations into the signaling pathways elicited by 1,25(OH)₂D₃ in HEK-293T cells

Recent studies in our laboratory have emphasized the crucial role played by upstream signaling pathways in the 1,25(OH)$_2$D$_3$-activated induction of the *CYP24* gene in transiently transfected COS-1 cells (Dwivedi *et al.*, 2002). In this work, the MAP kinase pathways ERK1/2/5 were shown to be important reflecting a non-genomic response to 1,25(OH)$_2$D$_3$ most likely through the interaction of nuclear VDR with signal transduction components at the membrane (see Chapter 1).

The identities of different signaling pathways involved in the 1,25(OH)$_2$D$_3$ mediated induction of the transfected *CYP24* promoter activity in HEK-293T cells were explored using the pharmacological inhibitors H89 (10μM), calphostin C (500nM) and PD98059 (50μM), which are specific inhibitors of PKA, PKC and ERK1/2/5 respectively. The results in Figure 4.16 showed that H89 and calphostin C do not affect basal expression of the *CYP24* promoter activity, but each reduce the 1,25(OH)$_2$D$_3$ mediated induction by 60-70%. The results indicate that PKA and PKC are not involved in basal expression of *CYP24* promoter activity, but contribute to 1,25(OH)$_2$D$_3$ responsiveness. PD98059 (50μM) (Figure 4.16) consistently reduced the *CYP24* basal promoter activity by 50% from 1.02 to 0.57, and also markedly reduced the 1,25(OH)$_2$D$_3$ mediated induction from 25-fold to 2-fold. The results imply that ERK1/2/5 MAP kinases are involved in basal expression as well as 1,25(OH)$_2$D$_3$ mediated induction of *CYP24* promoter activity.

To further confirm the involvement of the PKA signaling pathway, 100μM concentration of the cAMP analogue 8-chlorophenylthio-cAMP (8-CPT-cAMP) was employed in transiently transfected HEK-293T cells for 24 h. 8-CPT-cAMP alone activated the *CYP24* promoter by
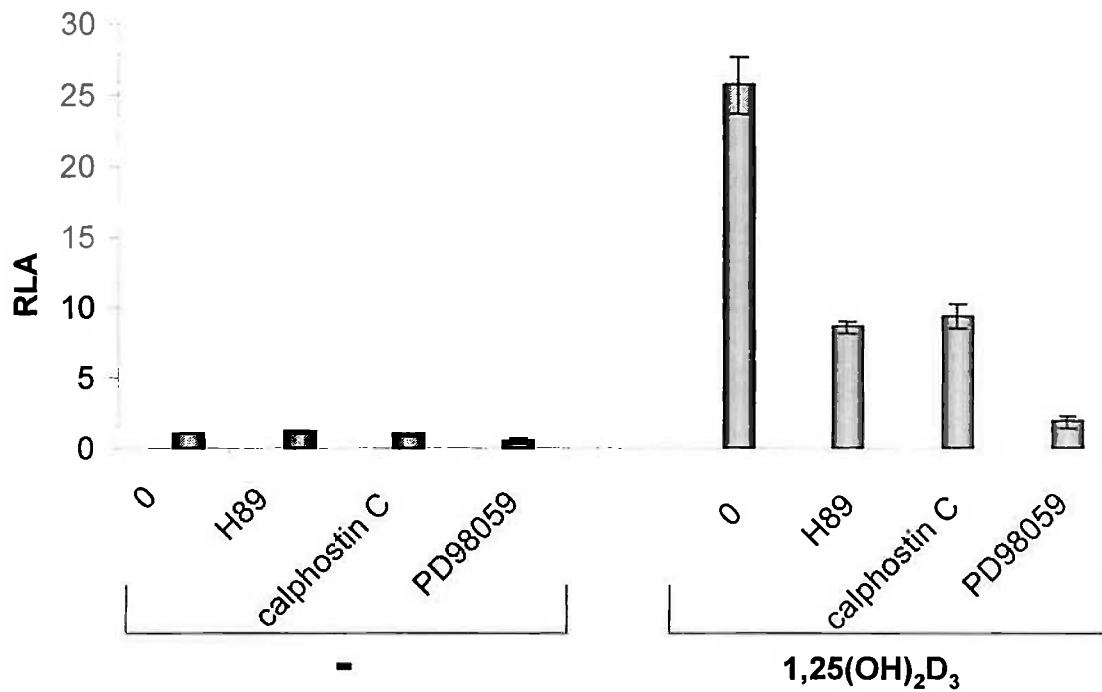
Figure 4.16 Effect of PKA, PKC and ERK1/2 MAP kinase inhibitors on the pCYP24WT(298)-Luc. HEK-293T cells were transiently transfected with plasmid pCYP24WT(298)-Luc and pre-incubated with H89, calphostin C and PD98059 for 1.5h before 24h incubation with $1,25(OH)_2D_3$ or ethanol. The relative luciferase activity (RLA) represents the mean ± S.D firefly luciferase/*Renilla* luciferase ratios of triplicate samples and similar results were observed in other two independent experiments.

about 3-fold and also could enhance vitamin D mediated induction from 25-fold to 35-fold (Figure 4.17A). It has been reported that both NF-Y and Sp1 are activated by PKA signal pathway (Osawa *et al.*, 1996; Zhong *et al.*, 2000). Therefore GC and CCAAT boxes in the promoter of *CYP24* gene are two possible responsive elements that could respond to cAMP/PKA. Forskolin (FSK, another PKA activator) was next employed in transiently transfected HEK-293T cells together with constructs mutated in the GC and CCAAT box. The results demonstrated that FSK weakly activated *CYP24*-298-Luc about 2.33 ± 0.06-fold induction, and this was reduced to 1.18 ± 0.24, 1.21 ± 0.28 and 1.01 ± 0.02 fold activation with p*CYP24*-mGC-Luc, p*CYP24*-mCCAAT-Luc and p*CYP24*-mGC+CCAAT-Luc constructs respectively. In an alternative approach, *Drosophila* SL3 cells were transiently cotransfected with p*CYP24*-WT-Luc (-298bp), p*CYP24*-mGC-Luc or p*CYP24*-mCCAAT, and an expression plasmid encoding the PKA catalytic subunit ($C\alpha$) under the control of the cytomegalovirus promoter (pCMV-PKA) (Ahlgren *et al.*, 1999). The results demonstrated that cotransfection of *Drosophila* SL3 cells with pCMV-PKA resulted in a 2.05-fold activation of p*CYP24*-WT-Luc reporter gene, and this was reduced to a 1.33-fold induction by p*CYP24*-mGC-Luc and a 1.48-fold induction by p*CYP24*-mCCAAT-Luc (Figure 4.17B). The data overall tentatively indicates that both the GC and CCAAT boxes could be targets of PKA activity.

It has recently been shown that the atypical PKC isoform, PKCζ, can phosphorylate Sp1 (Pal *et al.*, 1998). It was therefore of interest to determine if a dominant negative clone of PKCζ, PKCζ K281M could affect expression of the transfected *CYP24* promoter. It should be noted that calphostin C employed in Figure 4.16 would not inhibit this PKC isoform. PKCζ K281M was cotransfected at 200ng and 500ng with the p*CYP24*-WT-Luc construct. Over expression of PKCζ K281M had no effect on basal expression (data not shown). However $1,25(OH)_2D_3$ induced *CYP24* expression was consistently reduced by the dominant negative mutant (Figure 4.18). When the GC box was mutated, the mutant had no effect on $1,25(OH)_2D_3$ mediated
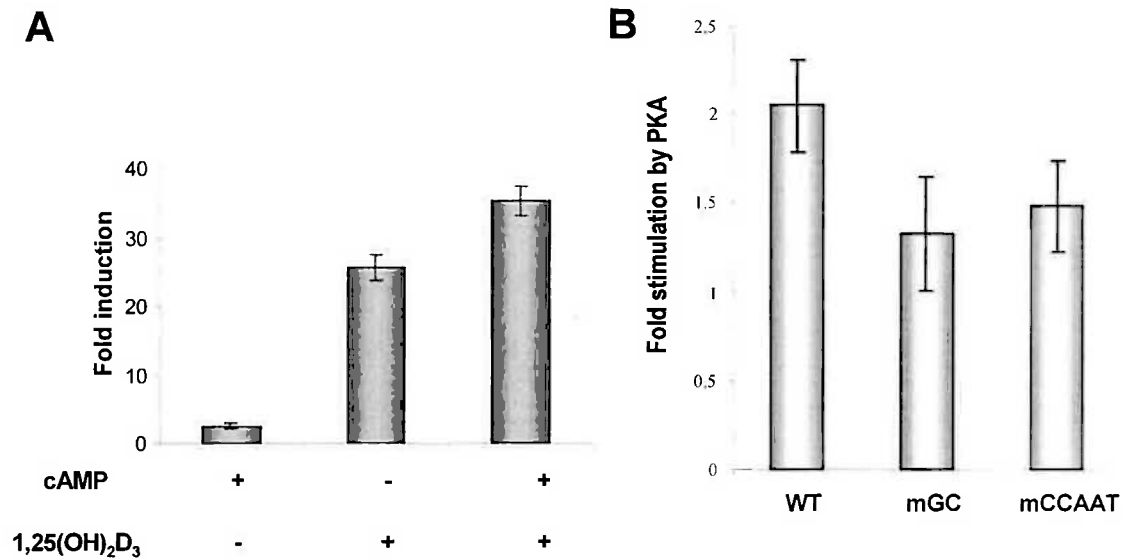
Figure 4.17 PKA signaling pathway which might elicited by 1,25(OH)₂D₃ acts through the GC and CCAAT sites. A, HEK-293T cells transiently transfected with p*CYP24*WT-Luc were treated with cAMP or/and 1,25(OH)₂D₃ for 24 h. The results are shown as fold induction, the ratio of relative luciferase activity from cAMP or/and 1,25(OH)₂D₃ treated cells to that from untreated cells. **B,** *Drosophila* SL3 cells were transiently transfected with p*CYP24*WT-Luc, p*CYP24*mGC-Luc or p*CYP24*mCCAAT-Luc, and an expression plasmid containing the PKA catalytic subunit (Cα) under the control of the cytomegalovirus promoter (pCMV-PKA). The results were shown as fold induction, the ratio of relative luciferase activity from pCMV-PKA transfected cells to that from mock DNA-transfected cells. Data are the representative of three independent experiments performed in triplicate.

fold induction (Figure 4.18). The data suggest that the isoform of $PKC\zeta$ acts solely through the GC box of the *CYP24* promoter to stimulate $1,25(OH)_2D_3$ mediated induction.

4.3 Discussion

Earlier EMSA studies in this laboratory established that an oligonucleotide encompassing the proximal VDRE-1 (from the rat *CYP24* promoter) bound VDR/RXR heterodimer to a much lesser extent than an oligonucleotide encompassing the upstream VDRE-2 (Kerry *et al.,* 1996). However, promoter analysis following mutagenesis of each site demonstrated that VDRE-1 contributed far more strongly to the $1,25(OH)_2D_3$ inductive response. To explain this it was suggested that transcription factors bound nearby to VDRE-1 might interact with either VDR/RXR or VDR/RXR bound coactivators to enhance the activity of this VDRE. In support of this hypothesis, an EBS was located immediately downstream from VDRE-1 and was found to bind Est-1 and to participate in $1,25(OH)_2D_3$ mediated induction although it was not involved in basal expression (See Figure 4.13; Dwivedi *et al.,* 2000). No transcription factor sites have been detected around VDRE-2. Recent studies have shown that $1,25(OH)_2D_3$ activates ERK-5 in COS-1 cells and this leads to phosphorylation of Ets-1 apparently increasing the activity of the protein and hence the activity of the promoter (Dwivedi *et al.,* 2002). Since the EBS does not contribute to basal expression, and the *CYP24* gene is widely expressed in different cell types, it was clear that other transcription factors present on the promoter must drive basal expression. A computer programme MatInspector (V2.2) revealed a putative GC box 18 bp downstream from the EBS at -128/-119 and further downstream a CCAAT box at -62/-51 located on the non-coding strand (Figure 4.1). The aim of this chapter was to investigate the functionality of the GC and CCAAT box sites in transiently transfected kidney and osteoblast cell lines expressing the first –298bp of rat promoter fused to the firefly luciferase reporter vector.
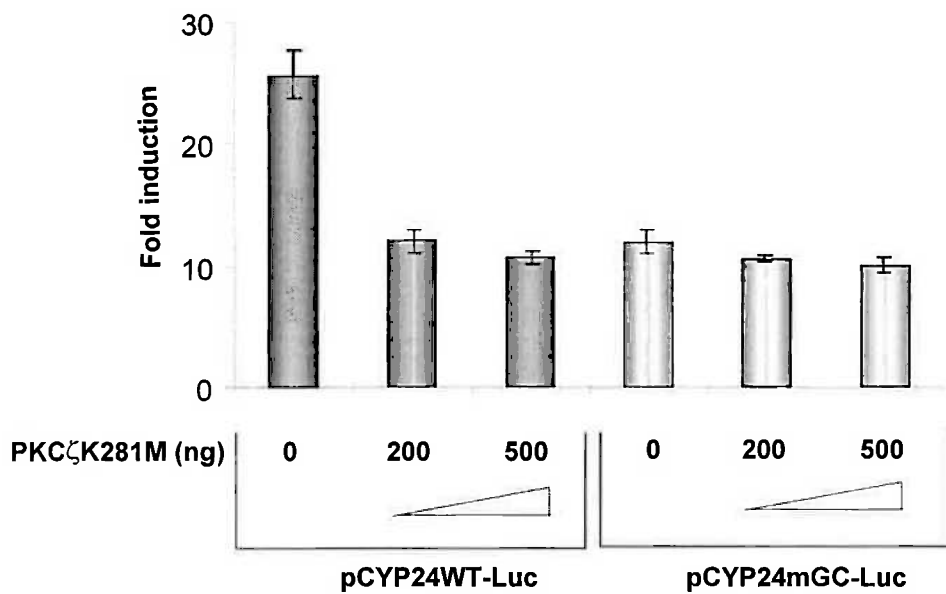
Figure 4.18 Effect of a dominant negative PKCζ, PKCζK281M, on the 1,25(OH)₂D₃ mediated induction of *CYP24* promoter activity. HEK-293T cells were transiently transfected with indicated amount of PKCζ K281M together with p*CYP24*WT(-298)-Luc or p*CYP24*mGC-Luc construct and treated with 1,25(OH)₂D₃ for 24h. The results represent as fold induction, the ratio of relative luciferase activity from 1,25(OH)₂D₃ treated cells to that from untreated cells. Data are the representative of three independent experiments performed in triplicate.

Site-directed mutagenesis of each site and both sites demonstrated that the GC and the CCAAT box sites contributed substantially to basal expression in the two cell lines. This finding was in keeping with the fact that the *CYP24* gene is expressed in most if not all cell types and that GC and CCAAT box sites are characteristic of house keeping gene promoters. The common transcription factor binding to GC boxes is Sp1. The location at -62/-51, the sequence and reverse orientation of the CCAAT box is strongly indicative of an NF-Y binding site (Mantovani, 1999). The consensus sequence for NF-Y binding sites is C G/A G/A CCAAT C/G A/G C A/C (Bucher *et al.*, 1990) and the CCAAT site in the *CYP24* promoter only differs in two positions (bold letter), TGGCCAATGAGC. EMSA using antibodies for Sp1 and NF-YB provided evidence for the binding of Sp1 to the GC box and the binding of NF-Y to the CCAAT box. Both proteins are ubiquitously expressed. NF-Y contains three subunits NF-YA, NF-YB and NF-YC. NF-YB and NF-YC form a dimer with a histone-fold like structure (Mantovani, 1999) that NF-YA interacts with and all three are required for interaction with DNA. Evidence for a functional role of Sp1 and NF-Y was demonstrated using the *Drosophila* SL3 cells which lacks endogenous Sp family members and NF-Y. In these experiments, exogenous Sp1 or NF-Y increased basal expression of the transfected *CYP24* promoter. NF-Y activation was entirely dependent on a functional CCAAT box and that of Sp1 was only partially dependent on the GC box at -114/-101 suggesting that there may be another site in the promoter. By analyzing the sequence of the –298 region, I also found another putative Sp1 binding site, GT box (-100/-88) but mutagenesis of this site did not alter the basal expression in HEK-293T cells. The finding that the GC and CCAAT box sites contributed substantially to basal expression (about 75% in both cell lines) was in marked contrast to the EBS that does not contribute to basal expression (Figure 4.13 and 4.14, and data in Dwivedi *et al.*, 2000). We have previously presented evidence that a repressor binds to unliganded VDR/RXR to inhibit basal expression of the transfected *CYP24* promoter (Dwivedi *et al.*, 1998). In support of this a dominant negative VDR that cannot bind to the repressor allowed Ets-1 to drive basal expression (Dwivedi *et al.*, 2000). We suggest that the

repressor prevent the nearby EBS from contributing to basal expression, by sterically interacting with Ets1 (Figure 4.19A). We further propose that GC and CCAAT box binding proteins can however still interact with the promoter and contribute to basal expression. Studies have shown that Sp1 interacts with the general transcription machinery, such as TATA-box binding protein (TBP) (Emili *et al.*, 1994) and some TBP-associated factors of TFIID (Tanese *et al.*, 1996) while NF-Y also interacts with TFIID through interactions with TBP (Bellorini *et al.*, 1997). Hence it is envisaged that in the presence of the repressor bound on VDR/RXR, basal expression is driven predominantly by Sp1 and NF-Y through interactions with TFIID at the TATA box (Figure 4.19A). In TATA less genes the CCAAT box is suggested to play a major role in the assembly of the basal transcription machinery and is generally located at a mean position of -66 (Mantovani, 1999). It is tempting to speculate that the TATA box in the *CYP24* promoter is weak and therefore requires input from the CCAAT box identified here to promote basal transcription.

There are numerous reports that a combination of a GC box and CCAAT box are important for promoter expression and that Sp1 can interact with NF-Y (Ge *et al.*, 20001; Roder et al, 1999; Yamada *et al.*, 2000). However in the present work, no evidence was seen for a cooperative interaction between Sp1 and NF-Y in their roles as stimulators of basal expression. Firstly, mutagenesis data in HEK-293T cells (Figure 4.3) does not imply synergy. Single mutations in the GC and CCAAT box lower the basal expression by 53% and 67% respectively, while simultaneous mutations in these sites lower expression by 80%. Similar data were seen in UMR106 cells (Figure 4.4). Experiments in *Drosophila* SL3 cells showed that while both sites stimulated the *CYP24* promoter together there was no synergy. The lack of cooperation may well reflect the distance (55bp) between the GC box and CCAAT box.

In the presence of $1,25(OH)_2D_3$, both the GC and CCAAT box sites are important contributors to induction. Their contribution is particular important in HEK-293T cells where mutagenesis of both sites lowers $1,25(OH)_2D_3$ from 23-fold to about 3-fold. In UMR106 cells the contributions of the GC and CCAAT box sites are not as pronounced with induction being
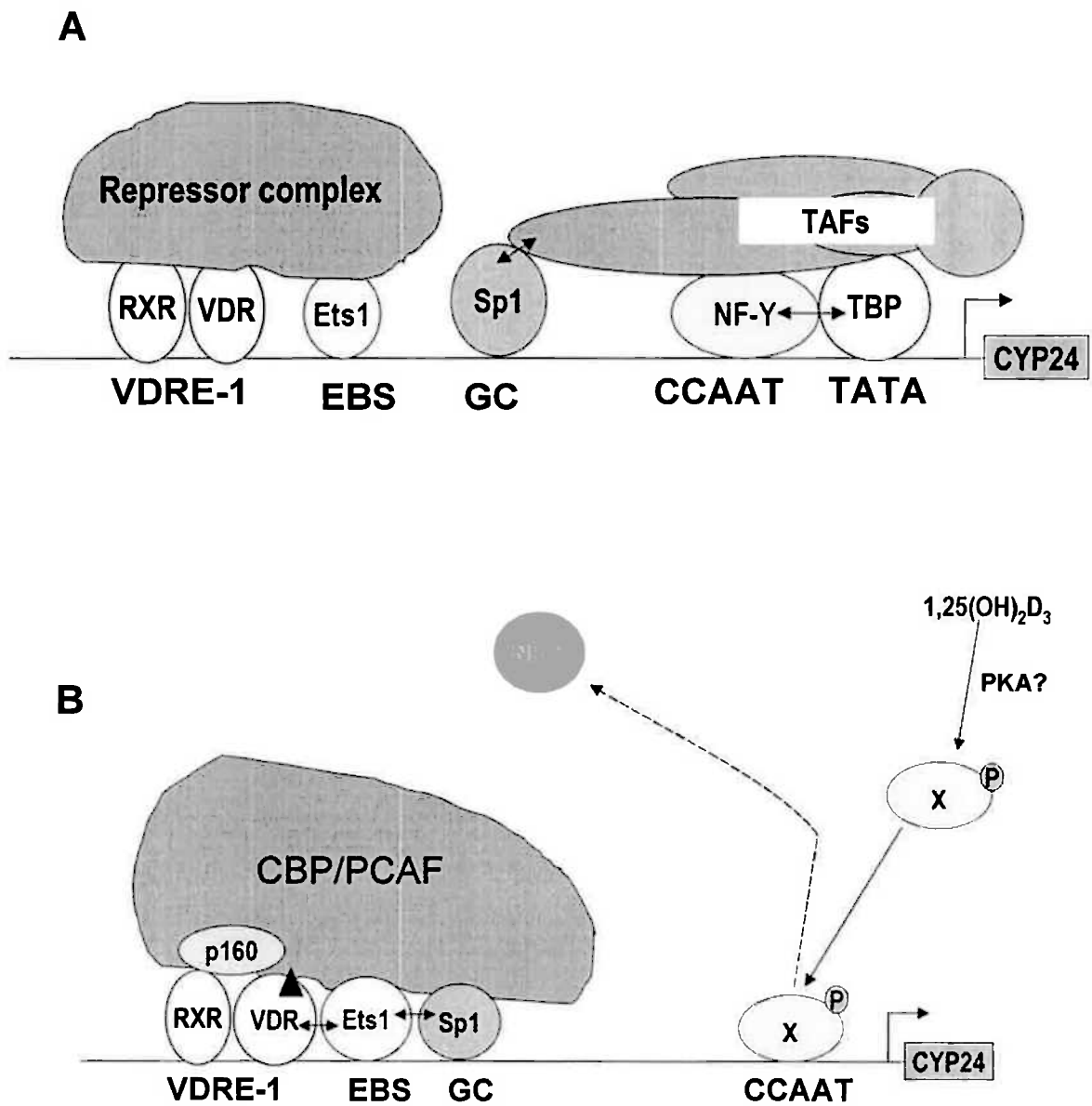
Figure 4.19 (A) A model for basal expression of *CYP24* gene and (B) a model for transcription regulation of *CYP24* gene by 1,25(OH)$_2$D$_3$ in HEK-293T cells.

lowered from 60-fold to about 24-fold when both sites are mutated. In the presence of 1,25(OH)$_2$D$_3$, we propose that the repressor is released from liganded VDR/RXR and the subsequently bound coactivator complex then interacts with Ets-1 and Sp1 and possibly the CCAAT binding protein (Figure 4.19B). Components of the coactivator complex could include a p160 family member together with CBP/PCAF.

There is evidence for these proposed interactions. As mentioned earlier EBS and VDRE-1 are separated by about two turns of the helix in the *CYP24* promoter and Ets-1 interacts with VDR in a two-hybrid assay indicating that a reaction is likely to take place on the promoter (Dwivedi *et al.*, 2002). Interactions between Sp1 and Ets1 have been reported (Dittmer *et al.*, 1997). While in the present study, the two-hybrid assay also provided evidence for an interaction of Sp1 with Ets-1. In addition, Ets1 and Sp1 are known to interact with coactivators, such as p300/CBP (Suzuki *et al.*, 2000; Shetty *et al.*, 1999; Yang *et al.*, 1998). Hence it is suggested that there are multiple interactions on the promoter where liganded VDR interacts with Ets-1 that in turn interacts with Sp1 generating an enhanceosome-like arrangement. In this model, it remains uncertain as to whether an interaction between Sp1 and RXR (as determined in the two hybrid assay) could take place within the context of the native promoter.

Whether the CCAAT binding protein can also interact with Sp1 present in the proposed enhanceosome-like assembly is not clear but its distance of 55bp downstream may preclude this. An interesting finding was made which suggests that at this CCAAT binding site, NF-Y binds initially to drive basal expression but is replaced by another protein in the presence of 1,25(OH)$_2$D$_3$. It was found that the dominant negative clone for NF-Y significantly lowered basal expression, thus supporting a role for NF-Y, but did not affect 1,25(OH)$_2$D$_3$ induction. The data raised the possibility that NF-Y may be replaced by an unknown CCAAT box protein (X) in the presence of hormone. That is, this protein X could be activated by hormone (eg by phosphorylation) and then compete with NF-Y for binding to the CCAAT box site (Fig 4.19B).

In this regard, several proteins in nuclear extracts were shown to bind to the CCAAT sequence in EMSAs (Figure 4.8). It is known that many proteins other than NF-Y can bind to a CCAAT sequence or inverted CCAAT sequence, such as CTF/NF1 (CCAAT transcription factor/Nuclear Factor 1) (Santoro *et al.*, 1988); C/EBP isoforms (CCAAT enhancer binding protein) (Lekstrom-Himes and Xanthopoulos, 1998), CDP (CCAAT displacement protein) (Barberis *et al.*, 1987) and YB-1/DbpB (DNA binding protein) (Diamond *et al.*, 2001). It seems that a C/EBP member is not involved since both C/EBPα and β antibodies failed to affect the formation of any binding complex. Interestingly recent preliminary experiments in our laboratory indicate an involvement of DbpB. It has been found that over expression of DbpB in HEK-293T cells resulted in a substantial increase in $1,25(OH)_2D_3$ mediated induction (from 22.23 ± 0.18 fold to 68.43 ± 5.57 fold), with no affect on the basal expression. Whether this protein binds to the CCAAT box remains to be determined.

Overall then the following picture is envisaged. In the absence of $1,25(OH)_2D_3$ there is basal expression in both cell lines and the basal expression is limited by a repressor bound to unliganded VDR/RXR that may sterically interfere with Ets-1 (Dwivedi *et al.*, 1998). Bound Sp1 and NF-Y contribute to basal expression through the direct interaction with TBP/TFIID on the TATA box (Figure 4.19A). In the presence of $1,25(OH)_2D_3$, the repressor is released allowing the binding of a large coactivator complex that includes CBP/p300 and p160 which interacts with Ets-1 (Yang *et al.*, 1998; Czuwara-Ladykowska *et al.*, 2002), Sp1 (Shetty *et al.*, 1999; Suzuki *et al.*, 2000). The interaction could result in transcriptional synergy. Such synergy could also result from protein-protein interactions between VDR and Ets-1 and in turn Ets-1 and Sp1.

An interesting question relates to the participation of VDRE-2. As mentioned so far no transcription factor binding sites have been identified located in the close vicinity of VDRE-2 and we suggest that a second coactivator complex binds to liganded VDR/RXR located on this VDRE. Previous studies in our laboratory (Kerry *et al.*, 1996) have noted transcriptional

synergy between the two VDREs in response to $1,25(OH)_2D_3$ in COS-1 cells; VDRE-2 contributes 3-fold, VDRE-1 contributes 6-fold, but together the level of induction is 18-fold. Similar results were also observed in HEK-293T cells and UMR106 cells (Data not shown). We propose that an interaction between the two coactivators bound on each VDRE in some way leads to transcriptional synergy with the contributions of the EBS and particularly the GC and CCAAT box sites critical for coactivator assembly/activation on VDRE-1.

In the final section of this chapter, the possible contributions of PKA, PKC and MAP kinase pathways to $1,25(OH)_2D_3$ induction of the *CYP24* –298 promoter were investigated in HEK-293T cells. In these experiments inhibitors H89 (PKA inhibitor), calphostin C (PKC inhibitor) and PD98059 (ERK1/2/5 inhibitor) were employed. The results from these inhibitors strongly suggests that PKA, PKC and ERK1/2/5 MAPK pathways are critical for $1,25(OH)_2D_3$ mediated induction of *CYP24* promoter in HEK-293T cells. In addition, cotransfection of a dominant negative form of PKCζ (PKCζ K281M), resulted in $1,25(OH)_2D_3$ mediated induction being significantly reduced suggesting that this isoform is involved in the induction process. As described in the introduction, it seems likely that nuclear VDR acting at the membrane is responsible for the non-genomic responses, that is, for the activation of PKA, PKC and MAP kinase pathways. There is evidence in muscle cells that nuclear VDR acts at the cytoplasm membrane and can couple with PKA and PKC pathways through a G protein (Morelli *et al.*, 1996; Vazquez *et al.*, 1997).

Therefore in the current study it can be proposed that liganded VDR in the cytoplasm activates G-protein coupled AC to increase the cAMP level and this would activate PKA (Figure 4.20A). Similarly the liganded VDR could also activate G-protein coupled stimulation of PLCβ as described in muscle cells (Morelli *et al.*, 1996). It is well known that activation of PLCβ leads to the hydrolysis of phosphatidylinositol bisphosphate (PIP2) and the subsequent formation of the second messenger, diacylglycerol (DAG) would mediate the activation of PKC (Figure 4.20B). Whether PLCβ is involved in the activation of PKC and activation of

89
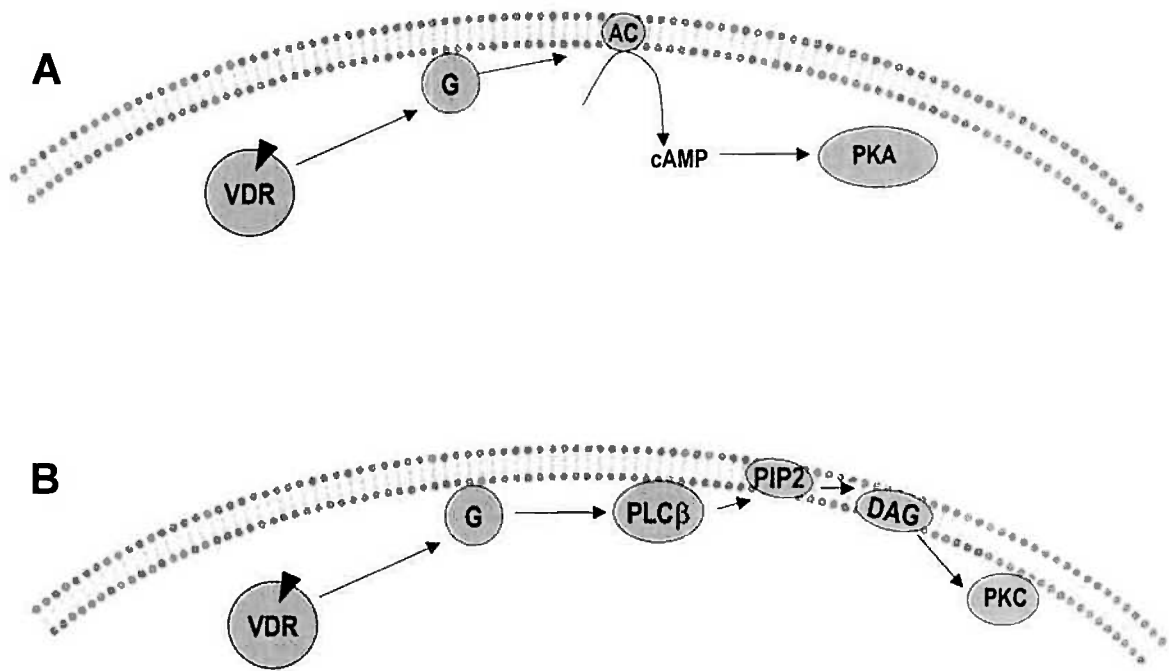
Figure 4.20   A proposed model for liganded VDR mediated non-genomic AC/PKA (A) and PLCβ/PKC (B) signalling system in HEK-293T cells.

CYP24 expression in HEK-293T cells has not been investigated. Stimulation of the MAP kinase (ie. ERK1/2) cascade by $1,25(OH)_2D_3$ can involve an increase of PKC activity (Buitrago *et al.*, 2003; Morelli *et al.*, 2000). In addition, activation of Raf-1 via Ras and PKC mediates $1,25(OH)_2D_3$ regulation of the MAP kinase pathway in muscle cells (Buitrago *et al.*, 2003). Therefore in the current study it is proposed that the $1,25(OH)_2D_3$ activated ERK MAP kinase cascade is dependent on Ras/Raf activated by PKC (Figure 4.20C).

Among all the PKC family members, the atypical member PKC ζ, belongs to the subfamily that are non-DAG dependent. Numerous studies have shown that PKC ζ is downstream of PI-3K (Catalti *et al.*, 2003; Calcerrada *et al.*, 2002; Martin *et al.*, 2001) and can be potently stimulated in vitro by the product of PI-3K (Diaz-Meco *et al.*, 1994). 3-Phosphoinositide-dependent kinase –1 (PDK-1) is known to be able to phosphorylated PKCζ at threonines 410 (Chou *et al.*, 1998). PDK-1 binds PtdIns 3,4,5-P3, a product of the PI-3K pathway (Vanhaesebroeck and Alessi, 2000). Therefore we propose that the PKC-PI3K-PDK1-PKCζ signaling transduction pathway exists in HEK-293T cells and is involved in $1,25(OH)_2D_3$ mediated induction (Figure 4.20D). Activated PKA, PKC isoforms and ERK MAP kinases resulting from above signaling cascades are then translocated into nucleus to modify the transcription factors. Clearly, the data obtained so far are only preliminary and more work is required to test the validity of this model.

An important question relates to the downstream targets of PKA, PKCζ and ERK1/2/5 activities. The GC and CCAAT boxes appear to be cAMP/PKA responsive. In the current work FSK, an activator of PKA pathways, stimulates weakly through both of these sites. This is also seen in *Drosophila* SL3 cells following over expression of CMV-PKA. It is possible that in response to $1,25(OH)_2D_3$, PKA phosphorylates NF-Y resulting in the dissociation of NF-Y from the CCAAT binding site and replacement by the protein X. Sp1 has been shown
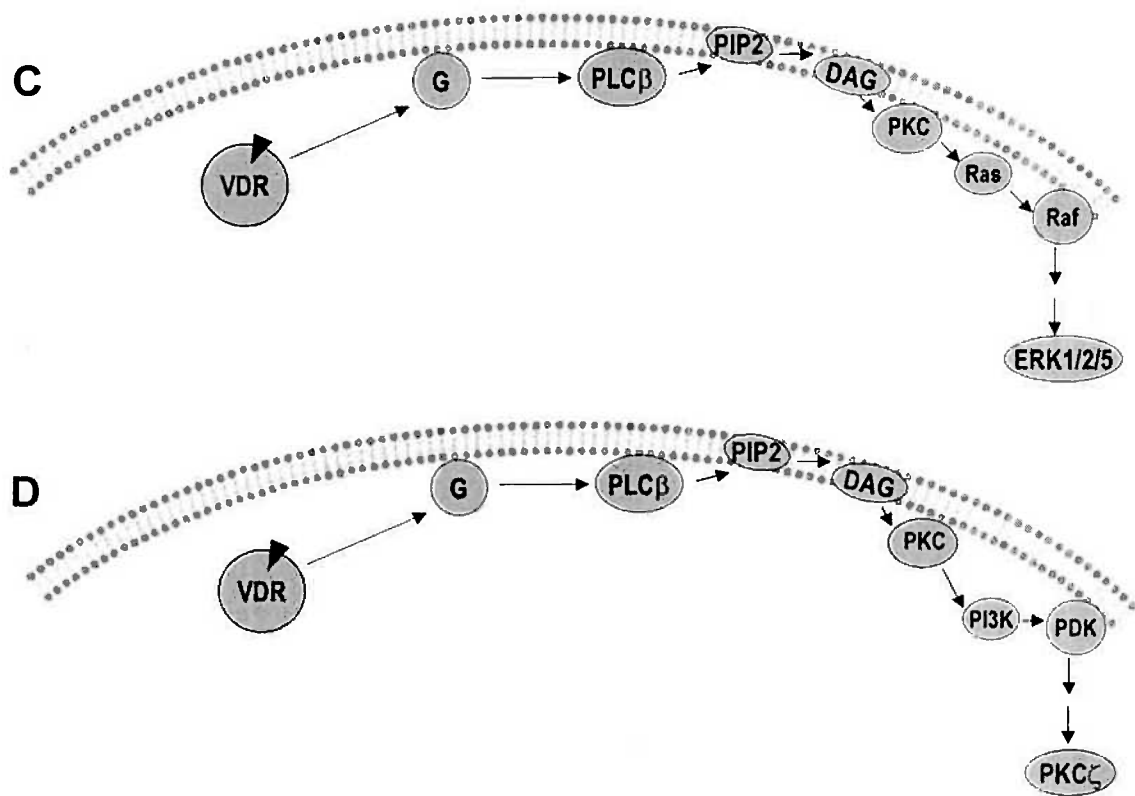
Figure 4.20   A   proposed   model   for   liganded   VDR   mediated   non-genomic ERK1/2/5 (C) and PI3K/PKCζ (D) signalling system in HEK- 293T cells.

to be phosphorylated by PKCζ (Pal *et al.,* 1998) and in the current study evidence is present for PKCζ involvement in $1,25(OH)_2D_3$ induction through the GC box (Figure 4.18). On this basis, it is suggested that $1,25(OH)_2D_3$ activates PKCζ which phosphorylates Sp1 leading to its activation. Finally the possible targets of ERK1/2/5 pathways, activated by $1,25(OH)_2D_3$ must be considered. Dwivedi et al (2002) showed that in response to $1,25(OH)_2D_3$ there is a rapid induction of ERK pathways in COS-1 cells where ERK1/2 phosphorylated RXR and ERK5 phosphorylated Ets-1. Other studies have shown that in addition to ERK5, ERK1/2 can also phosphorylate Ets-1 (Paumelle *et al.,* 2002). Sp1 is another possible target for the ERK1/2 MAP kinase signaling pathway since it can be phosphorylated on threonines 453 and 739 by ERK1/2 MAP kinase (Milanini-Mongiat *et al.,* 2002). Future work would need to address the phosphorylation status of RXR, Ets-1, Sp1 and the unknown CCAAT box protein (possibly DbpB) in response to $1,25(OH)_2D_3$.

✦ ✦

CHAPTER 5

Calcitonin stimulates expression of the rat 25-hydroxyvitamin D3-24 hydroxylase gene promoter in HEK-293 cells expressing calcitonin receptor: identification of signal pathways.

✦ ✦

5.1 Introduction

The ambient level of $1,25(OH)_2D_3$ represents a balance between its metabolic activation and inactivation (Jones *et al.*, 1998; Omdahl and May, 1997; Omdahl *et al.*, 2002). Bioactivation occurs predominantly in the kidney where the mitochondrial cytochrome P450 enzyme, 25-hydroxyvitamin D3 1α-hydroxylase (*1α(OH)ase*) catalyses the addition of a hydroxyl group at C-1 on 25-hydroxyvitamin D3 ($25(OH)D_3$) to generate $1,25(OH)_2D_3$ (Jones *et al.*, 1998; Omdahl *et al.*, 2002). Inactivation of $1,25(OH)_2D_3$ occurs via the C-23/C-24 oxidation pathways catalyzed by the multifunctional mitochondrial cytochrome P450 enzyme, 25-hydroxyvitamin D3 24-hydroxylase or *CYP24* present in the kidney and most other tissues (Jones *et al.*, 1998; Brown *et al.*, 1999b). *CYP24* initiates the inactivation and degradation of $1,25(OH)_2D_3$ through side chain hydroxylation at either C-23 or C-24, depending on the species (Omdahl *et al.*, 2002). Whilst $1,25(OH)_2D_3$ can be synthesized at extra-renal sites (Segersten *et al.*, 2002; Panda *et al.*, 2001b) where it likely acts in a paracrine/autocrine fashion, the production of $1,25(OH)_2D_3$ by the kidney is accepted as the primary source of endocrine-acting $1,25(OH)_2D_3$.

Circulating $1,25(OH)_2D_3$ together with the peptide hormone parathyroid hormone (PTH) are the key regulators of ambient calcium levels that must be controlled to support bone mineralization and other cellular processes. Small fluctuations in serum calcium are detected by the calcium sensing receptor (CaR) present on the cell surface of the PTH secreting parathyroid cells, with a lowered level of calcium resulting in increased PTH production (Brown and MacLeod, 2001; Brown, 2000). In turn, PTH through the PTH receptor (PTHR) located in the kidney cell plasma membrane up-regulates expression of renal *1α(OH)ase* and hence $1,25(OH)_2D_3$ synthesis (Jones *et al.*, 1998; Omdahl *et al.*, 2002). The resultant increased levels of PTH and $1,25(OH)_2D_3$ lead to increased serum calcium through enhanced kidney calcium reabsorption, stimulated bone resorption and intestinal calcium uptake (Brown and MacLeod, 2001).

A second peptide hormone, calcitonin secreted by the thyroid parafollicular C cells has been considered as a possible contributor to the maintenance of serum calcium (Inzerillo *et al.*, 2002; Pondel, 2000). Production of calcitonin by these cells in response to calcium differs fundamentally from that of PTH with increased serum calcium levels detected by the CaR resulting in the stimulation of calcitonin production (Brown and MacLeod, 2001). Calcitonin has hypocalcemic actions and is well known to inhibit osteoclastic bone resorption and stimulate renal calcium excretion in human and rodents (Jones *et al.*, 1998; Pondel, 2000). However, it is uncertain as to whether these actions of calcitonin contribute significantly to the maintenance of ambient calcium levels in human adults (Brown and MacLeod, 2001).

Alternatively, calcitonin could contribute to calcium homeostasis through regulating $1,25(OH)_2D_3$ levels. In this regard, Shinki *et al.* (1999) have presented evidence from experiments involving hormone administration to rats on dietary calcium, that calcitonin but not PTH induces renal *1α(OH)ase* under conditions of normal or high serum calcium. Administered PTH on the other hand induced *1α(OH)ase* only under hypocalcemic conditions. In other experiments Murayama *et al.* (1999) showed that calcitonin or PTH treatment of normal rats increased *1α(OH)ase* mRNA production in the kidney. *In vivo* experiments such as these are inherently difficult to interpret because in rodents, administration of pharmacological doses of calcitonin will result in hypocalcemia with PTH production, thus requiring parathyroid gland removal and hormone replacement.

In other studies employing transfected kidney cells, it has been shown that the promoter for human *1α(OH)ase* is up regulated both by calcitonin and also PTH (Murayama *et al.*, 1998 and 1999). In spite of the evidence for calcitonin stimulated expression of renal *1α(OH)ase*, no plausible physiological role for this finding has been proposed. There is no information on the regulation of the *CYP24* gene expression in kidney following calcitonin treatment although Beckman *et al.* (1994) reported its suppression of *CYP24* mRNA in the intestine of rats.

We have investigated whether rat *CYP24* promoter activity can be altered by calcitonin in transfected kidney cells. We show for the first time that calcitonin stimulates expression of the rat *CYP24* promoter and we have identified signal pathways and responsive promoter elements. We suggest that the induction of *CYP24* by calcitonin could have physiological relevance in the hypercalcemic state.

5.2   Results

Dual luciferase activity measurements in the presence of calcitonin.

As described earlier in the thesis, the dual luciferase assay can be employed to normalize for any transfection efficiency. Firefly luciferase activity is expressed relative to *Renilla* luciferase activity (driven by the thymidine kinase promoter). However it was repeatedly found that the level of *Renilla* luciferase was increased about 2-4 fold in the presence of salmon calcitonin at the 10nM concentration employed in this study. This concentration has been routinely used in cell culture experiments (Raggatt *et al.*, 2000; Evdokiou *et al.*, 2000). As an alternative control for transfection efficiency, I tested SV40-Luc, where *Renilla* luciferase is driven by the SV40 promoter, but this was also found to be induced by calcitonin. Therefore a ratio of luciferase activities could not be used to express *CYP24* promoter activity in the presence of calcitonin. For this reason, I have expressed the level of firefly luciferase activity in cell lysates relative to protein content and refer to this as luciferase activity (LA). Experiments have been repeated on at least two independent occasions. Within an experiment, transfections have been performed in triplicate and data averaged. The data presented in this chapter is from one representative experiment.

5.2.1   Calcitonin responsiveness of the rat *CYP24* gene promoter.

The construct pCYP24WT(298)-Luc contains 298bp of the rat *CYP24* gene promoter together with 74bp of 5' untranslated region, cloned upstream of the firefly luciferase cDNA sequence

as reporter (Hahn *et al.,* 1994). This region of the promoter has been intensively studied and encompasses two vitamin D response elements (VDREs) at -258/-244 and -150/-136 and a functional Ets-1 binding site (EBS) at -128/-119 (Kerry et al, 1996; Dwivedi et al, 2002). p*CYP24*WT-Luc was transiently transfected into human embryonic kidney cells (HEK-293T) stably expressing the insert-negative isoform of the human calcitonin receptor (CTR) and these cells are designated HR-12 (Raggatt *et al.,* 2000; Evdokiou *et al.,* 2000). The insert negative isoform of the human calcitonin receptor lacks a 16 amino acid insert present in the first intracellular loop of the other major expressed insert positive isoform and, as such, can activate phospholipase C (PLC), adenylate cyclase (AC) (Moore *et al.,* 1995; Pondel, 2000; Sexton *et al.,* 1999) and MAP kinase ERK1/2 pathways (Raggatt *et al.,* 2000; Chen *et al.,* 1998). Transfected cells were treated with salmon calcitonin at 10nM for 24 h (Figure 5.1).

It can be seen that the –298bp promoter construct is induced 4.67 ± 0.29 fold by calcitonin. In this and other experiments the pGL3 empty vector (promoter-less firefly luciferase construct) was found to be slightly responsive to calcitonin (about 1.5-fold) and luciferase activities in all experiments have been corrected for the value of the vector. A construct containing the entire 5' flanking region of -1400bp was also investigated but the level of calcitonin induction was similar to that for the –298bp construct (result not shown), strongly suggesting that all of the calcitonin responsive elements lie in the first –298bp of sequence. This region was investigated in detail.

5.2.2 Identification of calcitonin responsive sites in the –298bp promoter by transient transfections.

In addition to the Ets-1 site downstream of the proximal VDRE, an inverted CCAAT box at -62/-51 (5' GCTCATTGGCCA 3') and a GC box at -114/-101 (5' ACACCCGCCCCCCG 3') (Figure 5.1) have also been identified as described in Chapter 4. Site directed mutagenesis showed that while the EBS did not contribute to basal expression of p*CYP24*WT-Luc, the GC and CCAAT box sequences were particularly important with inactivation of these sites

	Basal (LA)	CT (LA)	Fold induction
pCYP24-WT-Luc	1.05 ± 0.14	4.92 ± 0.31	4.67 ± 0.29
pCYP24-mVDRE1+2-Luc	1.97 ± 0.29	8.32 ± 1.15	4.22 ± 0.58
pCYP24-mEBS-Luc	1.57 ± 0.23	6.85 ± 0.64	4.36 ± 0.41
pCYP24-mGC-Luc	0.33 ± 0.04	0.78 ± 0.14	2.53 ± 0.46
pCYP24-mCCAAT-Luc	0.14 ± 0.01	0.41 ± 0.09	2.57 ± 0.38
pCYP24-mGC+CCAAT-Luc	0.13 ± 0.003	0.23 ± 0.02	1.76 ± 0.17
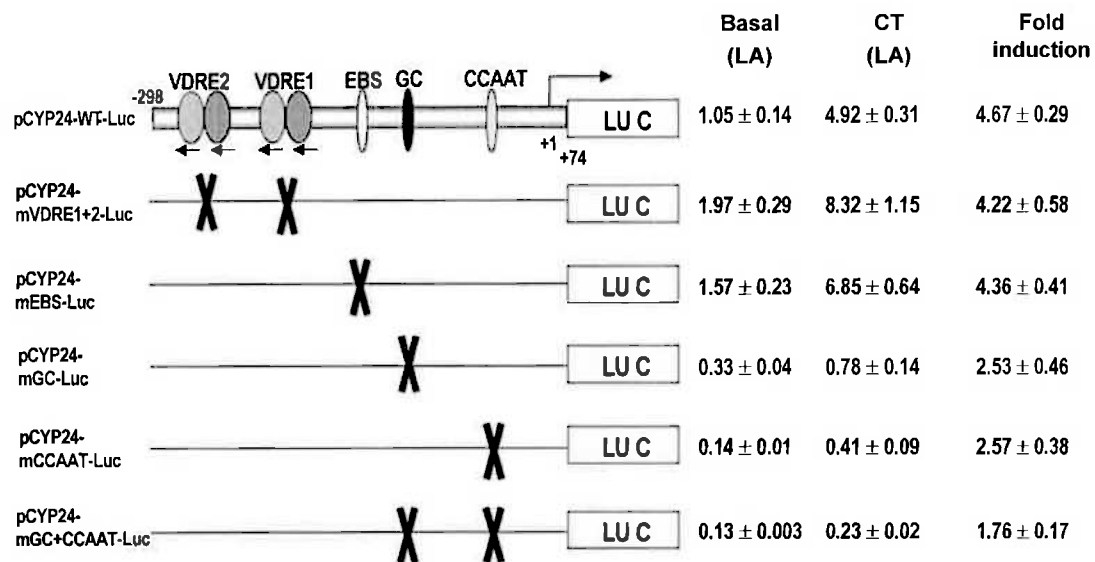
Figure 5.1 Transient transfection of wild type and mutant p*CYP24*-298-Luc constructs. The wild type and mutant promoter constructs were transiently transfected into HR-12 cells. The luciferase activity (LA) is the ratio of firefly luciferase activity normalized to the protein content. Following transfection, the cells were treated with calcitonin (CT) at 10nM for 24 hours. The luciferase activity (LA) represents the mean ± S.D firefly luciferase/protein content of triplicate samples. The fold induction shown is the ratio of luciferase activity from calcitonin treated cells to that from untreated cells. Data are the representative of two independent experiments performed in triplicate.

individually lowering basal expression from 1.05 to 0.33 and 0.14 respectively (Figure 5.1). This expression was lowered to 0.13 when both the GC and CCAAT box sites were mutated together (Figure 5.1). The increase in basal expression (from 1.05 to 1.97) observed when the two VDREs were mutated (Figure 5.1) has been previously observed in COS-1 cells and reflects repression by bound unliganded VDR/RXR (Polly *et al.*, 2000; Dwivedi *et al.*, 1998). An examination of these mutant constructs in the presence of calcitonin revealed that only the GC and CCAAT boxes, not the EBS and VDREs, contribute to calcitonin stimulation with inactivation of each site lowering induction by about 50% (from about 4.67-fold to 2.53 and 2.57-fold respectively) (Figure 5.1). Mutagenesis of both sites further lowered the fold induction by calcitonin to 1.76 (Figure 5.1). Thus the CCAAT and GC boxes are key elements underlying calcitonin responsiveness.

5.2.3 Functional role for the GC and CCAAT box in stably transfected cells.

The functional roles of the GC and CCAAT box elements were also investigated in pooled HR-12 cells stably expressing the wild type and mutated forms of p*CYP24*-298-Luc. Calcitonin induced p*CYP24*WT-Luc by 14.39-fold, a level higher than that observed in transiently transfected cells (Figure 5.2). Mutagenesis of the GC box and the CCAAT box lowered basal expression from 3.38 to 1.64 and 0.33 respectively. Calcitonin induction was markedly reduced with inactivation of either the GC or CCAAT box sites lowering expression by 80% and 95% respectively from 14.39 to 2.79 and 1.22 (Figure 5.2). Mutagenesis of both sites together was not examined in stably transfected cells. Hence, the GC and CCAAT box sites are major contributors to calcitonin inductions when stably integrated into the chromosome, as found in transient transfection analysis.

5.2.4 Characterization of the GC box binding protein

In Chapter 4, it was established that the GC box at -114/-101 binds Sp1, as determined by EMSA supershift analysis employing a monoclonal antibody to Sp1 (see earlier, Figure 4.6).

				Basal (LA)	CT (LA)	Fold induction
pCYP24-WT-Luc	-298	VDRE-2 VDRE-1	GC CCAAT Luciferase	3.38 ± 0.67	48.63 ± 3.34	14.39 ± 0 .99
pCYP24-mGC-Luc	-298		X Luciferase	1.64 ± 0.41	4.57 ± 0.55	2.79 ± 0.34
pCYP24-mCCAAT-Luc	-298		X Luciferase	0.33 ± 0.07	0.40 ± 0.03	1.22 ± 0.09
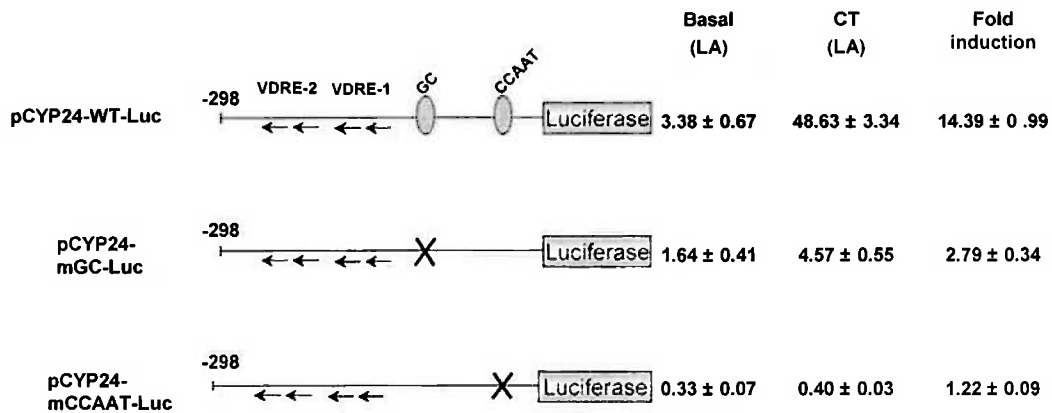
Figure 5.2 Stably transfected wild type and mutant pCYP24-298-Luc constructs. The procedure to generate stably transfected cells was described in section 2. 2 x 10^5 of pooled stably cells were seeded into 24-well plates in 400μl of DMEM containing 10% fetal calf serum and 1μg/ml puromycin and were allowed to grow overnight. Cells were treated with 10nM calcitonin (CT) in serum free IPMI 1640 medium for 24 hours. The luciferase activity from the stably transfected cells was normalized by the protein content in each sample. The values represent the mean ± S.D. of triplicate samples and similar data were seen in two other independent experiments.

In addition, the results from *Drosophila* SL3 cells in which Sp1 was over expressed showed that Sp1 could activate the *CYP24* promoter at least in part through this GC box (see earlier, Figure 4.10). It is concluded that Sp1 interacts with and functions through the GC box at -114/-101.

5.2.5 Characterization of the CCAAT box binding protein.

The sequence, orientation and position of the identified inverted CCAAT box at -62/-51, conforms to those sites shown to bind the ubiquitous transcription factor NF-Y that constitutes a trimer of NF-YA, B and C subunits (Mantovani, 1999). As described in Chapter 4, EMSA experiments have demonstrated that NF-Y present in nuclear extracts of HEK-293T cells can bind to this CCAAT box although several other proteins can also bind (Figure 4.8). To determine if NF-Y plays a role in *CYP24* promoter expression, NF-YA was over expressed in HR-12 cells together with p*CYP24*WT-Luc. However, there was no effect on promoter activity and this could reflect a high endogenous level (data not shown). Evidence presented in Chapter 4, clearly showed that over expression of NF-YA, B and C together in *Drosophila* SL3 cells increased expression of the promoter and that this was mediated through the CCAAT box (see earlier Figure 4.11). On this basis it was assumed that the CCAAT box is responsive to NF-Y.

When the dominant negative mutant for NF-YA, NF-YAm29 (Mantovani *et al.*, 1994) was expressed in HR-12 cells at 200ng, basal expression of p*CYP24*WT-Luc activity was strongly inhibited (from 0.23 ± 0.01 to 0.06 ± 0.01), while calcitonin mediated fold induction was moderately lowered from 5.33 ± 0.30 fold to 3.59 ± 0.10 fold (Figure 5.3). However at 200ng of the NF-YAm29, basal expression of p*CYP24*-mCCAAT-Luc was not altered (from 0.03 ± 0.001 to 0.03 ± 0.01) while calcitonin-mediated fold induction was not altered significantly (compare 2.57 ± 0.38 with 2.84 ± 0.52). The data suggests that NF-Y may play a role in both basal and calcitonin induced expression of p*CYP24*WT-Luc.
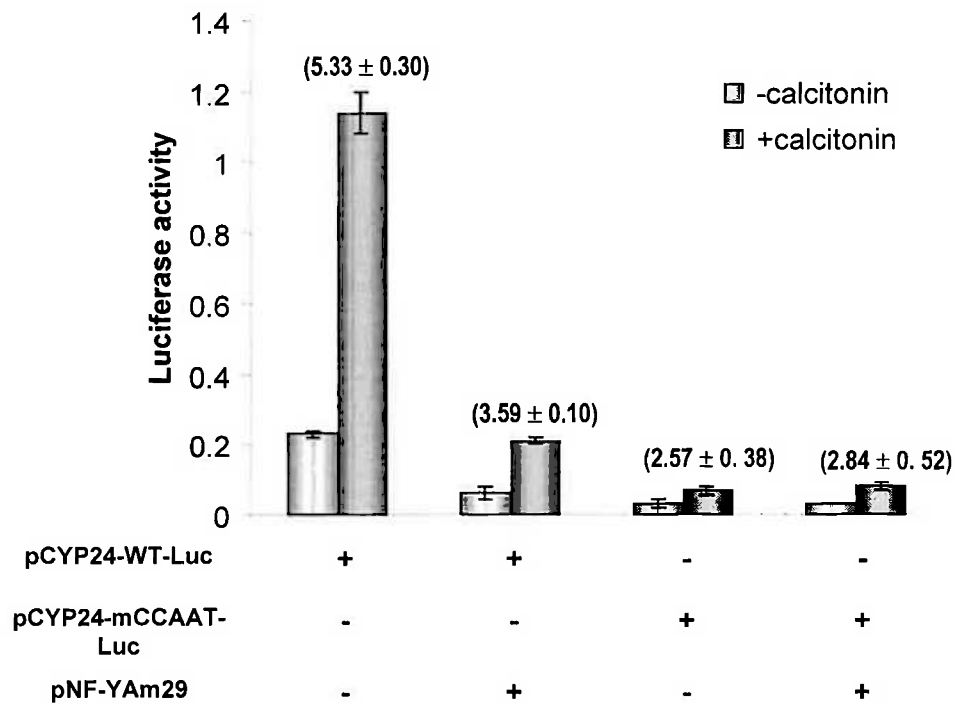
Figure 5.3 The effect of a dominant negative form of NF-Y, NF-YAm29 on basal and calcitonin induction of p*CYP24*-WT (298)-Luc or p*CYP24*-mCCAAT (298)-Luc constructs. HR-12 cells were cotransfected with 200ng of NF-YAm29 together with p*CYP24*-WT(298)-Luc or p*CYP24*-mCCAAT-Luc. After transfection overnight, the cells were treated with 10nM calcitonin for 24 h in serum free IPMI medium. The luciferase activity (LA) represents the mean ± S.D firefly luciferase/protein content of triplicate samples. The fold induction shown in the bracket is the ratio of luciferase activity from calcitonin treated cells to that from untreated cells. Data are the representative of two independent experiments performed in triplicate.

5.2.6 ERK1/2 pathways are not involved in calcitonin stimulation

In a previous study, it was established that treatment of HR-12 cells with calcitonin at 10nM resulted in a marked activation of ERK1/2 activity (Raggatt et al, 2000). The mechanism of this activation is not known, but is likely to be PKC dependent (Chabre *et al.*, 1992). We speculated that ERK1/2 activity may participate in calcitonin stimulation of the −298bp *CYP24* promoter particularly since our recent work has shown that this activity is important for induction of this promoter by $1,25(OH)_2D_3$ (Dwivedi *et al.*, 2002). However, the data do not provide evidence for the involvement of ERK1/2. Firstly, at 50µM, PD98059, a well-known ERK1/2 inhibitor, had no effect on basal or calcitonin-induced expression of p*CYP24*WT-Luc (Figure 5.4). Fold induction in the presence of calcitonin (4.67 ± 0.29) was not affected by inhibitor (4.42 ± 0.42).

Further support for the non-participation of the ERK1/2 pathway in calcitonin induction was obtained through over expression of the ERK1/2 dominant negative clone, ERK1K71R in HR-12 cells. At 200ng the mutant clone had no effect on calcitonin induction measured after 24h (fold induction in the absence and presence of the mutant was 4.67 ± 29 and 4.16 ± 0.27 respectively). In a control experiment the inhibitory effect of the mutant clone was confirmed. Induction of p*CYP24*WT-Luc by $1,25(OH)_2D_3$ at $10^{-7}M$ measured after 24h was lowered by ERK1K71R from 49.24 ± 3.90 to 32.87 ± 3.74, in keeping with results reported in COS-1 cells (Dwivedi *et al.*, 2002).

5.2.7 Involvement of PKA and PKC in calcitonin stimulation

It is well known that the activated insert negative isoform of the human calcitonin receptor can couple to protein kinase A (PKA) and protein kinase C (PKC) pathways through mechanisms involving G proteins (Shyu *et al.*, 1999; Chen *et al.*, 1998; Pondel, 2000). The data in Figure 5.5 show that the PKA inhibitor, H89, and the PKC inhibitor, calphostin C, did
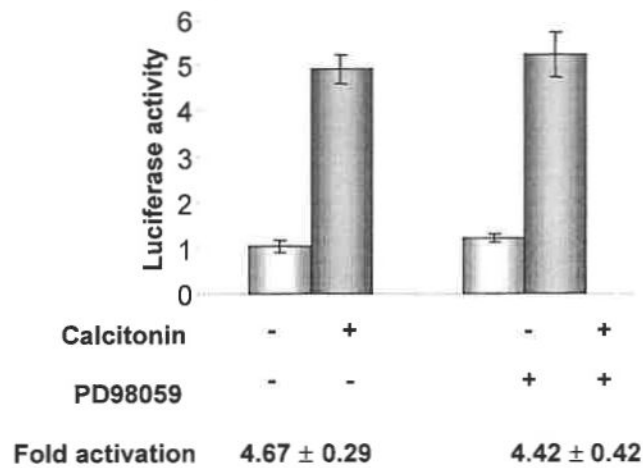
Figure 5.4 The effect of PD98059 at 50μM on calcitonin mediated induction of *CYP24* promoter activity in HR-12 cells. The cells were pretreated with 50μM PD 98059 before incubation with 10nM calcitonin for 24 h. The luciferase activity (LA) represents the mean ± S.D firefly luciferase/protein content of triplicate samples. The fold induction shown is the ratio of luciferase activity from calcitonin treated cells to that from untreated cells. Data are the representative of two independent experiments performed in triplicate.
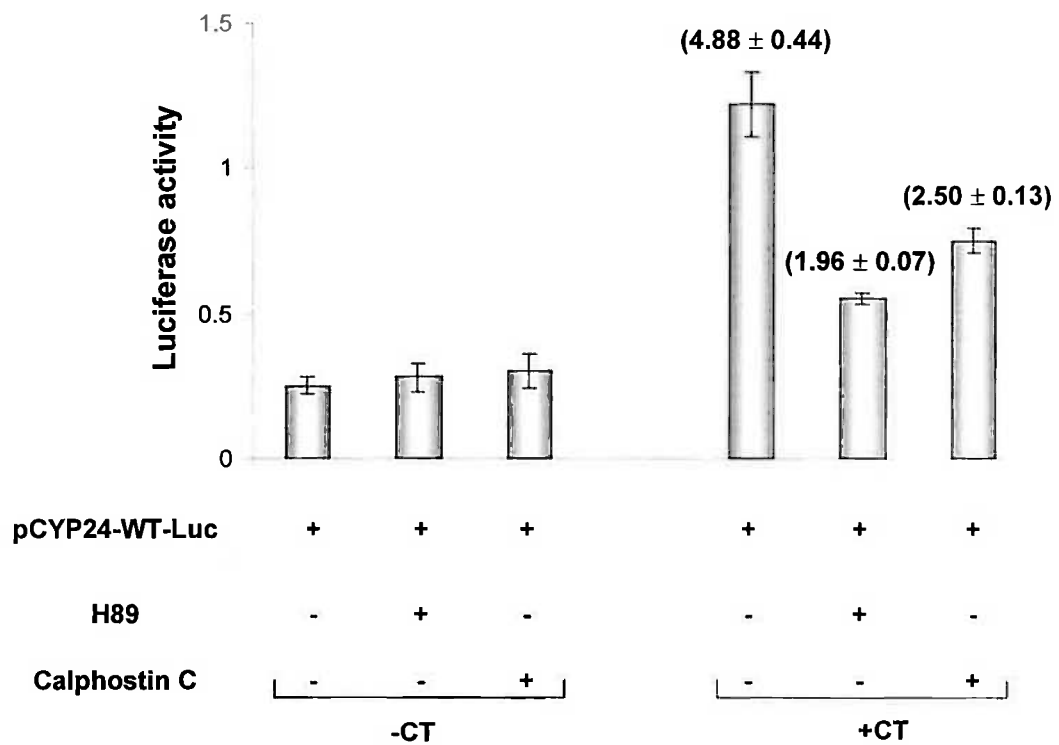
Figure 5.5 Effect of PKA and PKC inhibitors on calcitonin (CT) mediated induction of *CYP24* promoter activity. Cells were transiently transfected with p*CYP24*-WT (298)-Luc in HR-12 cells and after transfection were treated with 10 μM H89 or 100nM calphostin C for 1.5h prior to addition of 10nM CT for a further 24h incubation. The luciferase activity (LA) represents the mean ± S.D firefly luciferase/protein content of triplicate samples. The fold induction shown in the bracket is the ratio of luciferase activity from calcitonin or/and inhibitors treated cells to that from untreated cells. Data are the representative of two independent experiments performed in triplicate.

not affect basal activity of p*CYP24*WT-Luc in HR-12 cells. However, H89 and calphostin C markedly inhibited calcitonin-induced expression by 60% (from 4.88-fold to 1.96-fold) and 50% (from 4.88-fold to 2.50-fold) respectively (Figure 5.5). Hence PKA and PKC activities play a role in calcitonin-induced expression but not basal expression.

In support of PKA involvement, the PKA activator forskolin at 10µM moderately stimulated basal expression by 2.46 ± 0.49 fold. When the CCAAT box and GC box were mutated individually, fold induction by forskolin was lowered to 1.51 ± 0.36 and 1.40 ± 0.18 respectively; mutagenesis of both sites lowered induction to 1.16 ± 0.15 fold. Hence both the CCAAT and GC boxes appear to be target sites for PKA signaling. As mentioned in Chapter 4, activation of *CYP24* by over expression of CMV-PKA in *Drosophila* SL3 cells also occurs through the CCAAT and GC boxes.

5.2.8 PKCζ and *CYP24* promoter activity.

As mentioned in Chapter 4, the atypical PKC isoform PKCζ can bind and phosphorylate the zinc finger region of Sp1 (Pal *et al.*, 1998). Evidence was presented in Chapter 4 for a role of PKCζ in the $1,25(OH)_2D_3$ –dependent induction of the *CYP24* promoter in HEK-293T cells. It was of interest then to investigate whether PKCζ was involved in calcitonin-induced *CYP24* promoter expression in the HR-12 cells. A dominant negative clone of PKCζ, PKCζK281M (Soh *et al.*, 1999), when over expressed at 200ng, did not alter basal expression of p*CYP24*WT-Luc in HR-12 cells (data not shown). However, the dominant negative mutant consistently inhibited calcitonin induction by about 55% with an approximate 5.5-fold level of induction being reduced to about 2.4-fold (Figure 5.6). The data indicate that phosphorylation of Sp1 may be needed for calcitonin induction but not for basal expression of the *CYP24* promoter. PKCζ is known to be a downstream target of phosphoinositide 3-kinase (PI-3 kinase), (Catalti *et al.*, 2003; Calcerrada *et al.*, 2002; Martin *et al.*, 2001). There is evidence that PI-3 kinase can be activated as a result of interaction with Ras (Rodriguez-
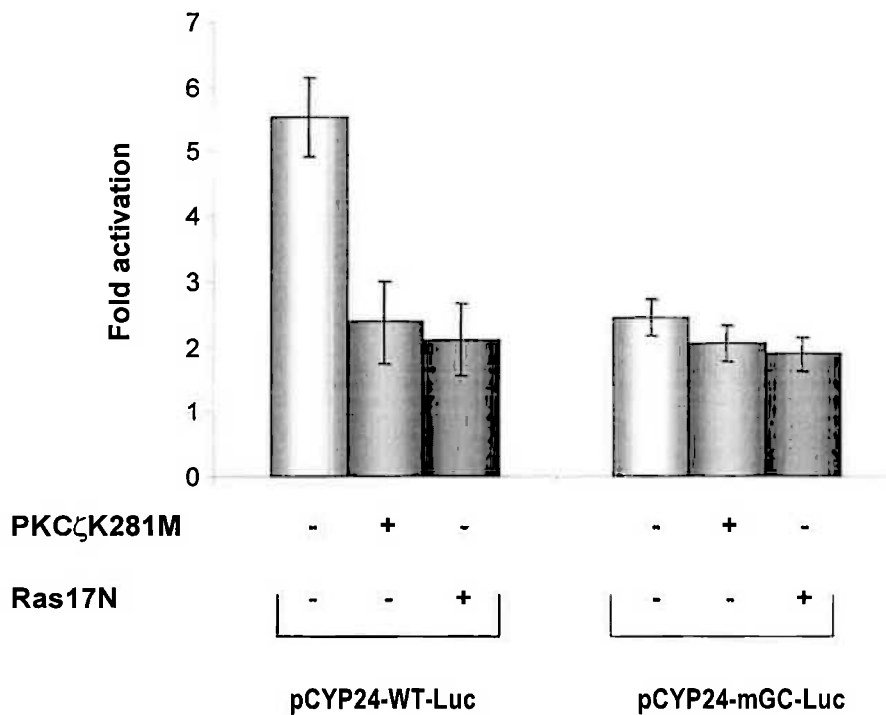
Figure 5.6 The effect of dominant negative mutants Ras17N and PKCζ K281M on calcitonin mediated induction of the *CYP24* promoter. HR-12 cells were transiently transfected with p*CYP24*-WT(298)-Luc or p*CYP24*mGC(298)-Luc constructs together either with 500ng of PKCζK281M or Ras17N and incubated with 10nM calcitonin. The results are present as fold induction, the ratio of luciferase activity from calcitonin treated cells to that from untreated cells (basal). Data are the representative of two independent experiments performed in triplicate.

Viciana *et al.,* 1994 and 1996) and a dominant negative clone of Ras (Ras17N) when tested at 200ng inhibited calcitonin induction of p*CYP24*WT-Luc by about 50% from 5.5-fold to 2.1-fold (Figure 5.6).

When the GC box was mutated in p*CYP24*mGC-Luc, there was no significant further lowering of calcitonin induction following over expression of the dominant negative mutant clone for PKCζ or for Ras indicating that these mutants act solely through the GC box (Figure 5.6). The data are compatible with a situation in which cross talk between Ras-PI3K-PKCζ acts on the GC box and contributes to calcitonin-induced but not basal expression of the *CYP24* promoter.

5.2.9 Synergistic effect of calcitonin and 1,25(OH)$_2$D$_3$ on *CYP24* promoter activity

As mentioned, 1,25(OH)$_2$D$_3$ markedly up regulates *CYP24* promoter expression (Chapter 4) and since in the current chapter it has been shown that calcitonin also stimulates this promoter, it was of interest to investigate the effect of simultaneous addition of both hormones. A marked synergistic response was observed (Figure 5.7). Calcitonin alone stimulates about 4.7-fold, 1,25(OH)$_2$D$_3$ stimulates 34-fold, but together there is about a 117-fold increase in promoter activity. Synergy was abolished when the two VDREs were mutated (Figure 5.7). However, a high level of synergy was maintained when the calcitonin responsive CCAAT and GC boxes, were mutated together (Figure 5.7). Hence the synergistic action is dependent on the VDREs. A high level of transcriptional synergy was also observed in cells stably transfected with p*CYP24*WT-Luc. Calcitonin alone was found to stimulate 60-fold, 1,25(OH)$_2$D$_3$ to stimulate 42-fold but together induction levels of about 897-fold were observed. This 9-fold level of synergy was almost abolished when the two VDREs were mutated (data not shown). The level of calcitonin mediated induction of wide type construct is noticed much higher than the 14-fold induction observed in Figure 5.2. The reason for this difference is not known.
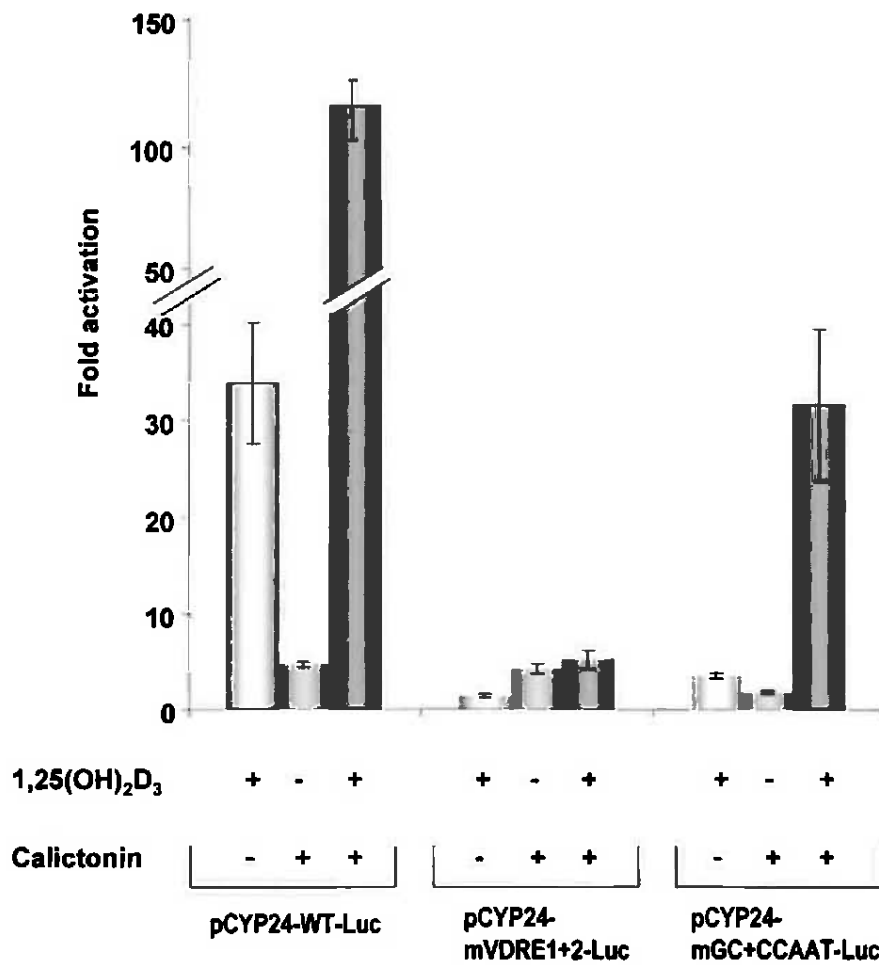
Figure 5.7 Synergistic effect of calcitonin and 1,25(OH)$_2$D$_3$ on the induction of pCYP24-WT-Luc in transiently transfected cells. HR-12 cells were transiently transfected with pCYP24-WT-Luc and treated with 10nM calcitonin or/and 10^{-7}M 1,25(OH)$_2$D$_3$ for 24 h. The luciferase activity represents the mean ± SD firefly luciferase/protein content of triplicate samples. The fold induction shown is the ratio of luciferase activity from calcitonin treated cells to that from untreated cells. Data are the representative of two independent experiments performed in triplicate.

5.3 Discussion

In this study, it has been shown for the first time that the −298bp promoter region of the rat *CYP24* gene can be significantly induced by calcitonin at 10nM. In transiently transfected HR-12 cells expressing the calcitonin receptor, levels of induction of about 4-fold were observed while in stably transfected cells levels of about 15-fold were seen. Mutagenesis analysis established the critical importance for both basal expression and calcitonin induction of a GC box and CCAAT box located at -114/-101 and -62/-51 respectively.

EMSA experiments demonstrated binding of Sp1 and NF-Y to the GC and CCAAT box sequences respectively (see chapter 4), while over expression analysis in *Drosophila* SL3 cells provided evidence for the functionality of these proteins at these sites (see chapter 4). Further evidence for the involvement of NF-Y in both the basal and calcitonin-induced expression was obtained using the dominant mutant NF-YAm29 that strongly inhibited basal expression and moderately inhibited calcitonin induction. The only transcription factor so far implicated in calcitonin action is Sp1, with calcitonin activation of the human p21 gene promoter showing a dependency on Sp1 binding sites (Evdokiou *et al.*, 2000). Hence, the involvement in calcitonin-mediated induction reported here of a of a CCAAT box binding protein, most likely NF-Y, represents only the second target transcription factor to be identified. Whether in response to calcitonin, Sp1 and NF-Y bound at the promoter interact physically as described on other promoters (Yamada *et al.*, 2000; Ge *et al.*, 2001), is not clear from the current studies.

As mentioned it has been reported that calcitonin treatment of HR-12 cells leads to a marked and prolonged activation of ERK1/2 (Raggatt *et al.*, 2000). Hence it was of particular interest to find that ERK1/2 activity is apparently not involved in calcitonin induction of the *CYP24* promoter. Neither the ERK1/2 inhibitor PD98059, nor the dominant negative mutant ERK1K71R affected calcitonin induction in HR-12 cells. At the concentration of PD98059

employed (50μM), it has been shown in these cells that the calcitonin activation of ERK1/2 is strongly inhibited (Raggatt *et al.,* 2000). While in these studies, it is not known whether calcitonin also activates ERK5, in addition to ERK1/2. Since PD98059 has been shown to also inhibit activation of ERK5 in other cell types (English and Cobb, 2002), it can be concluded that this MAP kinase is not involved in calcitonin induction of the promoter in HR-12 cells. In previous studies using COS-1 cells, evidence was presented for the activation of ERK1/2 and ERK5 by $1,25(OH)_2D_3$ treatment (Dwivedi *et al.,* 2002). RXR and Ets-1 were phosphorylated by ERK1/2 and ERK5 respectively and it was proposed that these phosphorylated proteins then stimulated expression of the $1,25(OH)_2D_3-$ induced promoter (Dwivedi *et al.,* 2002). However in the present work, neither Ets-1 nor VDR/RXR play any role in the calcitonin induced expression of the promoter. Overall, it can be concluded that calcitonin induction of the *CYP24* promoter is not dependent on any transcription factors or regulatory coactivator complexes bound to that promoter that require ERK1/2/5 for activity.

Experiments with specific inhibitors for PKA (H89) and PKC (calphostin C) provided evidence for the involvement of these signaling pathways in calcitonin induction. However neither pathway contributed to basal expression that remained unaffected by these inhibitors. In the presence of forskolin basal promoter expression was stimulated somewhat with both the GC and CCAAT box sites being target sites. This finding supported the idea that one action of calcitonin is to increase PKA activity and hence promoter expression through both the CCAAT and GC boxes. Perhaps phosphorylation of Sp1 and NF-Y enhances their activity in some way.

The role of PKC in calcitonin induction remains unclear but one possibility is that it is involved in the activation of Ras, PI-3 kinase and hence PKCζ as described in other cells (Vanhaesebroeck and Alessi, 2000; Rodriguez-Viciana *et al.,* 1994 and 1996). The atypical PKC isoform, PKCζ has received considerable attention recently since it interacts with and phosphorylates Sp1 and may lead to its activation (Rafty and Khachigian., 2001; Pal *et al.,*

2001). A kinase inactive mutant form of PKCζ, PKCζK281M (Soh *et al.*, 1999) was employed to investigate whether this isoform plays a role in *CYP24* promoter expression. Blocking PKCζ with this dominant negative mutant, had no effect on basal activity but consistently inhibited calcitonin induction and evidence indicated that this occurred through the GC site. The data suggest that PKCζ dependent- phosphorylation of Sp1 is important for calcitonin induction but not for basal activity.

As discussed in Chapter 4, PKCζ is known to be downstream of both Ras and PI-3 kinase (Catalti *et al.*, 2003; Calcerrada *et al.*, 2002; Martin *et al.*, 2001; Rodriguez-Viciana *et al.*, 1994 and 1996). There is evidence that PDK-1 (phosphoinositide-dependent protein kinase) can activate PKCζ but for this action, PDK-1 must be translocated to the cytoplasmic membrane through binding to a lipid product of PI-3 kinase (Vanhaesebroeck and Alessi, 2000). Additionally, PI-3 kinase can interact with Ras and can be activated as a result of this interaction (Rodriguez-Viciana *et al.*, 1994 and 1996). The finding here that Ras17N lowered calcitonin induction with this action predominantly occurring through the GC box is in keeping with the proposal that a Ras-PI-3 kinase-PDK-1- PKCζ pathway acts to phosphorylate Sp1. Future experiments are required to establish whether in fact Sp1 is phosphorylated *in vivo* in response to calcitonin and whether the dominant negative mutant for PKCζ can inhibit this. Also it will be important to determine whether calcitonin can activate Ras, PI-3 kinase and PKCζ and to establish the involvement of PDK-1.

A model compatible with the experimental findings is shown in Figure 5.8. In the absence of calcitonin, basal expression of the *CYP24* promoter is dependent on Sp1 bound at the GC box and NF-Y bound at the CCAAT box, as described in Chapter 4 for HEK-293T cells. It is proposed that calcitonin through a G protein coupled to the calcitonin receptor results in the activation of PKA, which may then phosphorylate Sp1 and NF-Y enhancing their transactivation ability. It is further proposed that activation of PKC occurs through another G protein coupled to the calcitonin receptor by a mechanism involving PLC β and DAG (Chabre

et al., 1992). PKC could then activate Ras and in turn the Ras-PI-3 kinase-PDK-1-PKCζ

pathway leading to the phosphorylation and activation of Sp1. While ERK1/2 is activated by

calcitonin, and possibly also ERK5, these ERKs play no role in the induction of the *CYP24*

promoter by calcitonin. It is further proposed that Ets-1, a target for ERK1/2/5, does not

contribute to basal or calcitonin activation because it is sterically prevented by the bound

corepressor complex (see Figure 5.8).

It is interesting to speculate on the possible physiological significance of the finding here that

the *CYP24* promoter is induced by calcitonin in kidney cell cultures. It is tempting to suggest

that calcitonin *in vivo* contributes to the control of circulating calcium levels through

regulating serum $1,25(OH)_2D_3$ levels. It is well accepted that $1,25(OH)_2D_3$ synthesized by the

kidney is important for maintenance of serum calcium (Omdahl *et al.,* 2002; Jones *et al.,*

1998). Hence in response to calcitonin an elevated renal *CYP24* production would lower the

production of $1,25(OH)_2D_3$ by the kidney. Such an action of calcitonin could be important in

the hypercalcemic state when a transient increase in serum calcium triggers the production of

calcitonin by the thyroid C cells (Brown and MacLeod, 2001).

While calcitonin alone induces expression of p*CYP24*WT-Luc, in the present work it was also

found that there was a marked cooperative effect in the presence of $1,25(OH)_2D_3$. In

transiently transfected cells an approximate 3-fold level of synergy was observed in the

presence of both hormones. Perhaps this synergy has physiological significance when the

hypercalcemic state is accompanied by elevated circulating $1,25(OH)_2D_3$. The synergy would

ensure that there is a rapid lowering of $1,25(OH)_2D_3$ production by the kidney commensurate

with lowered calcium intestinal uptake.

The mechanism for the transcription synergy may involve a greater activation of ERK1/2 in

the presence of both calcitonin and $1,25(OH)_2D_3$. Since ERK1/2 is critically important for

$1,25(OH)_2D_3$ induction most likely through phosphorylation and activation of RXR,VDR or

Ets-1, an increase in ERK1/2 activity will permit a synergistic response. The finding that

synergy is lost when the VDREs are mutated but not when the GC and CCAAT box sites are

mutated is in keeping with the proposal. Experiments are now required to determine the relative levels of ERK1/2 (and ERK5) in the presence of either or both hormones. It should be noted that calcitonin alone does not activate p*CYP24*WT-Luc through the VDREs even though ERK1/2 activities are likely to be increased (Raggatt *et al.*, 2000) because of the co-repressor complex bound to unliganded VDR/RXR (see Figure 5.8).

While in the current work, calcitonin induces *CYP24* expression in kidney cells, there are other reports from transient transfection assays that this hormone also induces the *1α(OH)ase* promoter (Murayama *et al.*, 1998). A situation in which both *1α(OH)ase* and *CYP24* activities are induced by calcitonin seems paradoxical. However as mentioned above calcitonin and $1,25(OH)_2D_3$ together result in a large synergistic activation of *CYP24* in kidney cells. This synergy could be responsible *in vivo* for ensuring that serum $1,25(OH)_2D_3$ levels are kept low in response to calcitonin production in the hypercalcemic state.

A comment should be made regarding the comparative actions of calcitonin and PTH on *1α(OH)ase* and *CYP24* expression in kidney cells. As described in chapter 3, PTH action on kidney cells results in a moderate two-fold induction of the *1α(OH)ase* promoter through a CCAAT site (5'AGGGATTGGCTG3') with the likely involvement of PKA. It might be expected that the *CYP24* promoter will respond to PTH through its active CCAAT box (5'GCTCATTGGCCA3'). However, no PTH induction of the *CYP24* promoter in kidney cells has been observed in our laboratory (data not shown) or elsewhere (Zierold *et al.*, 2000). Perhaps different proteins bind the CCAAT box sequences in the *1α(OH)ase* and *CYP24* promoter; although there is a common CCAAT sequence on the opposite strand the flanking regions show no homology (*1α(OH)ase*: 5'AGGGATTGGCTG3'; *CYP24*: 5'GCTCATTGGCCA3'). From a physiological point of view, stimulation of *CYP24* by PTH is not desirable since the role of PTH is to increase serum $1,25(OH)_2D_3$. Indeed there is evidence that PTH markedly lowers *CYP24* expression through altering the stability of the mRNA (Zierold *et al.*, 2000).
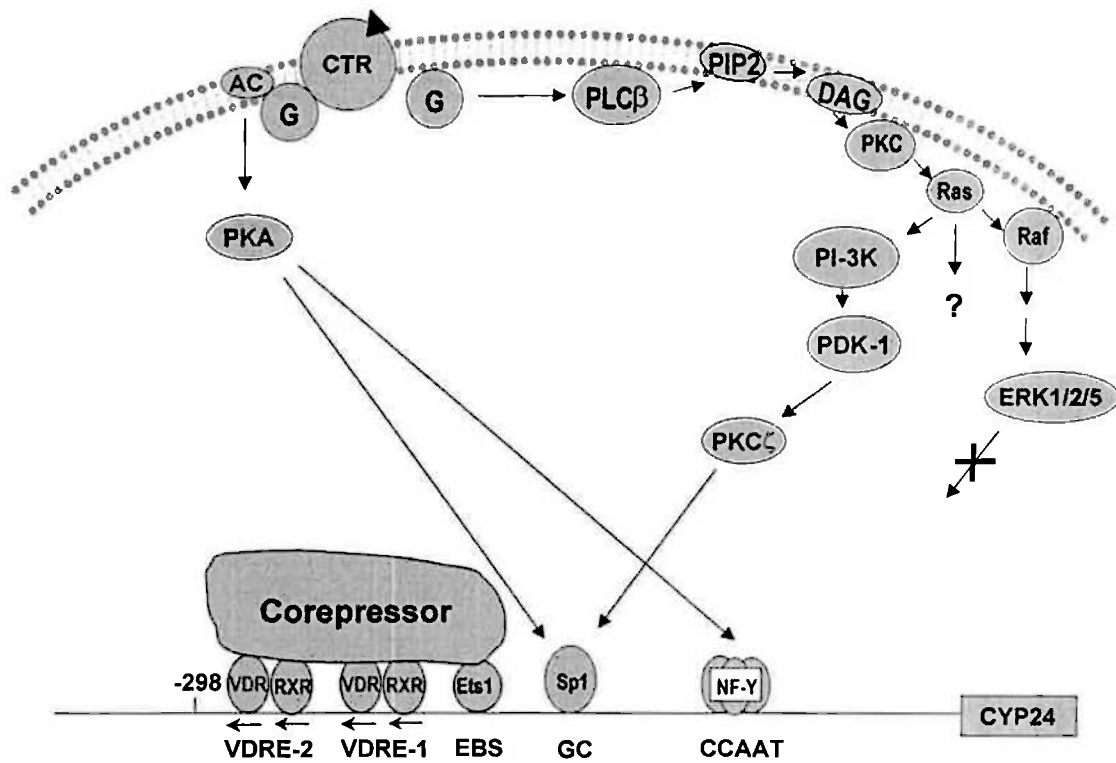
Figure 5.8 **Proposed molecular mechanism by which calcitonin induces the *CYP24* expression: the involvement of downstream CCAAT box and GC box sites in the promoter and PKA, PKC and PKC-Ras-PI-3K-PKCζ signaling pathways.**

In summary, it has been established that the *CYP24* gene promoter can be up-regulated in kidney cells by calcitonin, and this action can be further enhanced in the presence of $1,25(OH)_2D_3$. It is proposed that this regulation could be part of the process in which calcitonin regulates circulating calcium through controlling the production of renal $1,25(OH)_2D_3$.

+ +

CHAPTER 6

Summary and concluding discussion

+ +
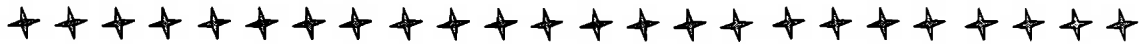
1,25(OH)$_2$D$_3$ plays important roles in calcium and phosphate homeostasis and bone mineralisation and recent evidence implicates the hormone in cell proliferation and differentiation, immune modulation, and bile acid metabolism. *1α(OH)ase* and *CYP24* are two crucial enzymes for 1,25(OH)$_2$D$_3$ synthesis and degradation respectively. Several physiological factors including 1,25(OH)$_2$D$_3$, parathyroid hormone (PTH) and calcitonin interact to finely regulate gene expression of the two enzymes resulting in the regulation of serum and tissue levels of 1,25(OH)$_2$D$_3$.

The study presented in this thesis has focused on the molecular mechanisms by which PTH regulates *1α(OH)ase* promoter expression , and 1,25(OH)$_2$D$_3$ and calcitonin regulate *CYP24* promoter expression.

It has been known for many years that PTH up-regulates renal *1α(OH)ase* gene expression under hypocalcemic conditions, resulting in enhanced 1,25(OH)$_2$D$_3$ production for the maintenance of calcium and phosphate homeostasis. However the molecular mechanism for the PTH response has not been examined in detail. In the current study analysis of the human *1α(OH)ase* gene promoter in transiently transfected kidney AOK-B50 cells, has established that the first 305bp of promoter is PTH responsive, a result also observed in stably transfected cells. By mutagenesis, three sites within the region from −66 to −135 were shown to contribute to basal expression: a distal GC box (GC-d), an EBS site and a proximal CCAAT (CCAAT-p) box. Sp1 interacted with the GC-d site physically and functionally as shown using electrophoretic mobility shift assays (EMSA) and over expression analysis in *Drosophila* SL3 cells. However, the protein that binds to the CCAAT-p box site to drive basal expression remains unknown with EMSA evidence suggesting that the protein is neither NF-Y nor a C/EBP protein. Importantly, the CCAAT-p box site only, was shown to contribute to PTH induction in the transfected cells. While experiments using a dominant negative mutant A-C/EBP suggested that a C/EBP member may be important for the PTH mediated induction,

EMSA experiments did not provide evidence for the binding of a C/EBP to the CCAAT site. However in these experiments, nuclear extracts from cells not treated with PTH were employed and future work should explore the possibility that a C/EBP protein that binds could be present in cells treated with PTH.

Studies into the contribution of various signaling pathways showed that while basal expression was independent of PKA, PKC and ERK1/2 MAP kinase activities, PTH mediated induction of the $1\alpha(OH)ase$ promoter activity was likely to be dependent on PKA activity. This conclusion is supported by other findings in kidney cells (Murayama *et al.*, 1999; Kong XF *et al.*, 1999; Henry, 1985). It is therefore proposed that PTH binds to the PTHR resulting in the activation of PKA, subsequent phosphorylation of the CCAAT binding protein, with enhanced transactivation of the $1\alpha(OH)ase$ gene. Identification of the protein that binds to the CCAAT box site is now required and this would permit an examination of the phosphorylation status of the protein following treatment with PTH. In the laboratory of our collaborator Dr Howard Morris, transgenic mice expressing 1500bp of the $1\alpha(OH)ase$ gene promoter fused to firefly luciferase have been developed. Interestingly, in response to hypocalcemia and vitamin D deficiency, the expression of the promoter in kidney cells is increased several fold, in keeping with the data obtained here. The transgenic model system would in the future permit the direct examination of the contribution of the CCAAT box site to PTH induction of the $1\alpha(OH)ase$ gene promoter.

It is generally agreed that the induction of renal *CYP24* gene expression by $1,25(OH)_2D_3$ will regulate serum $1,25(OH)_2D_3$ levels through reducing the production by the kidney and through removal of hormone from the serum. $1,25(OH)_2D_3$ also induces *CYP24* gene expression and activity in osteoblast cells. In these cells the local production of $1,25(OH)_2D_3$ is likely to be involved in osteoblast proliferation and differentiation and in mineralisation events. Hence osteoblast *CYP24* will be important for regulating the local concentration of

$1,25(OH)_2D_3$. In this thesis, I have examined $1,25(OH)_2D_3$ induction of *CYP24* in kidney HEK-293T cells and also in osteoblast UMR106 cells. The studies focused on the roles of the newly identified GC and CCAAT box sites identified in the *CYP24* promoter, downstream from the known functional Ets-1 binding site.

Basal expression of the *CYP24* promoter in both cell types was strongly dependent on the GC and CCAAT box sites. EMSA using antibodies for Sp1 and NF-YB provided evidence for the binding of Sp1 to the GC box and the binding of NF-Y to the CCAAT box. Both proteins are ubiquitous and this finding is in keeping with the fact that the *CYP24* gene is widely expressed. It is envisaged that while a repressor binds to unliganded VDR/RXR (Dwivedi *et al.*, 1998) and sterically interferes with Ets-1, Sp1 and NF-Y are able to bind to the promoter and are the major contributors for basal expression perhaps through interactions with TFIID at the TATA box both in HEK-293T and UMR106 cells.

In the presence of $1,25(OH)_2D_3$, both the GC and CCAAT box sites are major contributors to induction, as well as the EBS. In the present study, the mammalian two-hybrid assay provided evidence for an interaction of Sp1 with Ets-1. Hence it is envisaged that there are multiple interactions on the promoter where liganded VDR binds coactivators including p160 and CBP/p300. Interactions are then possible between VDR, Ets-1 and Sp1 and also with the coactivator complex. The CCAAT box binding protein may be too far downstream to interact with bound transcription factors but could interact with the coactivator complex. Such an enhanceosome-like interaction on the *CYP24* promoter would be expected to contribute to transcriptional synergy in the presence of $1,25(OH)_2D_3$. The identity of this CCAAT box binding protein that contributes to induction is intriguing. While NF-Y appears to play a role in basal expression it is not involved in $1,25(OH)_2D_3$ mediated induction in HEK-293T cells as shown using a dominant negative mutant NF-YAm29. This raises the possibility that another unknown protein (X) interacts with the CCAAT box in the presence of hormone. It seems that a C/EBP family member is not involved as shown by the over expression data and EMSA. The identity of the X protein requires to be further investigated although recent

preliminary experiments show that DbpB markedly stimulates 1,25(OH)$_2$D$_3$ induction raising the possibility that this protein is involved in the induction process. While NF-Y appears important for basal expression of the *CYP24* promoter in UMR106 cells, the identity of the CCAAT box protein that is involved in 1,25(OH)$_2$D$_3$ induction has not been investigated here. Experiments with HEK-293T cells revealed that PKA, PKC and ERK1/2/5 MAPK pathways are critical for 1,25(OH)$_2$D$_3$ mediated induction of the *CYP24* promoter as demonstrated by the use of pharmacological inhibitors. In addition, use of the dominant negative mutant PKCζ K281M provided evidence for the involvement of PKCζ (which is non-DAG dependent) in the induction process and specifically through the Sp1 site. There is evidence that liganded nuclear VDR acts at the cytoplasm membrane and couples with AC/PKA and PLCβ/PKC through a G protein (Morelli *et al.*, 1996; Vazquez *et al.*, 1997). Stimulation of the ERK1/2 MAP kinase cascade by 1,25(OH)$_2$D$_3$ can involve an increase of PKC and Ras (Buitrago *et al.*, 2002; Morelli *et al.*, 2000). Therefore in the current study it is proposed that the 1,25(OH)$_2$D$_3$ activated ERK MAP kinase cascade is dependent on Ras/Raf activated by PKC. It has been shown that PKCζ is downstream of PI-3K/PDK-1 (Catalti *et al.*, 2003; Chou *et al.*, 1998; Vanhaesebroeck and Alessi, 2001) and therefore it is proposed here that the PKC-PI-3K-PDK1-PKCζ pathway exists in HEK-293T cells.

In response to 1,25(OH)$_2$D$_3$, both the CCAAT and GC boxes appear to be two target sites for cAMP/PKA. The GC box is the only target site for PKCζ, and this agrees with recent work by others who have established that Sp1 can be phosphorylated by this PKC isoform (Pal *et al.*, 1998). The fact that PKCζ does not contribute to basal expression strongly indicates that PKCζ is activated by 1,25(OH)$_2$D$_3$ (via the PKC-PI-3K-PDK1-PKCζ pathway indicated earlier). Current studies in the laboratory are exploring this possibility and the involvement of the proposed pathway. In addition, the GC box could also be a target for ERK1/2 activity induced by 1,25(OH)$_2$D$_3$ since Sp1 has been found to be phosphorylated by ERK1/2 *in vitro* and *in vivo* (Milanini-Mongiat *et al.*, 2002). It would be interesting in the future to learn

whether the phosphorylation status of Sp1 can be altered by PKA, PKCζ and ERK1/2 induced by 1,25(OH)$_2$D$_3$.

In conclusion, a model can be proposed for the role of the GC and CCAAT box sites in the inductive process. In response to 1,25(OH)$_2$D$_3$, Sp1 is phosphorylated by PKCζ and perhaps also by PKA or ERK1/2 activities while NF-Y, involved in basal expression, is phosphorylated by PKA resulting in its dissociation and replacement by the protein X. Calcitonin has hypocalcemic actions and is well known to inhibit osteoclastic bone resorption and stimulate renal calcium excretion (Jones et al, 1998; Pondel, 2000). In an alternative hypothesis, calcitonin could contribute to calcium homeostasis through regulation of 1,25(OH)$_2$D$_3$ level. There is little information on the regulation of the $CYP24$ gene expression in kidney following calcitonin treatment although Beckman et $al.$ (1994) reported suppression of $CYP24$ mRNA in the intestine of rats.

The current study investigated whether rat $CYP24$ promoter activity can be altered by calcitonin in HR-12 cells and the relevant regulation mechanism. I have established that the – 298bp $CYP24$ promoter fused to luciferase is markedly induced by calcitonin in both transiently and stably transfected kidney cells. The GC box at -114/-101 and the CCAAT box at -62/-51 are critically involved in basal expression and calcitonin induction. The only transcription factor so far implicated in calcitonin action is Sp1 in the human p21 promoter (Evdokiou et $al.$, 2000). Hence, the likely involvement of NF-Y in calcitonin induction reported here would represent a novel finding.

PKA and PKC are involved in calcitonin-mediated induction of the $CYP24$ promoter activity as indicated by use of their specific inhibitors H89 and calphostin C. Of particular interest was the finding that ERK1/2/5 activities were not involved in calcitonin induction of the $CYP24$ promoter since calcitonin is known to strongly induce ERK1/2 activity and additionally these ERK activities are absolutely necessary for induction of this promoter by 1,25(OH)$_2$D$_3$.

In the current study, evidence was presented for the involvement of Ras and PKC ζ in the induction of *CYP24* promoter expression by calcitonin. In addition, the data raise the possibility that the Ras-PI3k-PKCζ signaling pathway acts on the GC box, as suggested for the action of $1,25(OH)_2D_3$. A model is proposed where calcitonin binds to calcitonin receptor resulting in the activation of PKA and PKC. PKA phosphorylates Sp1 and NF-Y as proposed in chapter 4. PKC activates Ras and then PI-3K-PDK-1-PKCζ cascade leading to phosphorylation of Sp1 and presumably its activation.

Calcitonin and $1,25(OH)_2D_3$ were found act in a synergistic fashion to induce *CYP24* gene promoter activity in both transient transfected and stably transfected cells. This synergy was almost abolished when the two VDREs were mutated, but was not affected when the GC and CCAAT boxes were mutated. It is suggested that a greater activation of ERK1/2 in the presence of both calcitonin and $1,25(OH)_2D_3$ is responsible for the synergy with the actions of ERK1/2 not involving either the GC or CCAAT box sites. Since ERK1/2 is critically important for $1,25(OH)_2D_3$ induction most likely through phosphorylation and activation of RXR and perhaps VDR and Ets-1, an increase in ERK1/2 activity will permit a synergistic response through these target proteins.

In conclusion, I have established for the first time that the promoter of the *CYP24* gene can be markedly stimulated by calcitonin, and this induction could be further enhanced in the presence of $1,25(OH)_2D_3$. These findings are extrapolated to an *in vivo* situation and it is suggested that induction of renal *CYP24* by calcitonin under hypercalcemic conditions could contribute to the lowering of $1,25(OH)_2D_3$ levels and hence serum calcium.

REFERENCES:

Abou-Samra A, Juppner H, Force T, Freeman M, Kong X, Schipani E, Urena P, Richards J, Bonventre J, Potts J (1992): Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: A single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium. *Proc Natl Acad Sci* USA. 89:2732-2736.

Ahlgren R, Suske G, Waterman M, Lund J (1999): Role of Sp1 in cAMP-dependent transcriptional regulation of the bovine CYP11A gene. *J Biol Chem*. 274:19422-19428.

Akeno N, Matsunuma A, Maseda T, Kawane T, Horiuchi N (2000): Regulation of vitamin D-1 alpha-hydroxylase and 24-hydroxylase expression by dexamethasone in mouse kidney. *J Endocrinol*. 164:339-348.

Akeno N, Saikatsu S, Kawane T, Horiuchi N (1997): Mouse vitamin D-24-Hydroxylase: molecular cloning, tissue distribution, and transcriptional regulation by 1α, 25-dihydroxyvitamin D3. *Endocrinolgy*. 138:2233-2240.

Akiyoshi-Shibata M, Sakaki T, Ohyama Y, Noshiro M, Okuda K, Yabusaki Y (1994): Further oxidation of hydroxycalcidiol by calcidiol 24-hydroxylase. A study with the mature enzyme expressed in Escherichia coli. *Eur J Biochem*. 224:335-343.

Akizawa T, Fukagawa M (1999): Modulation of parathyroid cell function by calcium ion in health and uremia. *Am J Med Sci*. 317:358-62.

Albertson D, Ylstra B, Segraves R, Collins C, Dairkee S, Kowbel D, Kuo W, Gray J, Pinkel D (2000): Quantitative mapping of amplicon structure by array CGH identifies CYP24 as a candidate oncogene. *Nat Genet*. 25:144-6.

Amling M, Priemel M, Holzmann T, Chapin K, Rueger J, Baron R, Demay M (1999): Rescue of the skeletal phenotype of vitamin D receptor ablated mice in the setting of normal mineral ion homeostasis: formal histomorphometric and biomechanical analyses. *Endocrinolgy*. 140:4982-4987.

Armbrecht H, Chen M, Hodam T, Boltz M (1997): Induction of 24-hydroxylase cytochrome P450 mRNA by 1,25-dihydroxyvitamin D and phorbol esters in normal rat kidney (NRK-52E) cells. *J Endocrinol*. 153:199-205.

Armbrecht H, Hodam T (1994): Parathyroid hormone and 1,25-dihydroxyvitamin D synergistically induce the 1,25-dihydroxyvitamin D-24-hydroxylase in rat UMR-106 osteoblast-like cells. *Biochem Biophys Res Commun*. 205:674-679.

Armbrecht H, Hodam T, Boltz M, Partridge N, Brown A, Kumar V (1998): Induction of the vitamin D 24-hydroxylase (CYP24) by 1,25-dihydroxyvitmin D3 is regulated by parathyroid hormone in UMR106 osteoblastic cells. *Endocrinology*. 139:3375-3381.

Armbrecht H, Okuda K, Wongsurawat N, Nemani RK, Chen ML, Boltz MA (1992): Characterization and regulation of the vitamin D hydroxylases. *Steroid Biochem. Molec. Bio*. 43:1073-1081.

Armbrecht H, Wongsurawat B, Zenser T, Davids B (1984): Effect of PTH and 1,25(OH)$_2$D$_3$ on renal 25(OH)D$_3$ metabolism, adenylate cyclase, and protein. *Am J Physiol*. 246:E102-107.

Astecker N, Reddy G, Herzig G, Vorisek G, Schuster I (2000): 1α,25-dihydroxy-3-epi-vitamin D$_3$ a physiological metabolite of 1α,25-dihydroxyvitamin D$_3$: Its production and metabolism in primary human keratinocytes. *Mol Cell Endocrinol*. 170:91-101.

113

Aubin J, Bonnelye E (2000): Osteoprotegerin and its ligand: a new paradigm for regulation of osteoclastogenesis and bone resorption. *Osteoporos Int.* 11:905-913.

Aubin J, Heersche J (1997): Vitamin D and osteoblasts. In Feldman D, Glorieux F, Pike J (eds): *"Vitamin D."* London: Academic Press Limited, pp 313-328.

Bar A, Hurwitz S, Maoz. A (1980): The 25-hydroxycholecalciferol 1 alpha-hydroxylase activity in chick cells: direct effect of parathyroid hormone. *FEBS lett.* 113:328-330.

Barberis A, Superti-Furga G, Busslinger M (1987): Mutually exclusive interaction of the CCAAT-binding factor and of a displacement protein with overlapping sequences of a histone gene promoter. *Cell.* 50:347-359.

Barletta F, Freedman L, Christakos S (2002): Enhancement of VDR-mediated transcription by phosphorylation: correlation with increased interaction between the VDR and DRIP205, a subunit of the VDR-interacting protein coactivator complex. *Mol Endocrinol.* 16:301-314.

Barry J, Leong G, Church W, Issa L, Eisman J, Gardiner E (2003): VDR Helix H10 SKIP/NCoA-62 Interaction. Interaction of SKIP/NCo-62, TFIIB and retinoid X receptor with vitamin D receptor Helix H10 residues. *J Biol Chem.* epub ahead of print.

Becker B, Hullett D, O'Herrin J, Malin G, Sollinger H, DeLuca H (2002): Vitamin D as immunomodulatory therapy for kidney transplantation. *Transplantation.* 74:1204-1206.

Beckman M, Goff J, Reinhardt T, Beitz D, Horst R (1994): *In vivo* regulation of rat intestinal 24-hydroxylase: potential new role of calcitonin. *Endocrinology.* 135:1951-1955.

Beckman M, Tadikonda P, Werner E, Prahl J, Yamada S, DeLuca H (1996): Human 25-hydroxyvitamin D3-24-hydroxylase, a multicatalytic enzyme. *Biochemistry.* 35:8465-8472.

Bellorini M, Lee D, Dantonel J, Zemzoumi K, Roeder R, Tora L, Mantovani R (1997): CCAAT binding NF-Y-TBP interactions: NF-YB and NF-YC require short domains adjacent to their histone fold motifs for association with TBP basic residues. *Nucleic Acids Res.* 25:2174-2181.

Bhalla A, Amento E, Krane S (1986): Differential effects of 1,25-dihydroxyvitamin D3 on human lymphocytes and monocyte/macrophages: inhibition of interleukin-2 and augmentation of interleukin-1 production. *Cell Immunol.* 98:311-322.

Bikle D (1992): Clinical counterpoint: vitamin D: new actions, new analogs, new therapeutic potential. *Endocr Rev.* 13:765-784.

Bikle D, Ng D, Tu C, Oda Y, Xie Z (2001): Calcium- and vitamin D-regulated keratinocyte differentiation. *Mol Cell Endocrinol.* 177:161-71.

Birnboim H, Doly J (1979): A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7:1513-1523.

Bjorkhem I, Moberg K (1995): Inborn errors in bile acid biosynthesis and storage of steroids other than cholesterol. In Scriver C, beandet A, Sly W, Valk D (eds): *"The Metabolic Basis of Inherited Disease."* New York: McGraw-Hill, pp 2073-99.

Bland R, Walker E, Hughes S, Stewart P, Hewison M (1999): Constitutive expression of 25-hydroxyvitmain D3-1α-hydroxylase in a transformed human proximal tubule cell line: Evidence for direct regulation of vitamin D metabolism by calcium. *Endocrinology.* 140:2027-2034.

Bland R, Zehnder D, Hughes S, Ronco P, Stewart P, Hewison M (2001): Regulation of vitamin D-1α-hydroxylase in a human cortical collecting duct cell line. *Kidney int.* 60:1277-1286.

Bollinger Bollag W, Bollag R (2001): 1,25-Dihydroxyvitamin D(3), phospholipase D and protein kinase C in keratinocyte differentiation. *Mol Cell Endocrinol.* 177:173-182.

Bolognese F, Wasner M, Dohna C, Gurtner A, Ronchi A, Muller H, Manni I, Mossner J, Piaggio G, Mantovani R, Engeland K (1999): The cyclin B2 promoter depends on NF-Y, a trimer whose CCAAT-binding activity is cell-cycle regulated. *Oncogene.* 18:1845-1853.

Bortman P, Folgueira M, Katayama M, Snitcovsky I, Brentani M (2002): Antiproliferative effects of 1,25-dihydroxyvitamin D3 on breast cells- a mini review. *Braz J Med Biol Res.* 35:1-9.

Brenza HL, Kimmel-Jehan C, Jehan F, Shinki T, Wakino S, Anazawa H, Suda T, DeLuca HF (1998): Parathyroid hormone activation of the 25-hydroxyvitamin D3-1α-hydroxylase gene promoter. *Proc Natl Acad Sci USA.* 95:1387-1391.

Bringhurst FR, Juppner H, Guo J, Urena P, Jr JTP, Kronenberg HM, Abou-Samra AB, Segre GV (1993): Cloned, stably expressed parathyroid hormone (PTH)/PTH-related peptide receptors activate multiple messenger signals and biological responses in LLC-PK1 kidney cells. *Endocrinology.* 132:2090-2098.

Brown A, Ritter C, Slatopolsky E, Muralidharan K, Okamura W, Reddy G (1999a): 1α,25-dihydroxy-3-epi-vitamin D3, a natural metabolite of 1α,25-dihydroxyvitamin D3, is a potent suppressor of parathyroid hormone secretion. *J Cell Biochem.* 73:106-113.

Brown AJ, Dusso A, Slatopolsky E (1999b): Vitamin D. *Am. J. Physiol.* 277:F157-F175.

Brown E (2000): The extacellular Ca^{2+}-sensing receptor central mediator of systemic calcium homeostasis. *Annu Rev Nutr.* 2000:507-533.

Brown E, MacLeod R (2001): Extracellular calcium sensing and extracellular calcium signaling. *Phyiol Rev.* 81:239-297.

Brown E, Pollak M (1998): The extracellular calcium-sensing receptor: Its role in health and disease. *Annu Rev Med.* 49:15-29.

Bucher P (1990): Weight matrix descriptions of four eukaryotic RNA polymerase II promoter elements derived from 502 unrelated promoter sequences. *J Mol Biol.* 212:563-578.

Buitrago C, Pardo V, De Boland A, Boland R (2003): Activation of RAF-1 through Ras and protein kinase C alpha mediates 1alpha,25(OH)2-Vitamin D3 regulation of the mitogen-activated protein kinase pathway in muscle cells. *J Biol Chem.* 278:2199-2205.

Buitrago C, Pardo V, De Boland A (2002): Nongenomic action of 1α,25(OH)2-vitamin D3. Activation of muscle cell PLCγ through the tyrosine kinase c-Src and PtdIns-kinase. *Eur J Biochem.* 269:2506-2515.

Calcerrada M, Miguel B, Martin L, Catalan R, Martinez A (2002): Involvement of phosphatidylinositol 3-kinase in nuclear translocation of protein kinase C zeta induced by C2-ceramide in rat hepatocytes. *FEBS Lett.* 514:361-365.

Campbell M, Elstner E, Holden S, Uskokovic M, koeffler H (1997): Inhibition of proliferation of prostate cancer cells by a 19-nor-hexafluoride vitamin D3 analogue involves the induction of p21wafl, p27kip1 and E-cadherin. *J Mol Endocrinol.* 19:15-27.

Campbell M, Gombart A, Kwok S, Park S, Koeffler H (2000): The anti-proliferative effects of 1alpha,25(OH)$_2$D$_3$ on breast and prostate cancer cells are associated with induction of BRCA1 gene expression. *Oncogene.* 19:5091-7.

Capiati D, Benassati S, Boland R (2002): 1,25(OH)$_2$-vitamin D$_3$ induces translocation of the vitamin D receptor (VDR) to the plasma membrane in skeletal muscle cells. *J Cell Biochem.* 86:128-135.

Carlberg C, Polly P (1998): Gene regulation by vitamin D$_3$: critical reviews in Eukaryotic gene expression. *Crit Rev Eukaryot Gene Expr.* 8:19-42.

Cataldi A, Di Pietro R, Centurione L, Rapino M, Santavenere E, Garaci F, Cocco L, Giuliani-Piccari G, Rana R (2003): Engagement of PI-3-kinase mediated Protein kinase C zeta activation in protecting Friend cells from ionizing radiation-induced apoptosis. *Int J Oncol.* 22:129-135.

Chabre O, Conklin B, Lin H, Lodish H, Wilson E, Ives H, Catanzariti L, Hemmings B, Bourne H (1992): A recombinant calcitonin receptor independently stimulates 3',5'-cyclic adenosine monophosphate and Ca^{2+}/inositol phosphate signaling pathways. *Mol Endocrinol.* 6:551-556.

Chang Z, Liu C (1994): Human thymidine kinase CCAAT-binding protein is NF-Y, whose A subunit expression is serum-dependent in human IMR-90 diploid fibroblasts. *J Biol Chem.* 269:17893-17898.

Chen K, DeLuca H (1995): Cloning of the human 1 alpha, 25-dihydroxyvitamin D3 24-hydroxylase gene promoter and identification of two vitamin D responsive elements. *Biochim Biophys Acta.* 1263:1-9.

Chen M, Boltz M, Armbrecht H (1993): Effects of 1,25-dihydroxyvitamin D3 and phorbol ester on 25-hydroxyvitamin D3 24-hydroxylase cytochrome P450 messenger ribonucleic acid levels in primary cultures of rat renal cells. *Endocrinology.* 132:1782-1788.

Chen Y, Shyu J, Santhanagopal A, Inoue D, David J, Dixon S, Horne W, Baron R (1998): The calcitonin receptor stimulates Shc tyrosine phosphorylation and Erk1/2 activation. Involvement of Gi, protein kinase C, and calcium. *J Biol Chem.* 273:19809-19816.

Chiba N, Suldan Z, Freedman L, Parvin J (2000): Binding of liganded vitamin D receptor to the vitamin D receptor interacting protein coactivator complex induces interaction with RNA polymerase II holoenzyme. *J Biol Chem.* 275:10719-22.

Chou M, Hou W, Johnson J, Graham L, Lee M, Chen C, Newton A, Schaffhausen B, Toker A (1998): Regulation of protein kinase C zeta by PI 3-kinase and PDK-1. *Curr Biol.* 8:1069-1077.

Christakos S, Raval-Pandya M, Wernyj R, Yang W (1996): Genomic mechanisms involved in the pleiotropic actions of 1,25 dihydroxyvitamin D$_3$. *Biochem J.* 316:361-371.

Cooke N, Haddad J (1989): Vitamin D binding protein (Gc-globulin). *Endocr Rev.* 10:294-307.

Costa E, Feldman D (1986): Homologous up-regulation of 1,25-dihydroxyvitamin D3 receptor in rats. *Biochem Biophys Res Commun.* 137:742-747.

Crofts L, Hancock M, Morrison N, Eisman J (1998): Multiple promoters direct the tissue-specific expression of novel N-terminal variant human vitamin D receptor gene transcripts. *Proc Natl Acad Sci USA* 95:10529-34.

Czuwara-Ladykowska J, Sementchenko V, Watson D, Trojanowska M (2002): Ets1 is an effector of the transforming growth factor β (TGF-β) signaling pathway and an antagonist of the profibrotic effects of TGF-β. *J Biol Chem.* 277:20399-20408.

Dardenne O, Prud'homme J, Arabian A, Glorieux FH, St-Arnaud R (2001): Targeted inactivation of the 25-hydroxyvitamin D-3-1 alpha-hydroxylase gene (CYP27B1) creates an animal model of pseudovitamin D-deficiency rickets. *Endocrinology.* 142:3135-3141.

DeLuca H, Cantorna M (2001): Vitamin D: its role and uses in immunology. *FASEB J.* 15:2579-2585.

Demay M, Gerardi J, DeLuca H, Kronenberg H (1990): DNA sequences in the rat osteocalcin gene that bind the 1,25-dihydroxyvitamin D3 receptor and confer responsiveness to 1,25-dihydroxyvitamin D3. *Proc Natl Acad Sci USA.* 87:369-373.

Diamond P, Shannon M, Vadas M, Coles L (2001): Cold shock domain factors activate the granulocyte-macrophage colony-stimulating factor promoter in stimulated Jurkat T cells. *J Biol Chem.* 276:7943-7951.

Diaz G, Paraskeva C, Thomas M, Binderup L, Hague A (2000): Apoptosis is induced by the active metabolite of vitamin D3 and its analogue EB1089 in colorectal adenoma and carcinoma cells: possible implications for prevention and therapy. *Cancer Res.* 60:2304-12.

Diaz-Meco M, Lozano J, Municio M, Berra E, Frutos S, Sanz L, Moscat J (1994): Evidence for the *in vitro* and *in vivo* interaction of Ras with protein kinase C zeta. *J Biol Chem.* 269:31706-31710.

Dittmer J, Pise-Masison CA, Clemens KE, Choi KS, Brady JN (1997): Interaction of human T-cell lymphotropic virus type I Tax, Ets1, and Sp1 in transactivation of the PTHrP P2 promoter. *J Biol Chem.* 272:4953-4958.

Dusso A, Kamimura S, Gallieni M, Zhong M, Negrea L, Shapiro S, Slatopolsky E (1997): gamma-Interferon-induced resistance to 1,25-(OH)$_2$D$_3$ in human monocytes and macrophages: a mechanism for the hypercalcemia of various granulomatoses. *J Clin Endocrinol Metab.* 82:2222-32.

Dwivedi P, Hii C, Ferrante A, Tan J, Der C, Omdahl J, Morris A, May B (2002): Role of MAP kinases in the 1,25-dihydroxyvitamin D3-induced transactivation of the rat cytochrome P450C24 (CYP24) promoter. Specific functions for ERK1/ERK2 and ERK5. *J Biol Chem.* 277:29643-29653.

Dwivedi P, Muscat G, Bailey P, Omdahl J, May B (1998): Repression of basal transcription by vitamin D receptor: evidence for interaction of unliganded vitamin D receptor with two receptor interaction domains in RIP13delta1. *J Mol Endocrinol.* 20:327-325.

Dwivedi P, Omdahl J, Kola I, Hume D, May B (2000): Regulation of rat cytochrome P450C24 (CYP24) gene expression: Evidence for functional cooperation of Ras-activated Ets transcription factors with the vitamin D receptor in 1,25-dihydroxyvitamin D3-mediated induction. *J Biol Chem.* 275:47-55.

Emili A, Greenblatt J, Ingles C (1994): Species-specific interaction of the glutamine-rich activation domains of Sp1 with the TATA box-binding protein. *Mol Cell Biol.* 14:1582-1593.

English J, Cobb M (2002): Pharmacological inhibitors of MAPK pathways. *Trends Pharmacol Sci.* 23:40-45.

Engstrom GW, Horst RL, Reinhardt TA, Littledike ET (1985): Effect of dietary phosphorus levels on porcine renal 25-hydroxyvitmin D-1 alpha- and 24R-hydroxylase activities and plasma 1,25-dihydroxyvitamin D3 concentration. *J Anim Sci.* 60:1005-1011.

Erben R, Soegiarto D, Weber K, Zeitz U, Lieberherr M, Gniadecki R, Moller G, Adamski J, Balling R (2002): Deletion of deoxyribonucleic acid binding domain of the vitamin D receptor abrogates genomic and nongenomic functions of vitamin D. *Mol Endocrinol.* 16:1524-37.

Evdokiou A, Raggatt L-J, Sakai T, Findlay DM (2000): Identification of a novel calcitonin-response element in the promoter of the human p21$^{WAF1/CIP1}$ gene. *J Mol Endocrinol.* 24:195-206.

Feldman D, Zhao X, Krishnan A (2000): Vitamin D and prostate cancer. *Endocrinology.* 141:5-9.

Fleet J, Eksir F, Hance K, Wood R (2002): Vitamin D-inducible calcium transport and gene expression in three Caco-2 cell lines. *Am J Physiol Gastrointest Liver Physiol.* 283:G618-625.

Force T, Bonventre J, Flannery M, Gorn A, Yamin M, Goldring S (1992): A cloned porcine renal calcitonin receptor couples to adenylyl cyclase and phospholipase C. *Am J Physiol.* 262:F1110-F1115.

Franceschi R, Romano P, Park K (1988): Regulation of type I collagen synthesis by 1,25-dihydroxyvitamin D3 in human osteosarcoma cells. *J Biol Chem.* 263:18938-18945.

Fraser D, Kodicek E (1973): Regulation of 25-hydroxycholecalciferol-1-hydroxylase activity in kidney by parathyroid hormone. *Nat New Biol.* 241:163-166.

Fu G, Lin D, Zhang M, Bikle D, Shackleton C, Miller W, Portale A (1997): Cloning of human 25-hydroxyvitamin D3-1α-hydroxylase and mutations causing vitamin D-dependent rickets type 1. *Mol Endocrinol.* 11:1961-1970.

Fukase M, Birge SJ, Rifas L, Avioli L, Chase L (1982): Regulation of 25 hydroxyvitamin D3 1-hydroxylase in serum-free monolayer culture of mouse kidney. *Endocrinology.* 110:1073-1075.

Galante L, Colston KW, S.J.Macauley, Macintyre I (1972): Effect of calcitonin on vitamin D Metabolism. *Nature.* 238:271-273.

Garcion E, Sindji L, Montero-Menei C, Andre C, Brachet P, Darcy F (1998): Expression of inducible nitric oxide synthase during rat brain inflammation: regulation by 1,25-dihydroxyvitamin D3. *Glia.* 22:282-94.

Garcion E, Wion-Barbot N, Montero-Menei C, Berger F, Wion D (2002): New clues about vitamin D functions in the nervous system. *Trends Endocrinol Metab.* 13:100-105.

Gascon-Barré M, Demers C, Ghrab O, Theodoropoulos C, Lapointe R, Jones G, Valiquette L, Ménard D (2001): Expression of CYP27A, a gene encoding a vitamin D-25 hydroxylase in human liver and kidney. *Clin Endocrinol.* 54:107-115.

Ge Y, Konrad MA, Matherly LH, Taub JW (2001): Transcriptional regulation of the human cystathionine beta-synthase -1b basal promoter: synergistic transactivation by transcription factors NF-Y and Sp1/Sp3. *Biochem J.* 357:97-105.

Glorieux F, St-Arnaud R (1998): Molecular cloning of (25-OHD)-1 alpha-hydroxylase: An approach to the understanding of vitamin D pseudo-deficiency. *Recent Prog Horm Res.* 53:341-349.

Gniadecki R, Gajkowska B, Hansen M (1997): 1,25-dihydroxyvitamin D3 stimulates the assembly of adherens junctions in keratinocytes: involvement of protein kinase C. *Endocrinology*. 138:2241-8.

Gorn A, Rudolph S, Flannery M, Morton C, Weremowicz S, Wang T, Krane S, Goldring S (1995): Expression of two human skeletal calcitonin receptor isoforms cloned from a giant cell tumour of bone. The first intracellular domain modulates ligand binding and signal transduction. *J Clin Invest*. 95:2680-2691.

Guzey M, Kitada S, Reed J (2002): Apoptosis induction by 1alpha,25-dihydroxyvitamin D3 in prostate cancer. *Mol Cancer Ther*. 1:667-77.

Hahn C, Kerry D, Omdahl J, May B (1994): Identification of vitamin D responsive elements in the promoter of the rat cytochrome p450 24 gene. *Nucleic Acids Res*. 22:2410-2417.

Henry H, Dutta C, Cunningham N, Blanchard R, Tang C, Marchetto G, Chou S (1992): The cellular and molecular regulation of $1,25(OH)_2D_3$ production. *J Steroid Biochem Mol Biol*. 41:401-407.

Henry H, Luntao E (1989): Interactions between intracellular signals involved in the regulation of 25-hydroxyvitamin D3 metabolism. *Endocrinology*. 124:2228-2234.

Henry HL (1985): Parathyroid hormone modulation of 25-hydroxyvitamin D_3 metabolism by cultured chick kidney cells is mimicked and enhanced by forskolin. *Endocrinology*. 116:503-510.

Henry HL (1997): The 25-hydroxyvitamin D 1 α-hydroxylase. In Feldman D, Glorieux FH, Pike JW (eds): "*Vitamin D*." London: Academic Press Limited, pp 57-68.

Hewison M, Zehnder D, Bland R, Stewart PM (2000): 1α-hydroxylase and the action of vitamin D. *J Mol Endocrinol*. 25:141-148.

Hisatake J-I, Kubota T, Hisatake Y, Uskokovic M, Tomoyasu S, Koeffler H (1999): 5,6 trans-16-ene-vitamin D3: a new class of potent inhibitors of proliferation of prostate, breast, and myeloid leukemic cells. *Cancer Res*. 59:4023-4029.

Ho H, Gilbert M, Nussenzveig D, Gershengorn M (1999): Glycosylation is important for binding to human calcitonin receptors. *Biochemistry*. 38:1866-1872.

Hoenderop J, Nilius B, Bindels. R (2002): Molecular mechanism of active Ca^{2+} reabsorption in the distal nephron. *Annu Rev Physiol*. 64:529-549.

Hsieh J-C, Jurutka PW, galligan MA, Terpening CM, Haussler CA, Samuels DS, Shimizu Y, Shimizu N, Haussler MR (1991): Human vitamin D receptor is selectively phosphorylated by protein kinase C on serine 51, a residue crucial to its trans-activation function. *Proc Natl Acad Sci USA*. 88:9315-9319.

Hsu J, Feldman D, McNeal J, Peehl D (2001): Reduced 1alpha-hydroxylase activity in human prostate cancer cells correlates with decreased susceptibility to 25-hydroxyvitamin D3-induced growth inhibition. *Cancer Res*. 61:2852-6.

Ikegame M, Rakopoulos M, Zhou H, Houssami S, Martin T, Moseley J, Findlay D (1995): Calcitonin receptor isoforms in mouse and rat osteoclasts. *J Bone Miner Res*. 10:59-65.

Inoue T, Kamiyama J, Sakai T (1999): Sp1 and NF-Y synergistically mediate the effect of vitamin D3 in the p27kip1 gene promoter that lacks vitamin D response elements. *J Biol Chem*. 274:32309-32317.

Inzerillo A, Zaidi M, Huang C (2002): Calcitonin: the other thyroid hormone. *Thyroid.* 12:791-798.

Issa LL, Leong GM, Eisman JA (1998): Molecular mechanism of vitamin D receptor action. *Inflamm Res.* 47:451-475.

Jackson S, Ericsson J, Osborne T, Edwards P (1995): NF-Y has a novel role in sterol-dependent transcription of two cholesterogenic genes. *J Biol Chem.* 270:21445-21448.

James S, Mackay A, Colston K (1996): Effects of 1,25 dihydroxyvitamin D3 and its analogues on induction of apoptosis in breast cancer cells. *J Steroid Biochem Mol Biol.* 58:395-401.

Jehan F, DeLuca H (1997): Cloning and characterization of the mouse vitamin D receptor promoter. *Proc Natl Acad Sci USA.* 94:10138-10143.

Jehan F, DeLuca H (2000): The mouse vitamin D receptor is mainly expressed through an Sp1-driven promoter *in vivo. Arch Biochem Biophys.* 377:273-283.

Jensen S, Madsen M, Lukas J, Binderup L, Bartek J (2001): Inhibitory effects of 1 alpha, 25-dihydroxyvitamin D(3) on the G(1)-S phase-controlling machinery. *Mol Endocrinol.* 15:1370-1380.

Jimenez-Lara AM, Aranda A (1999): Lysine 246 of the vitamin D receptor is crucial for ligand-dependent interaction with coactivators and transcriptional activity. *J Biol Chem.* 274:13503-13510.

Jones G, Strugnell SA, DeLuca. HF (1998): Current understanding of the molecular actions of vitamin D. *Physiol Rev.* 78:1193-1231.

Jurutka P, Hsieh J (1993): Phosphorylation of the human 1,25-dihydroxyvitamin D3 receptor by cAMP-dependent protein kinase, *in vitro,* and in transfected COS-7 cells. *Biochem Biophys Res Commun.* 191:1089-1096.

Jurutka PW, Hsieh J-C, Nakajima S, Haussler CA, Whitfield GK, Haussler MR (1996): Human vitamin D receptor phosphorylation by casein kinase II at Ser-208 potentiates transcriptional activation. *Proc Natl Acad Sci USA.* 93:3519-3524.

Kasuga H, Hosogane N, Matsuoka K, Mori I, Sakura Y, Shimakawa K, Shinki T, Suda T, Taketomi S (2002): Characterization of transgenic rats constitutively expressing vitamin D-24-hydroxylase gene. *Biochem Biophys Res Commun.* 297:1332-1338.

Kato S, Yoshizazawa T, Kitanaka S, Murayama A, Takeyama K (2002): Molecular genetics of vitamin D-dependent hereditary rickets. *Horm Res.* 57:73-78.

Kawashima H, Torikai S, Kurokawa K (1981): Calcitonin selectively stimulates 25-hydroxyvitamin D3-1α-hydroxylase in proximal straight tubule of rat kidney. *Nature.* 291:327-329.

Kerry DM, Dwivedi PP, Hahn CN, Morris HA, Omdahl JL, May BK (1996): Transcriptional synergism between vitamin D-responsive elements in the rat 25-hydroxyvitamin D3 24-hydroxylase(CYP24) promoter. *J Biol Chem.* 271:29715-29721.

Kifor O, Diaz R, Butters R, Brown E (1997): The Ca2+-sensing receptor (CaR) activates phospholipases C, A2, and D in bovine parathyroid and CaR-transfected, human embryonic kidney (HEK293) cells. *J Bone Miner Res.* 12:715-25.

Kifor O, MacLeod R, Diaz R, Bai M, Yamaguchi T, Yao T, Kifor I, Brown E (2001): Regulation of MAP kinase by calcium-sensing receptor in bovine parathyroid and CaR-transfected HEK293 cells. *Am J Physiol Renal Physiol.* 280:F291-302.

Kitanaka S, Takeyama K-I, Murayama A, Sato T, Okumura K, Nogami M, Hasegawa Y, Niimi H, Yanagisawa J, Tanaka T, Kato S (1998): Inactivating mutations in the 25-hydroxyvitamin D_3 1α-hydroxylase gene in patients with pseudovitamin D-deficiency rickets. *N Engl J Med.* 338:653-661.

Kitazawa R, Kitazawa S (2002): Vitamin D3 augments osteoclastogenesis via vitamin D-responsive element of mouse RANKL gene promoter. *Biochem Biophys Res Comm.* 290:650-655.

Kong XF, Zhu XH, Pei YL, Jackson DM, Holick MF (1999): Molecular cloning, characterization, and promoter analysis of the human 25-hydroxyvitamin D_3-1α-hydroxylase gene. *Proc Natl Acad Sci USA.* 96:6988-6993.

Kong Y, Yoshida H, Sarosi I, Tan H, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos A, Van G, Itie A, Khoo W, Wakeham A, Dunstan C, Lacey D, Mak T, Boyle W, Penninger J (1999): OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature.* 397:315-323.

Korkor A, Gray R, Henry H, Kleinman J, Blumenthal S, Garancis J (1987): Evidence that stimulation of $1,25(OH)_2D_3$ production in primary cultures of mouse kidney cells by cyclic AMP requires new protein synthesis. *J Bone Miner Res.* 2:517-24.

Krishnan A, Feldman D (1997): Regulation of vitamin D receptor abundance. In Feldman D, Glorieux F, Pike J (eds): *"Vitamin D."* London, UK: Academic Press, pp 179-200.

Krylov D, Olive M, Vinson C (1995): Extending dimerization interfaces: the bZIP basic region can form a coiled coil. *EMBO J.* 14:5329-37.

Kuestner R, Elrod R, Grant F, Hagen F, Kuijper J, Matthewes S, O'Hara P, Sheppard P, Stroop S, Thompson D, Whitmore T, Findlay D, Houssami S, Sexton P, Moore E (1994): Cloning and characterization of an abundant subtype of the human calcitonin receptor. *Mol Pharmacol.* 46:246-255.

Lacey D, Timms E, Tan H, Kelley M, Dunstan C, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian Y, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle W (1998): Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell.* 93:165-176.

Leeuwen JV, Pols H (1997): Vitamin D: Anticancer and differentiation. In Feldman D, Glorieux FH, Pike JW (eds): *"Vitamin D."* London, UK: Academic Press Limited, pp 1089-1105.

Lekstrom-Himes J, Xanthopoulos K (1998): Biological role of the CCAAT/Enhancer-binding protein family of transcription factors. *J Biol Chem.* 273:28545-28548.

Lemire J (1997): The role of vitamin D in immunosuppression: lessons from autoimmunity and transplantation. In Feldman D, Glorieux F, Pike J (eds): *"Vitamin D."* London, UK: Academic Press Limited, pp 1167-1181.

Li J, Sodek H (1993): Cloning and characterization of the rat bone sialoprotein gene promoter. *Biochem J.* 289:625-629.

Li Y, Amling M, Pirro A, Priemel M, Meuse J, Baron R, Delling G, Demay M (1998): Normalization of mineral ion homeostasis by dietary means prevents hyperparathyroidism, rickets, and osteomalacia, but not alopecia in vitamin D receptor-ablated mice. *Endocrinolgy.* 139:4391-6.

Liang F, Schaufele F, Gardner DG (2001): Functional interaction of NF-Y and Sp1 is required for type a natriuretic peptide receptor gene transcription. *J Biol Chem.* 276:1516-1522.

Lokeshwar B, Schwartz G, Selzer M, Burnstein K, Zhuang S, Block N, Binderup L (1999): Inhibition of prostate cancer metastasis *in vivo*: A comparison of 1,23-dihydroxyvitamin D (Calcitriol) and EB1089. *Cancer Epidemiol Biomarkers Prev.* 8:241-248.

Lowe K, Maiyar A, Norman A (1992): Vitamin D-mediated gene expression. *Crit Rev Eukaryot Gene Expr.* 2:65-109.

Lu Z, Jehan F, Zierold C, DeLuca H (2000): Isolation and characterization of the chicken vitamin D receptor gene and its promoter. *J Cell Biochem.* 77:92-102.

Mackey S, Heymont J, Kronenberg H, Demay M (1996): Vitamin D receptor binding to the negative human parathyroid hormone vitamin D response element does not require the retinoid X receptor. *Mol Endocrinol.* 10:298-305.

Majeski S, Skopinska M, Marczak M, Szmurlo A, Bollag W, Jablonska S (1996): Vitamin D is a potent inhibitor of tumor cell-induced angiogenesis. *J Invest Derm Sym Proc.* 1:97-101.

Makin G, Lohnes D, Byford V, Ray R, Jones G (1989): Target cell metabolism of 1,25-dihydroxyvitamin D3 to calcitroic acid. Evidence for a pathway in kidney and bone involving 24-oxidation. *Biochem J.* 262:173-180.

Makishima M, Lu T, Xie W, Whitfield G, Domoto H, Evans R, Haussler M, Mangelsdorf D (2002): Vitamin D receptor as an intestinal bile acid sensor. *Science.* 296:1313-1316.

Malloy P, Weisman Y, Feldman D (1994): Hereditary 1 apha-25-dihydroxyvitamin D-resistant rickets resulting from a mutation in the vitamin D receptor deoxyribonucleic acid-binding domain. *J Clin Endocrinol Metab.* 78:313-316.

Malloy P, Zhu W, Zhao X, Pehling G, Feldman D (2001): A novel inborn error in the ligand-binding domain of the vitamin D receptor causes hereditary vitamin D-resistant rickets. *Mol Genet Metab.* 73:138-148.

Mannstadt M, Juppner H, Gardella TJ (1999): Receptors for PTH and PTHrP: their biological importance and functional properties. *Am J Physiol.* 277:F665-F675.

Manolagas S, Yu X, Girasole G, Bellido T (1994): Vitamin D and the hematolymphopoietic tissue: a 1994 update. *Semin Nephrol.* 14:129-143.

Mantovani R (1999): The molecular biology of the CCAAT-binding factor NF-Y. *Gene.* 239:15-17.

Mantovani R, Li X, Pessara U, Hooft van Huisjduijnen R, Benoist C, Mathis D (1994): Dominant negative analogs of NF-YA. *J Biol Chem.* 269:20340-20346.

Mantovani R, Pessara U, Tronche F, Li X-Y, Knapp A-M, Pasquali J-L, Benoist C, Mathis D (1992): Monoclonal antibodies to NF-Y define its function in MHC class II and albumin gene transcription. *EMBO J.* 11:3315-3322.

Martin A, San-Antonio B, Fresno M (2001): Regulation of nuclear factor kappa B transactivation. Implication of phosphatidylinositol 3-kinase and protein kinase C zeta in c-Rel activation by tumor necrosis factor alpha. *J Biol Chem.* 276:15840-9.

Martin TJ (1999): Calcitonin, an update. *Bone*. 24:63S-65S.

Marziali G, Perrotti E, Ilari R, Coccia E, Mantovani R, Testa U, Battistani A (1999): The activity of the CCAAT-box binding factor NF-Y is modulated through the regulated expression of its A subunit during monocyte to macrophage differentiation: regulation of tissue-specific genes through a ubiquitous transcription factor. *Blood*. 93:519-526.

McLeod J, Cooke N (1989): The vitamin D-binding protein, α-fetoprotein, albumin multigene family: detection of transcripts in multiple tissues. *J Biol Chem*. 264:21760-21769.

Mendel C (1989): The free hormone hypothesis: a physiologically-based mathematical model. *Endocr Rev*. 10:232-274.

Messerlian S, Gao X, St-Arnaud R (2000): The 3-epi- and 24-oxo-derivatives of 1α, 25 dihydroxyvitamin D_3 stimulate transcription through the vitamin D receptor. *J Steroid Biochem Mol Biol*. 72:29-34.

Milanini-Mongiat J, Pouyssegur J, Pages G (2002): Identification of two p42/p44 mitogen activated kinases phosphorylation sites on Sp1: their implication in Vascular Endothelial Growth Factor gene transcription. *J Biol Chem*. 277:20631-20639

Miller WL, Portale AA (2000): Vitamin D 1α-hydroxylase. *Trends Endocrinol Metab*. 11:315-319.

Miyamoto K, Kesterson R, Yamamoto H, Taketani Y, Nishiwaki E, Tatsumi S, Inoue Y, Morita K, Takeda E, Pike J (1997): Structural organization of the human vitamin D receptor chromosomal gene and its promoter. *Mol Endocrinol*. 11:1165-1179.

Moallem E, Kilav R, Silver J, Naveh-Many T (1998): RNA protein binding and post-transcriptional regulation of parathyroid hormone gene expression by calcium and phosphate. *J Biol Chem*. 273:5253-5259.

Moffatt K, Johannes W, Hedlund T, Miller G (2001): Growth inhibitory effects of 1alpha, 25-dihydroxyvitamin D(3) are mediated by increased levels of p21in the prostatic carcinoma cell line ALVA-31. *Cancer Res*. 61:7122-7129.

Monkawa T, Yoshida T, Wakino S, Shinki T, Anazawa H, DeLuca HF, Suda T, Hayashi M, Saruta T (1997): Molecular cloning of cDNA and genomic DNA for human 25-hydroxyvitamin D3 1α-hydroxylase. *Biochem Biophys Res Commun*. 239:527-533.

Moore E, Kuestner R, Stroop S, Grant F, Matthewes S, Brady C, Sexton P, Findlay D (1995): Functionally different isoforms of the human calcitonin receptor result from alternative splicing of the gene transcript. *Mol Endocrinol*. 9:959-968.

Morelli S, Boland R, de Boland AR (1996): $1\alpha,25(OH)_2$-vitamin D_3 stimulation of phospholipases C and D in muscle cells involves extracellular calcium and a pertussis-sensitive G protein. *Mol Cell Endocrinol*. 122:207-211.

Morelli S, Buitrago C, Vazquez G, de Boland AR, Boland R (2000): Involvement of tyrosine kinase activity in $1\alpha,25(OH)_2$-vitamin D_3 signal transduction in skeletal muscle cells. *J Biol Chem*. 275:36021-36028.

Morimoto S, Kumahara Y (1985): A patient with psoriasis cured by 1α-hydroxyvitamin D3. *Med J* Osaka Univ. 35:51-54.

Murayama A, Kim M, Unno K, Takeyama K, Kato S (2001): A novel mechanism of the vitamin D dependent transcriptional repression through the human 25-hydroxyvitamin D3 1 alpha-hydroxylase in nVDRE. *J Bone Miner Res.* 16:S229 (Abstract).

Murayama A, Takeyama K, Asahina T, Kitanaka S, Kato S (2000): Cloning of a novel transcription factor mediating the negative vitamin D responsiveness through the human 25-hydroxyvitamin D3 1α-hydroxylase nVDRE. *J Bone Mineral Res.* 15:S199 (Abstract).

Murayama A, Takeyama K, Kitanaka S, Kodera Y, Hosoya T, Kato S (1998): The promoter of the human 25-hydroxyvitamin D_3 1α-hydroxylase gene confers positive and negative responsiveness to PTH, calcitonin, and 1alpha,25(OH)$_2$D$_3$. *Biochem Biophys Res Commun.* 249:11-16.

Murayama A, Takeyama K, Kitanaka S, Kodera Y, Kawaguchi Y, Hosoya T, Kato S (1999): Positive and negative regulations of the renal 25-hydroxyvitamin D_3 1α-hydroxylase gene by parathyroid hormone, calcitonin, and 1α,25(OH)$_2$D$_3$ in intact animals. *Endocrinology.* 140:2224-2231.

Naro F, Perez M, Migliaccio S, Galson D, Orcel P, Teti A, Goldring S (1998): Phospholipase D- and protein kinase C isoenzyme-dependent signal transduction pathways activated by the calcitonin receptor. *Endocrinology.* 139:3241-3248.

Naveilhan P, Neveu I, Baudet C, Ohyama K, Brachet P, Wion D (1993): Expression of 25(OH) vitamin D_3 24-hydroxylase gene in glial cells. *Neuroreport.* 5:255-7.

Nemere I, Dormanen M, Hammond M, Okamura W, Norman A (1994): Identification of a specific binding protein for 1α,25-dihydroxyvitaminD3 in basal-lateral membranes of chick intestinal epithelium and relationship to transcaltachia. *J Biol Chem.* 269:23750-23756.

Nemere I, Schwartz Z, Pedrozo H, Sylvia V, Dean D, Boyan B (1998): Identification of a membrane receptor for 1,25-dihydroxyvitamin D_3 which mediates rapid activation of protein kinase C. *J Bone Miner Res.* 13:1353-1359.

Nishimura A, Shinki T, Jin C, Ohyama Y, Noshiro M, Okuda K, Suda T (1994): Regulation of messenger ribonucleic acid expression of 1 alpha,25-dihydroxyvitamin D_3-24-hydroxylase in rat osteoblasts. *Endocrinology.* 143:1794-1799.

Noda M, Vogel R, Craig A, Prahl J, DeLuca H, Denhardt D (1990): Identification of a DNA sequence responsible for binding of the 1,25-dihydroxyvitamin D3 receptor and 1,25-dihydroxyvitamin D3 enhancement of mouse secreted phosphoprotein 1 (Spp1-1 or osteopontin) gene expression. *Proc Natl Acad Sci USA.* 87:9995-9999.

Nolte R, Wisely G, Westin S, Cobb J, Lambert M, Kurokawa R, Rosenfeld M, Willson T, Glass C, Milburn M (1998): Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-gamma. *Nature.* 395:137-143.

Norman A, Bishop J, Bula C, Olivera C, Mizwicki M, Zanello L, Ishida H, Okamura W (2002): Molecular tools for study of genomic and rapid signal transduction responses initiated by 1α,25(OH)2-vitaminD3. *Steroids.* 67:457-466.

Ohyama Y, Hayashi S, Okuda K (1989): Purification of 25-hydroxyvitamin D_3 24-hydroxylase from rat kidney mitochondria. *FEBS lett.* 255:405-408.

Ohyama Y, Noshiro M, Eggertsen G, Gotoh O, Bjorkhem I, Okuda K (1992): Structural characterization of the gene encoding rat 25-hydroxyvitamin D3 24-hydroxylase. *Biochemistry.* 32:76-82.

Ohyama Y, Noshiro M, Okuda K (1991): Cloning and expression of cDNA encoding 25-hydroxyvitmin D3 24-hydroxylase. *FEBS Lett.* 278:195-198.

Ohyama Y, Okuda K (1991): Isolation and characterization of a cytochrome P-450 from rat kidney mitochondria that catalyzes the 24-hydroxylation of 25-hydroxyvitamin D3. *J Biol Chem.* 266:8690-8695.

Omdahl J, May B (1997): 24-hydroxylase. In Feldman D, Glorieux FH, Pike JW (eds): "*Vitamin D.*" London, UK: Academic Press Limited, pp 69-85.

Omdahl JL, Bobrovnikova EA, Choe S, Dwivedi PP, May BK (2001): Overview of regulatory cytochrome P450 enzymes of the vitamin D pathway. *Steroids.* 66:381-389.

Omdahl JL, Morris HA, May BK (2002): Hydroxylase enzymes of the vitamin D pathway: Expression, function, and regulation. *Annu Rev Nutr.* 22:139-166.

Ono T, Tanaka H, Yamate T, Nagai Y, Nakamura T, Seino Y (1996): 24R,25-dihydrovitamin D3 promotes bone formation without causing excessive resorption in hypophosphatemic mice. *Endocrinology.* 137:2633-2637.

Osawa H, Robey R, Printz R, Granner D (1996): Identification and characterization of basal and cyclic AMP response elements in the promoter of the rat hexokinase II gene. *J Biol Chem.* 271:17296-17303.

Overbergh L, Decallonne B, Valckx D, Verstuyf A, Depovere J, Laureys J, Rutgeerts O, Saint-Arnaud R, Bouillon R, Mathieu C (2000): Identification and immune regulation of 25-hydroxyvitamin D-1-alpha-hydroxylase in murine macrophages. *Clin Exp Immunol.* 120:139-146.

Pal S, Claffey K, Cohen H, Mukhopadhyay D (1998): Activation of Sp1-mediated vascular permeability factor/vascular endothelial growth factor transcription requires specific interaction with protein kinase C zeta. *J Biol Chem.* 273:26277-26280.

Pal S, Datta K, Khosravi-Far R, Mukhopadhyay D (2001): Role of protein kinase Czeta in Ras-mediated transcriptional activation of vascular permeability factor/vascular endothelial growth factor expression. *J Biol Chem.* 276:2395-2403.

Palmer H, Gonzalez-Sancho J, Espada J, Berciano M, Puig I, Baulida J, Quintanilla M, Cano A, Herreros Ad, Lafarga M, Munoz A (2001): Vitamin D(3) promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of beta-catenin signaling. *J Cell Biol.* 154:369-387.

Panda DK, Miao D, Tremblay ML, Sirois J, Farookhi R, Hendy GN, Goltzman D (2001a): Targeted ablation of the 25-hydroxyvitamin D 1alpha -hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction. *Proc Natl Acad Sci USA.* 98:7498-7503.

Panda DK, Kawas SA, Seldin MF, Hendy GN, Goltzman D (2001b): 25-hydroxyvitamin D 1α-hydroxylase: structure of the mouse gene, chromosomal assignment, and developmental expression. *J Bone Mineral Res.* 16:46-56.

Paumelle R, Tulasne D, Kherrouche Z, Plaza S, Leroy C, Reveneau S, Vandenbunder B, Fafeur V, Tulashe D, Reveneau S (2002): Hepatocyte growth factor/scatter factor activates the ETS1 transcription factor by a RAS-RAF-MEK-ERK signaling pathway. *Oncogene* 21:2309-19.

Pearman AT, chou W, Bergman K, Pulumati M, Partridge N (1996): Parathyroid hormone induces c-fos promoter activation in osteoblastic cells through phosphorylated cAMP response element (CRE)-binding protein binding to the major CRE. *J Biol Chem.* 271:25715-25721.

Pedrozo HA, Boyan BD, Mazock J, Dean DD, Gomez R, Schwartz Z (1999): TGFβ1 regulates 25-hydroxyvitmain D3 1α- and 24-hydroxylase activity in cultured growth plate chondrocytes in a maturation - dependent manner. *Calcif Tissue Int.* 64:50-56.

Pike J (1997): The vitamin D receptor and its gene. In Feldman D, Glorieux FH, Pike JW (eds): *"Vitamin D."* London: Academic Press Limited, pp 105-125.

Polly P, Herdick M, Moehren U, Baniahmad A, Heinzel T, Carlberg C (2000): VDR-Alien: a novel, DNA-selective vitamin D(3) receptor-corepressor partnership. *FASEB J.* 14:1455-63.

Pols H, Schilte H, Herrmann-Erlee N, Visser T, Birkenhager J (1986): The effect of 1,25-dihydroxyvitamin D3 on growth, alkaline phosphatase and adenylate cyclase of rat osteoblast-like cells. *Bone Miner.* 1:397-405.

Pondel M (2000): Calcitonin and calcitonin receptors: bone and beyond. *Int J Exp Path.* 81:405-422.

Rachez C, Freedman L (2000): Mechanism of gene regulation by vitamin D3 receptor: a network of coactivator interactions. *Gene.* 246:9-21.

Rafty L, Khachigian L (2001): Sp1 phosphorylation regulates inducible expression of platelet-derived growth factor B-chain gene via atypical protein kinase C-zeta. *Nucleic Acids Res.* 29:1027-1033.

Raggatt L, Evdokiou A, Findlay D (2000): Sustained activation of Erk1/2 MAPK and cell growth suppression by the insert-negative, but not the insert-positive isoform of the human calcitonin receptor. *J Endocrinol.* 167:93-105.

Rasmussen H, Wong M, Bikle D, Goodman D (1972): Hormonal control of the renal conversion of 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol. *J Clin Invest* 51:2502-2504.

Reddy G, Muralidharan K, Okamura W, Tserng K, McLane J (1994): Metabolism of 1α,25-dihydroxyvitamin D3 and one of its A-ring diastereomer 1α,25-dihydroxy-3-epivitamin D3 in neonatal human keratinocytes. In Norman A, Bouillon R, Thomasset M (eds): *"Vitamin D: A pluripotent steroid hormone: Structural studies, molecular endocrinology and clinical applications."* New York: Walter de Gruyter, pp 172-173.

Renaud J, Rochel N, Ruff M, Vivat V, Chambon P, Gronemeyer H, Moras D (1995): Crystal structure of the RAR-gamma ligand-binding domain bound to all-trans retinoic acid. *Nature.* 378:681-689.

Rigby W, Denome S, Fanger M (1987): Regulation of lymphokine production and human T lymphocyte activation by 1,25-dihydroxyvitamin D3. Specific inhibition at the level of messenger RNA. *J Clin Invest.* 79:1659-1664.

Ritchie H, Hughes M, Thompson E, Malloy P, Hochberg Z, Feldman D, Pike J, O'Malley B (1989): An ochre mutation in the vitamin D receptor gene causes hereditary 1,25-dihydroxyvitamin D3-resistant rickets in three families. *Proc Natl Acad Sci USA.* 86:9783-9787.

Rochel N, Wurtz J, Mitschler A, Klaholz B, Moras D (2000): The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand. *Mol Cell.* 5:173-179.

Roder K, Wolf SS, Larkin KJ, Schweizer M (1999): Interaction between the two ubiquitously expressed transcription factors NF-Y and Sp1. *Gene.* 234:61-69.

Rodriguez-Viciana P, Warne P, Dhand R, Vanhaesebroeck B, Gout I, Fry M, Waterfield M, Downward J (1994): Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature.* 370:508-509.

Rodriguez-Viciana P, Warne P, Vanhaesebroeck B, Waterfield M, Downward J (1996): Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation. *EMBO J.* 15:2442-51.

Rosen H, Reshef A, Maeda N, Lippoldt A, Shpizen S, Triger L, Eggertsen G, Bjorkhem I, Leitersdorf E (1998): Markedly reduced bile acid synthesis but maintained levels of cholesterol and vitamin D metabolites in mice with disrupted sterol 27-hydroxylase gene. *J Biol Chem.* 273:14805-12.

Rut A, Hewison M, Kristjansson K, Luisi B, Hughes M, O'Riordan J (1994): Two mutations causing vitamin D resistant rickets: Modelling on the basis of steroid hormone receptor DNA-binding domain crystal structures. *Clin Endocrinol.* 41:581-590.

Safadi F, Thornton P, Magiera H, Hollis B, Gentile M, Haddad J, Liebhaber S, Cooke N (1999): Osteopathy and resistance to vitamin D toxicity in mice null for vitamin D binding protein. *J Clin Invest.* 103:239-251.

Sakai Y, Demay M (2000): Evaluation of keratinocyte proliferation and differentiation in vitamin D receptor knockout mice. *Endocrinology.* 141:2043-2049.

Sakai Y, Kishimoto J, Demay M (2001): Metabolic and cellular analysis of alopecia in vitamin D receptor knockout mice. *J Clin Invest.* 107:961-966.

Sambrook J, Fritsch EF, Maniatis T (1989): "Molecular Cloning- A Laboratory Manual." New York: Cold Spring Harbor Laboratory.

Santoro C, Mermod N, Andrews P, Tjian R (1988): A family of human CCAAT-box-binding proteins active in transcription and DNA replication: cloning and expression of multiple cDNAs. *Nature.* 334:218-224.

Sawada N, Sakaki T, Ohta M, Inouye K (2000): Metabolism of vitamin D(3) by human CYP27A1. *Biochem Biophys Res Commun.* 273:977-984.

Scaglione-Sewell B, Bissonnette M, Skarosi S, Abraham C, Brasitus T (2000): A vitamin D3 analog induces a G1-phase arrest in CaCo-2 cells by inhibiting cdk2 and cdk6: roles of cyclin E, p21Waf1, and p27Kip1. *Endocrinology* 141:3931-9.

Segersten U, Correa P, Hewison M, Hellman P, Dralle H, Carling T, Akerstrom G, Westin G (2002): 25-hydroxyvitamin D3-1 alpha-hydroxylase expression in normal and pathological parathyroid glands. *J Clin Endocrinol Metab.* 87:2967-2972.

Selvamurugan N, Chou W-Y, Pearman AT, Pulumatii MR, Partridge NC (1998): Parathyroid hormone regulates the rat collagenase-3 promoter in osteoblastic cells through the cooperative interaction of the activator protein-1 site and the runt domain binding sequence. *J Biol Chem.* 273:10647-10657.

Seo E, Einhorn T, Norman A (1997): An essential vitamin D metabolite for both normal bone integrity and healing of tibial fracture in chicks. *Endocrinolgy.* 138:3864-3872.

Seo E, Norman A (1997): Three-fold induction of renal 25-hydroxyvitaminD3 -24-hydroxylase activity and increased serum 24,25-dihydroxyvitamin D3 levels are correlated with the healing process after chick tibial fracture. *J Bone Miner Res.* 12:598-606.

Sexton P, Findlay D, Martin T (1999): Calcitonin. *Curr Med Chem.* 6:1067-1093.

Shaffer P, Gewirth D (2002): Structural basis of VDR-DNA interactions on direct repeat response elements. *EMBO J* 21:2242-52.

Shao A, Wood R, Fleet J (2001): Increased vitamin D receptor level enhances 1,25-dihydroxyvitamin D-3-mediated gene expression and calcium transport in Caco-2 cells. *J Bone Miner Res.* 16:615-624.

Shetty S, Takahashi T, Matsui H, Ayengar R, Raghow R (1999): Transcriptional autorepression of Msx1 gene is mediated by interactions of Msx1 protein with a multi-protein transcriptional complex containing TATA-binding protein, Sp1 and cAMP-response-element-binding protein-binding protein (CBP/p300). *Biochem J.* 339:751-758.

Shigematsu T, Horiuchi N, Ogura Y, Miyahara T, Suda T (1986): Human parathyroid hormone inhibits renal 24-hydroxylase activity of 25-hydroxyvitamin D_3 by a mechanism involving adenosine 3',5'-monophosphate in rats. *Endocrinology.* 118:1583-1589.

Shinki T, Jin C, Nishimura A, Nagai Y, Ohyama Y, Noshiaro M, Okuda K, Suda T (1992): Parathyroid hormone inhibits 25-hydroxyvitamin D_3-24-hydroxylase mRNA expression stimulated by 1α,25-dihydroxyvitami D_3 in rat kidney but not in intestine. *J Biol Chem.* 267:13757-13762.

Shinki T, Shimada H, Wakino S, Anazawa H, Hayashi M, Saruta T, DeLuca H, Suda T (1997): Cloning and expression of rat 25-hydroxyvitamin D_3-1α-hydroxylase cDNA. *Proc Natl Acad Sci USA.* 94:12920-12925.

Shinki T, Ueno Y, Deluca HF, Suda T (1999): Calcitonin is a major regulator for the expression of renal 25-hydroxyvitamin D_3-1α-hydroxylase gene in normocalcemic rats. *Proc Natl Acad Sci USA.* 96:8253-8258.

Shinpo K, Kikuchi S, Sasaki H, Moriwaka F, Tashiro K (2000): Effect of 1,25-dihydroxyvitamin D(3) on cultured mesencephalic dopaminergic neurons to the combined toxicity caused by L-buthionine sulfoximine and 1-methyl-4-phenylpyridine. *J Neurosci Res.* 62:374-82.

Shyu J-F, Inoue D, Baron R, Horne WC (1996): The deletion of 14 amino acids in the seventh transmembrane domain of a naturally occurring calcitonin receptor isoform alters ligand binding and selectively abolishes coupling to phospholipase C. *J Biol Chem.* 271:31127-31134.

Siu-Caldera M, Zou L, Ehrlich M, Schwartz E, Ishizuka S, Reddy G (1995): Human osteoblasts in culture metabolize both 1 alpha, 25-dihydroxyvitamin D3 and its precursor 25-hydroxyvitamin D3 into their respective lactones. *Endocrinology.* 136:4195-4203.

Siu-Caldera ML, Sekimoto H, Weiskopf A, Vouros P, Muralidharan KR, Okamura WH, Bishop J, Norman AW, Uskokovic MR, Schuster I, Reddy GS (1999): Production of 1α,25-dihydroxy-3-epi-vitamin D3 in two rat osteosarcoma cell lines (UMR106 and ROS17/2.8): existence of the C-3 epimerization pathway in ROS17/2.8 cells in which the C-24 oxidation pathway is not expressed. *Bone.* 24:457-463.

Skjodt H, Gallagher J, Beresford J, Couch M, Poser J, Russell R (1985): Vitamin D metabolites regulate osteocalcin synthesis and proliferation of human bone cells *in vitro. J Endocrinol.* 105:391-396.

Skowronski R, Peehl D, Feldman D (1993): Vitamin D and prostate cancer: 1,25 dihydroxyvitamin D3 receptors and actions in human prostate cancer cell lines. *Endocrinology.* 132:1952-60.

Soh J, Lee E, Prywes R, Weinstein I (1999): Novel roles of specific isoforms of protein kinase C in activation of the c-fos serum response element. *Mol Cell Biol.* 19:1313-24.

Solomon C, White J, Kremer R (1999): Mitogen-activated protein kinase inhibits 1,25-dihydroxyvitamin D3-dependent signal transduction by phosphorylating human retinoid X receptor α. *J Clin Invest.* 103:1729-1735.

St-Arnaud R (1999): Targeted inactivation of vitamin D hydroxylases in mice. *Bone.* 25:127-129.

St-Arnaud R, Arabian A, Travers R, Barletta F, Raval-pandya M, Chapin K, Depovere J, Mathieu C, Christakos S, Demay MB, Glorieux FH (2000): Deficient mineralization of Intramembranous bone in vitamin D-24-hydroxylase-ablated mice is due to elevated 1,25-dihydroxyvitamin D and not to the absence of 24,25-dihydroxyvitamin D. *Endocrinology.* 141:2658-2666.

St-Arnaud R, Glorieux FH (1997): Vitamin D and bone development. In Feldman D, Glorieux FH, Pike JW (eds): *"Vitamin D."* London, UK: Academic press, pp 293-303.

St-Arnaud R, Messerlian S, Moir JM, Omdahl JL, Glorieux FH (1997): The 25-hydroxyvitamin D 1-alpha-hydroxylase gene maps to the pseudovitamin D-deficiency rickets (PDDR) disease locus. *J Bone Miner Res.* 12:1552-1559.

Suda T, Takahashi N (1997): Vitamin D and osteoclastogenesis. In Feldman D, Glorieux FH, Pike JW (eds): *"Vitamin D."* London: Academic Press Limited, pp 329-340.

Sunn K, Cock T, Crofts L, Eisman J, Gardiner E (2001): Novel N-terminal variant of human VDR. *Mol Endocrinol.* 15:1599-1609.

Suske G (1999): The Sp-family of transcription factors. *Gene.* 238:291-300.

Suzuki T, Kimura A, Nagai R, Horikoshi M (2000): Regulation of interaction of the acetyltransferase region of p300 and the DNA-binding domain of Sp1 on and through DNA binding. *Genes Cells.* 5:29-41.

Swarthout J, D'Alonzo R, Selvamurugan N, Partridge N (2002): Parathyroid hormone-dependent signaling pathways regulating genes in bone cells. *Gene.* 282:1-17.

Takeyama K, Kitanaka S, Sato T, Kobori M, Yanagisawa J, Kato S (1997): 25-hydroxyvitamin D3 1α-hydroxylase and vitamin D synthesis. *Science.* 277:1827-1830.

Tanaka Y, DeLuca HF (1983): Stimulation of 1,25-dihydroxyvitamin D3 production by 1,25-dihydroxyvitamin D3 in the hypocalcaemic rat. *Biochem J.* 214:893-897.

Tanaka Y, DeLuca HF (1984): Rat renal 25-hydroxyvitamin D3 1- and 24-hydroxylases: their *in vivo* regulation. *Am J Physiol* 246:E168-E173.

Tanese N, Saluja D, Vassallo M, Chen J, Admon A (1996): Molecular cloning and analysis of two subunits of the human TFIID complex: hTAFII130 and hTAFII100. *Proc Natl Acad Sci USA.* 93:13611-13616.

Thys-Jacobs S, Chan F, Koberle L, Bilezikian J (1997): Hypercalcemia due to vitamin D toxicity. In Feldman D, Glorieux FH, Pike JW (eds): *"Vitamin D."* London, UK: Academic Press Limited, pp 883-901.

Toell A, Polly P, Carlberg C (2000): All natural DR3-type vitamin D response elements show a similar functionality *in vitro*. *Biochem J.* 352:301-309.

Valor LM, Campos-Caro A, Carrasco-Serrano C, Ortiz JA, Ballesta JJ, Criado M (2002): Transcription factors NF-Y and Sp1 are important determinants of the promoter activity of the bovine and human neuronal nicotinic receptor β4 subunit genes. *J Biol Chem.* 277:8866-8876.

Van Cromphaut S, Dewerchin M, Hoenderop J, Stockmans I, Van Herck E, Kato S, Bindels R, Collen D, Carmeliet P, Bouillon R, Carmeliet G (2001): Duodenal calcium absorption in vitamin D receptor-knockout mice: functional and molecular aspects. *Proc Natl Acad Sci* USA 98:13324-13329.

van den Bemd GJ, Chang GT (2002): vitamin D and vitamin D analogs in cancer treatment. *Curr Drug Targets*. 3:85-94.

Vanhaesebroeck B, Alessi D (2000): The PI3K-PDK1 connection: more than just a road to PKB. *Biochem J*. 346:561-576.

Vasseur S, Mallo GV, Garcia-Montero A, Ortiz EM, Fiedler F, Canepa E, Moreno S, Iovanna JL (1999): Structural and functional characterization of the mouse p8 gene: promotion of transcription by the CAAT-enhancer binding protein α(C/EBPα) and C/EBPβ trans-acting factors involves a C/EBP *cis*-acting element and other regions of the promoter. *Biochem J*. 343:377-383.

Vazquez G, De Boland A, Boland R (1997): 1 alpha, 25-$(OH)_2$ vitamin D_3 stimulates the adenylyl cyclase pathway in muscle cells by a GTP-dependent mechanism which presumably involves phosphorylation of G alpha i. *Biochem Biophys Res Commun*. 234:125-128.

Verheijen MH, Defize LH (1997): Parathyroid hormone activates mitogen-activated protein kinase via a cAMP-mediated pathway independent of Ras. *J Biol Chem*. 272:3423-3429.

Walters M (1992): Newly identified actions of the vitamin D endocrine system. *Endocr Rev*. 13:719-764.

Wang Q, Luo X, Studzinski G (2000): Cyclin-dependent kinase 6 is the principal target of p27/Kip1 regulation of the G1-phase traverse in 1,25-dihydroxyvitamin D3-treated HL60 cells. *Cancer Res*. 57:2851-5.

Wang X, Zhang M, Miller W, Portale A (2002): Novel gene mutations in patients with 1 alpha-hydroxylase deficiency that confer partial enzyme activity *in vitro*. *J Clin Endocrinol Metab*. 87:2424-2430.

Wang Y, Chiang Y, Su T, Hayashi T, Morales M, Hoffer B, Lin S (2000): Vitamin D(3) attenuates cortical infarction induced by middle cerebral arterial ligation in rats. *Neuropharmacology*. 39:873-80.

White P, Cooke N (2000): The multifunctional properties and characteristics of vitamin D-binding protein. *TEM*. 11:320-327.

Whitlatch L, Young M, Schwartz G, Flanagan J, Burnstein K, Lokeshwar B, Rich E, Holick M, Chen T (2002): 25-Hydroxyvitamin D-1alpha-hydroxylase activity is diminished in human prostate cancer cells and is enhanced by gene transfer. *J Steroid Biochem Mol Biol*. 81:135-40.

Wikvall K (2001): Cytochrome P450 enzymes in the bioactivation of vitamin D to its hormonal form. *Int J Mol Med*. 7:201-209.

Wu G, Fan R, Li W, Ko T, Brattain M (1997): Modulation of cell cycle control by vitamin D3 and its analogue, EB 1089, in human breast cancer cells. *Oncogene*. 15:1555-1563.

Wu S, Finch J, Zhong M, Slatopolsky E, Grieff M, Brown A (1996): Expression of the renal 25-hydroxyvitamin D-24-hydroxylase gene: regulation by dietary phosphate. *Am J Physiol Renal Physiol*. 271:F203-208.

Xia L, Zhang Y (2001): Sp1 and Ets family transcription factors regulate the mouse Mta2 gene expression. *Gene*. 268:77-85.

Xie Z, Munson SJ, Huang N, Schuster I, Portale AA, Miller WL, Bikle DD (2002): The mechanism of 1,25-dihydroxyvitamin D3 auto-regulation in keratinocytes. *J Biol Chem.* 277:32516-32526.

Yamada K, Tanaka T, Miyamoto K, Noguchi T (2000): Sp family members and nuclear factor-Y cooperatively stimulate transcription from the rat pyruvate kinase M gene distal promoter region via their direct interactions. *J Biol Chem.* 275:18129-18137.

Yang C, Shapiro L, Rivera M, Kumar A, Brindle P (1998): A role for CREB binding protein and p300 transcriptional coactivators in Ets-1 transactivation functions. *Mol Cell Biol.* 18:2218-2229.

Yang W, Friedman P, Kumar R, Omdahl J, May B, Siu-Caldera M, reddy G, Christakos S (1999): Expression of 25(OH)D$_3$ 24-hydroxylase in distal nephron: coordinate regulation by 1,25(OH)$_2$D$_3$ and cAMP or PTH. *Am J Physiol.* 276:E793-E805.

Ylikomi T, Laaksi I, Lou Y, Martikainen P, Miettinen S, Pennanen P, Purmonen S, Syvala H, Vienonen A, Tuohimaa P (2002): Antiproliferative action of vitamin D. *Vitam Horm.* 64:357-406.

Yoshida N, Yoshida T, Nakamura A, Monkawa T, Hayashi M, Saruta T (1999): Calcitonin induces 25-hydroxyvitamin D3 1alpha -hydroxylase mRNA expression via protein kinase C pathway in LLC-PK1 cells. *J Am Soc Nephrol.* 10:2474-2479.

Yoshida T, Monkawa T, Tenenhouse H, Goodyer P, Shinki T, Suda T, Wakino S, Hayashi M, Saruta T (1998): Two novel 1α-hydroxylase mutations in French-Canadians with vitamin D dependency rickets type I. *Kidney Int.* 54:1437-1443.

Yoshida T, Yoshida N, Monkawa T, Hayashi M, Saruta T (2001): Dietary phosphorus deprivation induces 25-hydroxyvitamin D(3) 1alpha-hydroxylase gene expression. *Endocrinology.* 142:1720-1726.

Yoshida T, Yoshida N, Nakamura A, Monkawa T, Hayashi M, Saruta T (1999): Cloning of porcine 25-hydroxyvitamin D3 1 α-hydroxylase and its regulation by cAMP in LLC-PK1 cells. *J Am Soc Nephrol.* 10:963-970.

Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, Kawakami T, Arioka K, Sato H, Uchiyama Y, Masushige S, Fukamizu A, Matsumoto T, Kato S (1997): Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. *Nat Genet.* 16:391-396.

Zehnder D, Bland R, Walker E, Bradwell A, Howie A, Hewison M, Stewart P (1999): Expression of 25-hydroxy-vitamin D3-1α hydroxylase in the human kidney. *J Am Soc Nephrol.* 10:2465-2473.

Zehnder D, Bland R, Williams M, McNinch R, Howie A, Stewart P, Hewison M (2001): Extrarenal expression of 25-hydroxyvitamin D-3-1 alpha-hydroxylase. *J Clin Endocrinol Metab.* 86:888-894.

Zhen X, Wei L, Wu Q, Zhang Y, Chen Q (2001): Mitogen-activated protein kinase p38 mediates regulation of chondrocyte differentiation by parathyroid hormone. *J Biol Chem.* 276:4879-4885.

Zhong Z, Hammani K, Bae W, DeClerck Y (2000): NF-Y and Sp1 cooperate for the transcriptional activation and cAMP response of human tissue inhibitor of metalloproteinases-2. *J Biol Chem.* 275:18602-18610.

Zierold C, Darwish H, DeLuca H (1995): Two vitamin D response elements function in the rat 1,25-dihydroxyvitamin D 24-hydroxylase promoter. *J Biol Chem.* 270:1675-1678.

Zierold C, Mings J, DeLuca H (2001): Parathyroid hormone regulates 25-hydroxyvitamin D3-24-hydroxylase mRNA by altering its stability. *Proc Natl Acad Sci USA.* 98:13572-13576.

Zierold C, Reinholz G, Mings J, Prahl J, DeLuca H (2000): Regulation of the porcine 1,25-dihydroxyvitamin D3-24-hydroxylase (CYP24) by 1,25-dihydroxyvitamin D3 and parathyroid hormone in AOK-B50 cells. *Arch Biochem Biophys.* 381:323-327.

Addendum

1. **Chapter 3, p65**: It should be noted that AOK-B50 cells were treated with H89 (30µM), calphostin C (0.5µM), PD98059 (10µM) or SB203580 (10µM) prior to PTH treatment.

2. **Chapter 3, p66**: The EMSA-based conclusions in this chapter should be considered preliminary until EMSA experiments with extracts from PTH-treated cells are undertaken.