



**Regulation of the 25-Hydroxyvitamin D<sub>3</sub> 24-Hydroxylase  
(CYP24) gene promoter expression by a novel binding site in  
response to 1,25(OH)<sub>2</sub>D<sub>3</sub>**

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

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**August 2003**

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## DECLARATION

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To the best of my knowledge, this thesis contains no material that has been previously published by any other person, except where due reference has been made in the text.

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Signature.

Date.....01/08/03.....

**Josef S Kaplan**

## ACKNOWLEDGEMENTS

I am forever in debt to Professor Peter Rathjen, Associate Professor Barry Egan and Associate Professor Brian K May for permission to undertake a Ph.D. in the Department of Biochemistry which is of a high standard of leadership, commitment, and quality of work, but also fun.

There are no words to describe the thanks that go out to the BKM lab. Brian, your valuable contribution to my project will always be warmly remembered, as will your tireless support of my writing. Prem, thank-you for your time, guidance and technical advice you have taught me over the past few years. Remember Prem, the EBS will never be as strong as the CBS. To past members of our lab, Tim, Satish, and Ingrid, thank-you for all your valuable discussions to science, related and unrelated topics, and for just being there during the ups and downs of during my Ph.D. To my fellow Ph.D. students Tania and Sophia. Well Tania, what can I say, the lab hasn't been the same without you. You were always there organising lab functions, bringing cake and "on the phone". Your presence always brought a smile to our faces. We have missed you dearly and it was fun working with you. To Sophia, I don't know anyone else who was as excited as her whenever she heard any kind of news, exclaiming "Oh really". I have enjoyed your friendship and working with you also in the lab. Thank-you also for all your help with my thesis preparations. I wish you, XJ and Cheryl every success for the future. To other past and present honour students of the lab. It has been a fun and exciting experience to help guide you guys through one of your toughest years of your life. To Barbara, I've enjoyed your friendship and working with you also. Who knew that someone in the lab as quiet and shy as you would be performing live stage shows in front of huge audiences. Thank-you for all your help concerning reading and editing of some chapters for my thesis and may the future bring you good luck and fortune. A big thank-you goes out to the entire staff and students of the Biochemistry department. Thank-you for all your support, friendship and the pleasant working environment over the past few years. A special thanks must also go out the TC staff who over the years have not only provided glassware and reagents, but also a friendly place to go and have a chat.

I must also say a special thanks to my mum Eva, and my brother John, for their continuous love, support, encouragement and understanding throughout the course of my studies. Well guys, its finally all over.

To my wife, Sandra. I don't know if words will ever describe how much I have appreciated your love and support, throughout this long haul. You've managed to stay by my side, and in the process become my wife during the ups and downs of my Ph.D. whilst completely renovating our house together. I also thank-you for not minding too much and staying up late (as much as you hated it) and helping me read and edit my thesis. Thanks Sandra, xxxx

## ABREVIATIONS

Abbreviations are as described in the Journal of Biological Chemistry "Instructions to Authors" 1989. Additional abbreviations are listed below.

1,25D	1,25(OH) <sub>2</sub> D <sub>3</sub> or 1,25-dihydroxyvitamin D <sub>3</sub>
25(OH)D <sub>3</sub>	25-hydroxyvitamin D <sub>3</sub>
24,25(OH) <sub>2</sub> D <sub>3</sub>	24,25-dihydroxyvitamin D <sub>3</sub>
CYP1 $\alpha$	25-hydroxyvitamin D <sub>3</sub> 1 $\alpha$ -hydroxylase
CYP24	25-hydroxyvitamin D <sub>3</sub> 24-hydroxylase
PTH	Parathyroid Hormone
VDR	vitamin D <sub>3</sub> receptor
RXR	Retinoid-X-receptor
VDRE	vitamin D response element
EBS	Ets-1 Binding site
CHEP	CYP24-hydroxylase enhancing protein
CBS	CHEP binding site
NR	Nuclear Receptor
OR	Orphan receptor
ROR $\alpha$	Retinoid-related orphan receptor
RORE	ROR $\alpha$ response element
EMSA	Electrophoretic Mobility shift assay
TK	Thymidine Kinase
DR-3	Direct repeat separated by 3 base pairs
PMA	Phorbol 12-Myristate 13-Acetate
AP-1	Activating Protein-1

## THESIS SUMMARY

1,25(OH)<sub>2</sub>D<sub>3</sub> (1,25D) plays an important role in many different physiological processes including calcium homeostasis, modulation of immune function and control of cellular proliferation and differentiation. The 25-Hydroxyvitamin D<sub>3</sub> 1 $\alpha$ -Hydroxylase (CYP27B1) and the 25-Hydroxyvitamin D<sub>3</sub> 24-Hydroxylase (CYP24) are the two rate-limiting enzymes involved in the synthesis and degradation of 1,25D respectively. Various factors regulate expression of CYP27B1 and CYP24 enzymes resulting in the maintenance of homeostatic levels of serum and cellular 1,25D. The aim of this thesis was to examine the role of an apparently novel transcription factor binding site in 1,25D mediated expression of the rat CYP24 gene promoter in osteoblast and kidney cells.

Mutagenesis of the sequence 5'-TGTCGGTCA-3' located at -171/-163 upstream of VDRE-1 was shown to be critical for 1,25D dependent activation but not basal expression, of -298 bp and -186 bp CYP24 promoter constructs with this dependence being greater in ROS 17/2.8 cells than in 293T cells. Mutagenesis of the CBS lowered 1,25D mediated CYP24 promoter activity to ~80% and ~50% in ROS 17/2.8 cells and 293T cells respectively. The reduction in CYP24 promoter activity upon mutagenesis of the CBS correlates to a loss of protein binding from this site in EMSA experiments using nuclear extracts prepared from ROS 17/2.8 cells and 293T cells. This sequence is referred to as the CYP24 hydroxylase enhancing protein (CHEP) binding site (CBS). UV cross-linking experiments demonstrated that CHEP appears to be a DNA binding protein of ~60 kDa. The cloning of the CBS (5'-TGTCGGTCA-3') upstream of VDRE-1 in heterologous thymidine kinase (TK) promoter constructs demonstrated that this sequence alone is sufficient to enhance the activity of this VDRE-1. Furthermore, the CBS did not contribute to basal expression of the TK-promoter constructs and alone was unresponsive to 1,25D demonstrating that the CBS does not form part of a vitamin D<sub>3</sub> response element (VDRE). Database searches of transcription factor binding sites failed to identify this sequence as a known transcription factor binding site. Closer inspection of this sequence revealed that the CBS had sequence characteristics similar to monomeric orphan receptors, members of the nuclear receptor superfamily of transcription factors. The 5'-TGT-3' sequence could

constitute the 5'-flanking sequence and the 5'-CGGTCA-3' the core half-site thus resembling the consensus nuclear receptor half-site (5'-AGGTCA-3'). Over-expression of the known monomeric orphan receptors SF-1,  $ERR\alpha$ ,  $ROR\alpha1$ ,  $Rev-erb\alpha$  and NGFI-B did not enhance fold 1,25D dependent induction of native -186 bp CYP24 promoter constructs containing VDRE-1. Surprisingly, exogenous expression of  $ROR\alpha1$  reduced both basal and 1,25D induced transcriptional activity of -186 bp CYP24 promoter constructs. Furthermore the use of pharmacological antagonists of  $ERR\alpha$ ,  $ERR\beta$ , and  $ERR\gamma$  receptor transcriptional activity, and studies with supershifting antibodies recognising all  $ROR\alpha$  isoforms in EMSA experiments did not provide evidence for the CBS being an orphan receptor. Overall the data strongly suggested that the CBS is not a monomeric orphan receptor binding site.

The phorbol ester, phorbol 12-myristate 13-acetate (PMA) together with 1,25D synergistically activates CYP24 gene expression in kidney but not in bone cells. In other studies and also in this study, mutagenesis of the Ets-1 binding site (EBS) present in the CYP24 promoter reduced the fold PMA and 1,25D dependent transcriptional synergy by approximately 50%. Strikingly, mutagenesis of the CBS completely abolished PMA induced 1,25D dependent transcriptional synergy of -186 bp CYP24 promoter constructs in 293T cells. Together with the EBS, these are the first reports to describe the identity of transcription factors involved in the activation of the CYP24 promoter in response to PMA. Over-expression of c-Jun but not c-Fos, known downstream targets of PMA signalling, potentiated 1,25D CYP24 promoter activity that was dependent upon a functional CBS. EMSA analysis using supershifting antibodies against all seven members of the AP-1 family of transcription factors did not detect any supershifted complexes binding to the CBS oligonucleotide probe using nuclear extracts prepared from 293T cells either untreated or treated with 1,25D. Interestingly, the binding of CHEP to the CBS oligonucleotide was unaltered in nuclear extracts prepared from 293T cells treated with PMA alone, 1,25D alone and PMA and 1,25D together. EMSA experiments using unlabeled competitor control AP-1 oligonucleotide and oligonucleotide probes encompassing a sequence similar to the CBS shown to bind AP-1 very weakly competed for the binding of CHEP to the CBS oligonucleotide in 293T nuclear extracts. AP-1 protein binding to a control AP-1 oligonucleotide but not the CBS oligonucleotide probe, was detected in nuclear

extracts prepared from untransfected and c-Jun and c-Fos transfected 293T cells. The data suggested that whilst the CBS is PMA responsive, the CBS does not appear to bind the AP-1 protein. It is envisaged that 1,25D potentiated CYP24 promoter activity in the presence of over-expressed c-Jun, may be the result of c-Jun “piggy-backing” with the protein bound at the CBS.

1,25D functions both in the kidney and bone to induce CYP24 gene expression which represents a pivotal feedback mechanism preventing the possibility of toxicity from occurring as a result of excessive 1,25D levels. A unique feature of the CYP24 gene promoter is the presence of two VDREs termed VDRE-1 and VDRE-2. In response to high 1,25D levels, transcriptional synergism arises between these VDREs ensuring the rapid removal of 1,25D. Mutagenesis of the CBS completely abrogates 1,25D dependent transcriptional synergy between VDRE-1 and VDRE-2 in ROS 17/2.8 cells. This is the first report to describe a transcription factor distinct from VDR/RXR that participates in 1,25D mediated synergy. The CBS mediates its transcriptional effects through VDRE-1 and not the more distal, VDRE-2 which may be a reflection of the ~80 bp distance separating these two binding sites. Replacement of VDRE-1 with VDRE-2 in –186 bp CYP24 promoter constructs demonstrated that 1,25D dependent activation of this VDRE is still dependent on a functional CBS. The mechanism underlying the transcriptional synergy is unclear. It is envisaged that CHEP together with the neighboring Ets-1 interacts with the coactivator complex bound at VDRE-1 so that this complex may interact with a separate coactivator complex bound at VDRE-2. Alternatively, the interaction of the coactivator complex stabilised by CHEP and possibly Ets-1 and the RNA polymerase II machinery and synergy may arise from an increased interaction between these two complexes. Exogenous expression of the coactivators SRC-1, GRIP-1 and CBP, in cells transiently transfected with –186 bp CYP24 promoter constructs, did not compensate for the absence of a functional CBS. These data further supported the role for CHEP in interaction and/or stabilisation of the coactivator complex bound at VDRE-1. Mutagenesis of the CBS reduces 1,25D mediated induction of -186 bp CYP24 promoter constructs in ROS 17/2.8 cells by ~50% compared with the ~80% reduction upon mutagenesis of the CBS. This is presumably a reflection of the important role CHEP plays through interacting with the coactivator complex bound at VDRE-1.

The expression of -298 bp CYP24 promoter constructs containing both VDREs is reliant upon a functional CBS over a wide range of 1,25D concentrations. At very low levels of 1,25D, there appears to be a switch in dependency from the CBS at high levels of 1,25D to the EBS at very low levels of 1,25D. The molecular basis for this phenomenon is unclear but *in vivo* it is envisaged that the presence of CHEP during high levels of 1,25D would ensure that transcriptional synergy occurs between VDRE-1 and VDRE-2 resulting in the rapid catabolism of potentially toxic levels of 1,25D. Collectively, these studies have defined a critical role played by an apparently novel transcription factor binding site in 1,25D mediated CYP24 gene expression.

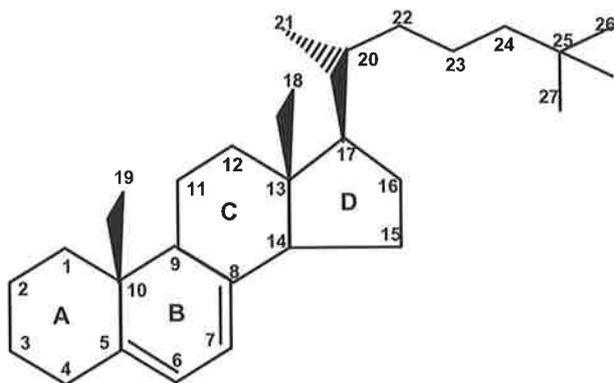
## CHAPTER 1

# Introduction

# 1. Introduction

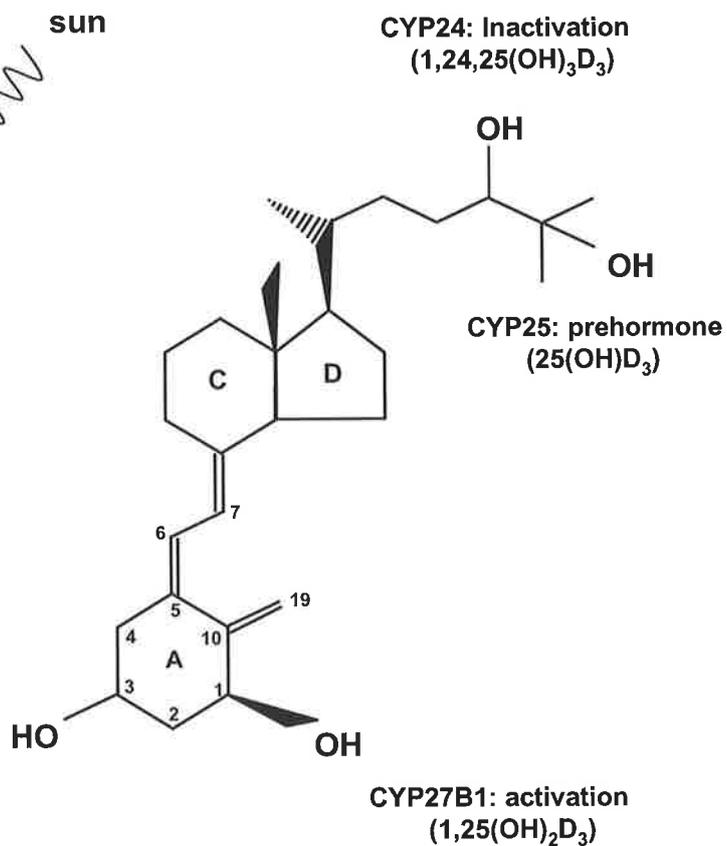
## 1.1 Brief Overview of 1,25(OH)<sub>2</sub>D<sub>3</sub> metabolism and action

The biologically active vitamin D<sub>3</sub> molecule, 1,25(OH)<sub>2</sub>D<sub>3</sub> (1,25D), is a seco-steroid hormone where the carbon bond is cleaved between C-9 and C-10 in ring B of the cholesterol molecule (Figure 1.1). The synthesis of active 1,25D begins in the basal epidermal layer of the skin where ultra-violet light induced photolysis followed by a series of non-enzymatic reactions, converts 7-dehydrocholesterol to the precursor vitamin D<sub>3</sub> (Figure 1.2). Alternatively, the precursor vitamin D<sub>3</sub> can be obtained through a dietary intake of fatty fish oil, or vitamin D<sub>3</sub>-fortified milk (Breslau and Zerwekh, 1997). Vitamin D<sub>3</sub> enters the circulation and is transported to the liver by the vitamin D binding protein (DBP). In the liver, the cytochrome P450 enzyme, 25-hydroxylase (CYP25), hydroxylates carbon-25 of vitamin D<sub>3</sub> to form 25-hydroxyvitamin D<sub>3</sub> [25(OH)D<sub>3</sub>] (Gascon-Barre, 1997). The activity of 25-hydroxylation appears to be loosely regulated and not under a tight regulatory control. The 25-hydroxyvitamin D<sub>3</sub> is then transported to the kidney by DBP where it is further hydroxylated. The kidney is the major organ responsible for the synthesis of circulatory 1,25D. The biotransformation of 25(OH)D<sub>3</sub> to 1,25(OH)<sub>2</sub>D<sub>3</sub> is tightly controlled by another cytochrome P450 enzyme, 25-hydroxyvitamin D<sub>3</sub> 1 $\alpha$ -hydroxylase (CYP27B1 or CYP1 $\alpha$ ), which hydroxylates C-1 of 25(OH)D<sub>3</sub> (Henry, 1997; Jones *et al.*, 1998). A third important cytochrome P450, 25-hydroxyvitamin D<sub>3</sub> 24-hydroxylase (CYP24) catalyses the hydroxylation of 25(OH)D<sub>3</sub> to 24,25(OH)<sub>2</sub>D<sub>3</sub> and of 1,25D to 1,24,25(OH)<sub>3</sub>D<sub>3</sub>. This represents the first step in their catabolism CYP24 via the C-24/C-23 oxidation pathway (Omdahl *et al.*, 2002; Omdahl and May, 1997). The acquisition of an hydroxyl group at C-24 commits these substrates to metabolic inactivation resulting in the formation of a 23-COOH water soluble product called calcitric acid which is filtered and excreted by the kidney. Other C-23 hydroxylated metabolites are produced by CYP24 and this is discussed later in further detail. CYP27B1 and CYP24 therefore catalyse the rate-limiting steps in the activation and degradation of active 1,25D, respectively, and these two enzymes are crucial for regulating ambient levels of serum and cellular 1,25D.



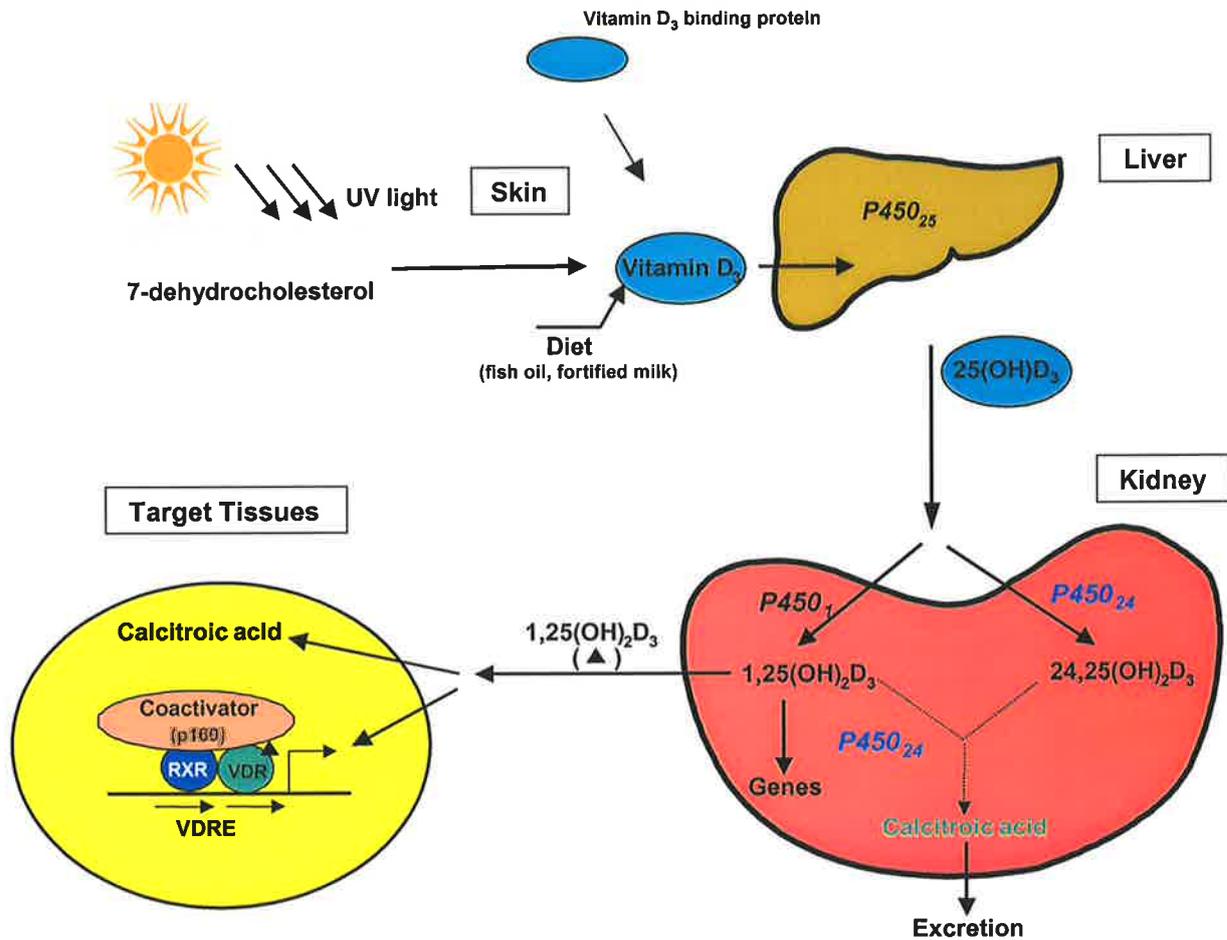
**7-Dehydrocholesterol**

sun



**Figure 1.1 Structure of major vitamin D<sub>3</sub> metabolites.**

The chemical structures of the major vitamin D<sub>3</sub> metabolites are shown with the sites of hydroxylation by associated cytochrome P540 enzymes that either direct vitamin D<sub>3</sub> bioactivation (hydroxylations catalysed by CYP25 and CYP27B1) and side-chain oxidation (CYP24). Also shown is sunlight induced fission of the carbon bond between C-9 and C-10 to which 1,25D derives its seco-steroid nature.



**Figure 1.2 General overview of vitamin D<sub>3</sub> synthesis and metabolism.**

Vitamin D<sub>3</sub> is synthesized in the skin from 7-dehydrocholesterol and is transported to the kidney by binding to the vitamin D<sub>3</sub> binding protein (DBP). In the kidney, vitamin D<sub>3</sub> is hydroxylated to form 25(OH)D<sub>3</sub> (1,25D) which is carried to the kidney where it has two fates. Firstly, 25(OH)D<sub>3</sub> entering the liver is bioactivated to form 1,25(OH)<sub>2</sub>D<sub>3</sub> following hydroxylation of C-1 by CYP27B1. Secondly, the majority of 25(OH)D<sub>3</sub> is hydroxylated at C-24 by the 25-hydroxyvitaminD<sub>3</sub> 24-hydroxylase (CYP24) that initiates its metabolism via the C-24 oxidation pathway. The product of the C-24 oxidation pathway is calcitroic acid which is filtered and excreted by the kidneys. Active 1,25D exerts its biological effects by binding the vitamin D<sub>3</sub> receptor (VDR) which heterodimerises with its partner protein RXR. Liganded VDR/RXR heterodimers bind to vitamin D<sub>3</sub> (VDRE) response elements in the promoter regions of 1,25D target genes to activate or repress transcription.

1,25D exerts its biological effects via a genomic mechanism and a non-genomic mechanism. The genomic mechanism of 1,25D target gene activation involves 1,25D binding to the vitamin D receptor (VDR). The properties of VDR are described later. VDR binds as a heterodimer with the retinoid-X-receptor (RXR) to vitamin D response elements (VDREs) in gene promoters. VDREs generally consist of two direct repeats of the consensus 5'-AGGTCA-3' separated by three nucleotides (DR-3). The binding of 1,25D results in a conformational change in VDR allowing the dissociation of a corepressor (Dwivedi *et al.*, 1998; Polly *et al.*, 2000) (discussed later), facilitating the interactions of VDR with coactivator proteins such as members of the p160 family (MacDonald *et al.*, 2001). These coactivators can modulate chromatin structure (Collingwood *et al.*, 1999) and make protein-protein contacts with the basal transcriptional machinery. Non-genomic signalling by 1,25D involves the activation of signalling pathways and will be discussed later.

## **1.2 Physiological roles of 1,25D**

1,25D, has important pleiotropic roles and can affect the cellular physiology of various organs and cell types. 1,25D is principally involved in calcium and phosphorus homeostasis, bone remodelling, muscle function, immunity, endocrine secretions, neurotransmission and cellular proliferation and differentiation, and recently a newly defined role in secondary bile acid metabolism. The following sections will briefly discuss the roles of 1,25D.

### **1.2.1 1,25D and Calcium Homeostasis**

As mentioned, the kidney is the major source of circulating, endocrine-acting 1,25D that functions as the principle regulator of calcium homeostasis. When serum levels of calcium are low, parathyroid hormone (PTH) levels increase. PTH increases serum 1,25D concentrations by up regulating renal CYP27B1 expression and down regulating renal CYP24 expression (discussed in later sections). 1,25D increases calcium absorption by the intestine, inhibits renal calcium excretion and also mobilises calcium from bone in order to maintain correct serum calcium levels (DeLuca and Zierold, 1998, Jones *et al.*, 1998). 1,25D, in a VDR dependent manner, acts to increase mRNA and protein levels of

the calcium binding proteins Calbindin-D<sub>9K</sub> and Calbindin-D<sub>28K</sub> involved in the translocation of calcium in the intestine and kidney, respectively (Hoenderop *et al.*, 1999; Li *et al.*, 2001). VDR null mice show reduced mRNA levels of intestinal Calbindin-D<sub>9K</sub> and kidney epithelial calcium channel (EcaC) mRNA (Hoenderop *et al.*, 2002; Song *et al.*, 2002). However, VDR null mice fed a high calcium diet can normalise serum calcium and prevent osteomalacia (Song *et al.*, 2003). Similarly, CYP27B1 knockout mice show decreased levels of ECaC, Calbindin-D<sub>9K</sub>, Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCX1) and Ca<sup>2+</sup>-ATPase (PMCA1b) proteins (Hoenderop *et al.*, 2002). 1,25D repletion of CYP27B1 knockout mice results in the increased expression of the above mentioned calcium transport proteins and normalisation of serum calcium (Hoenderop *et al.*, 2002). Therefore 1,25D signalling through the VDR strongly correlates with the expression of genes involved in calcium homeostasis.

### **1.2.2 Inhibition of cell proliferation and induction of cell differentiation**

This section will briefly describe some aspects of the role of 1,25D in modulating the proliferation and differentiation states of normal and cancerous cell types.

The activation or repression by 1,25D of specific proto-oncogenes or tumour suppressor genes related to cellular proliferation and differentiation has been observed for many normal and cancer cell types. For example, such genes include p27<sup>kip1</sup>, p21<sup>waf/cip1</sup>, c-myc, laminin, cyclin C, c-Fos, c-Jun, ornithine decarboxylase, and members of the transforming growth factor family (TGF-β) (Minghetti *et al.*, 1998; Freedman, 1999). 1,25D is a potent inhibitor of cellular proliferation as well as an inducer of cellular differentiation. The differentiation of myeloid leukemic cells into macrophages is a well-studied system. Rots *et al.* (1999) demonstrated that 1,25D treatment of the myelomonocytic U937 cells results in an early proliferative burst followed by growth inhibition and subsequent differentiation into macrophages. This was attributed to an increase in cyclin A, D1 and E proteins during the proliferative burst. Protein levels of p21 and p27 (cyclin dependent kinase inhibitors-CKI) were also elevated but persisted during the proliferative phase and subsequent inhibition of cell growth. The ectopic expression of p21 and p27 in the absence of 1,25D results in U937 cells expressing

monocyte/macrophage specific markers (Rots *et al.*, 1999). MCF-7 cells when treated with 1,25D are growth arrested in the G1-S phase of the cell cycle and no biphasic expression of cyclin dependent kinase inhibitors is observed (Rots *et al.*, 1999; Jensen *et al.*, 2001). Concomitant to the growth arrest in the G1-S phase, c-myc expression was also decreased (Jensen *et al.*, 2001). Therefore, growth arrest and differentiation clearly involves the induction of different cyclins and cyclin dependent kinase inhibitors. Similarly, 1,25D treatment of squamous cell carcinomas both *in vitro* and *in vivo* undergo growth arrest at the G0-G1 phase of the cell cycle (Hershberger *et al.*, 1999; Akutsu *et al.*, 2001). The up-regulation of p27 mRNA and protein and a subsequent decrease in p27 mRNA and protein contributed to the observed growth arrest. Many established prostate cancer cell lines such as LNCaP, PC-3, ALVA-31 and DU-145 are growth inhibited by 1,25D (Chen *et al.*, 1997; Hedlund *et al.*, 1996; Zhuang and Burnstein, 1998; Schwartz *et al.*, 1997) and are induced to undergo apoptosis by down regulating Bcl-2 and Bcl-X<sub>L</sub> proteins (Blutt *et al.*, 2000). 1,25D inhibits the proliferation and stimulates the differentiation of keratinocytes. Keratinocytes can locally produce 1,25D (Xie *et al.*, 2002) which promotes the differentiation of keratinocytes in a phospholipase D and PKC dependent manner (Bollinger Bollag and Bollag, 2001). 1,25D also promotes the proliferation of keratinocytes under situations of stress and injury via enhancing keratinocyte growth factor (KGF) expression and action (Gamady *et al.*, 2003). 1,25D has been shown inhibit the expression of a number of genes such as EGF-R, c-myc and IL-2, IL-6, IL-8, TNF- $\alpha$ , IFN $\gamma$ , GM-CSF which play a role in the inflammation and proliferation of keratinocytes and T-cells (DiSepio *et al.*, 1999). It is interesting to note that CYP27B1 knockout mice do not develop alopecia (hair loss). This phenotype is observed only in VDR null mice and is not due to an intrinsic inability of keratinocytes to differentiate or proliferate, but rather appears to be an abnormality in the initiation of the hair cycle (Sakai and Demay, 2000). VDR null mice expressing a transgene under the control of the keratinocyte specific keratin 24 gene promoter rescues the alopecia phenotype (Chen *et al.*, 2001a). Therefore it appears that VDR is important for the prevention of alopecia.

The major side effects of 1,25D treatment of patients with cancers is the high incidence of hypercalcemia (excess calcium in the blood) and hypercalcinuria (excess calcium in the urine) (Colston, 1997). These risks have prompted the search for synthetic vitamin D analogs that retain their antiproliferation ability but

have a reduced hypercalcemic/calcinuric activity profile. One such example is EB1089 (a dimethyl side chain analog). EB 1089 has been shown in preclinical trials using rats, to reduced tumour mass, volume and incidence of carcinogen induced mammary tumours (Colston, 1992). As a consequence, EB 1089 is in clinical trials for the treatment of breast cancer (Jones *et al.*, 1998). Another 1,25D analog, MC 903, has been used to treat psoriasis for nearly 15 years and is first line therapy for this disease (Sutton and MacDonald, 2003; DiSepio *et al.*, 1999). More recently a more potent analog, TX 522, a 14 epianalog, has been developed for the treatment of psoriasis due to its low calcemic properties and is currently under clinical investigation (phase II trials) for the treatment of psoriasis (Verlinden *et al.*, 2001).

### 1.2.3 Modulation of the immune system

1,25D interacts with mature monocytes and macrophages enhancing their immune function and improving host defence against bacterial infection and tumour cell growth (Brown *et al.*, 1999). CYP27B1 is expressed in precursor monocytes, peripheral macrophages and granulomatoses and CYP24 is present in peripheral monocytes (Dusso *et al.*, 1997; Omdahl and May, 2002). The VDR is expressed in most cells of the immune system indicating a role for this 1,25D in immunity (Griffin *et al.*, 2003). The principle action of 1,25D on the immune system is to act as an immunosuppressive agent. This is accomplished by decreasing the rate of proliferation and activity of T cells and B cells and inducing the availability of suppressor T cells (Lemire, 1997). An important action of these immunosuppressive effects in animal models is the therapeutic treatment for the control of systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, juvenile (type I) diabetes, experimental autoimmune encephalomyelitis in mice and transplant rejection (DeLuca and Cantorna, 2001; Hullet *et al.*, 1998; Brown *et al.*, 1999; Jones *et al.*, 1998; Griffin *et al.*, 2003). 1,25D has an inhibitory effect on inflammatory T cell activity by stimulating the production of TGF- $\beta$  and interleukin 4 (IL-4) (DeLuca and Cantorna, 2001) or decreasing T cell proliferation by inhibiting IL-2 and interferon- $\gamma$  production (Alroy *et al.*, 1995). Therefore, these results suggest potential therapeutic applications for the treatment of various autoimmune diseases in humans by 1,25D.

#### 1.2.4 Function of 1,25D in bone

Bone remodelling occurs throughout life and consists of cycles of bone formation by osteoblasts and bone resorption by osteoclasts. VDR is present in osteoblast and osteoclast cells (Suda and Takahashi, 1997; Aubin and Heersche, 1997). The role of 1,25D in bone formation has been confirmed but the direct action of 1,25D on bone is under investigation. It is well known that a deficiency of 1,25D causes rickets in children and osteomalacia in adults (Thomas and Demay., 2000; Glorieux, 1997; Kato *et al.*, 2002). This was confirmed in CYP27B1 knockout and VDR null mice which develop skeletal abnormalities (Panda *et al.*, 2001; Dardenne *et al.*, 2001; Van Cromphaut *et al.*, 2001; Amling *et al.*, 1999; Li *et al.*, 1997). Similarly, CYP24 knockout mice develop abnormal bone mineralisation phenotypes that appear to be a result of exposure of bone cells to excessive 1,25D (St-Arnaud *et al.*, 2000). These results suggest important functions for 1,25D in normal bone growth and mineralisation.

The actions of 1,25D on osteoblast and osteoclast activity resulting in bone formation and resorption, respectively, are complicated and will only be dealt with briefly. 1,25D stimulates the differentiation of osteoblast cells from immature mesenchymal stem cells to a mature differentiated osteoblast phenotype. These mature osteoblast cells express genes involved in matrix formation and bone mineralisation such as osteopontin, osteocalcin, and alkaline phosphatase (Aubin and Heersche, 1997; Skjodt *et al.*, 1985; Demay *et al.*, 1990; Pols *et al.*, 1986; Noda *et al.*, 1990; Owen *et al.*, 1991). Genes such as those for the osteopontin and osteocalcin are up-regulated by 1,25D through a vitamin D<sub>3</sub> response element (VDRE) (discussed later) localised in these promoters (Demay *et al.*, 1990; Noda *et al.*, 1990). In osteoclasts, the carbonic anhydrase-II and  $\beta$ -integrin genes for example, similarly contain VDREs in their promoters and are up-regulated in response to 1,25D (Cao *et al.*, 1993; Quelo *et al.*, 1994). 1,25D additionally stimulates osteoclastogenesis (osteoclast cell formation) and osteoclast resorptive activity. The mechanism of action of 1,25D on osteoclastogenesis and osteoclast activity appears to be indirect. Recent studies have shown that osteoblasts/stromal cells express receptor activator of NF-kappaB ligand (RANKL) and together with 1,25D, supports the differentiation of osteoclasts (Kitazawa and Kitazawa, 2002; Lacey *et al.*, 1998; Suda and Takahashi, 1997). Osteoclast precursors which

express activator of NF-kappaB (RANK) recognise RANKL through cell-cell interaction and in the presence of macrophage colony stimulating factor (M-CSF) differentiate into bone resorbing osteoclasts (Kong *et al.*, 1999a; Suda *et al.*, 2003). The outcome resulting from 1,25D regulation of osteoblast and osteoclast activity is balanced bone formation and resorption. 1,25D is still recommended as a dietary supplement in conjunction with other pharmacological therapy for the treatment of decreased bone mass or osteoporosis (Sutton and MacDonald, 2003). Recently, a potent analog 2-methylene-19-nor-(20S)-1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> (2MD) potently stimulates bone formation *in vitro* and markedly improves bone mass in ovariectomised rats without inducing hypercalcemia (Shevde *et al.*, 2002). The ability of 2MD to increase bone mass was attributed to its unknown ability to selectively act on bone in preference to the intestine and to increase osteoclast formation via up-regulating the expression of RANKL (Shevde *et al.*, 2002). Therefore such studies using potent, bone selective 1,25D analogs should lead to useful therapies for the treatment of bone diseases.

### **1.2.5 1,25D and secondary bile acid metabolism**

Recently, it has been demonstrated that 1,25D liganded VDR can activate the expression of the CYP3A gene (Thummel *et al.*, 2001), a P450 enzyme involved in catabolism of the secondary bile acid lithocholic acid (LCA) in the liver and the intestine. LCA is a toxin that promotes colorectal carcinogenesis (Debruyne *et al.*, 2002) whereas 1,25D is protective against colon cancer (Lamprecht *et al.*, 2001). Makishima *et al.* (2002) demonstrated that LCA bound to and activated the VDR through association with the steroid receptor coactivator, SRC-1. Therefore 1,25D activation of CYP3A expression via the VDR, promotes the detoxification of the secondary bile acids and protection from these hepatotoxic and enteric carcinogens. Hence 1,25D plays a protective role via a novel mechanism to protect against secondary bile acid-induced colon cancer.

### **1.3 The Nuclear Receptor (NR) Superfamily and VDR**

VDR is a member of the nuclear receptor superfamily family (Moras and Gronemeyer, 1998; Mangelsdorf *et al.*, 1995). The NR superfamily is divided into

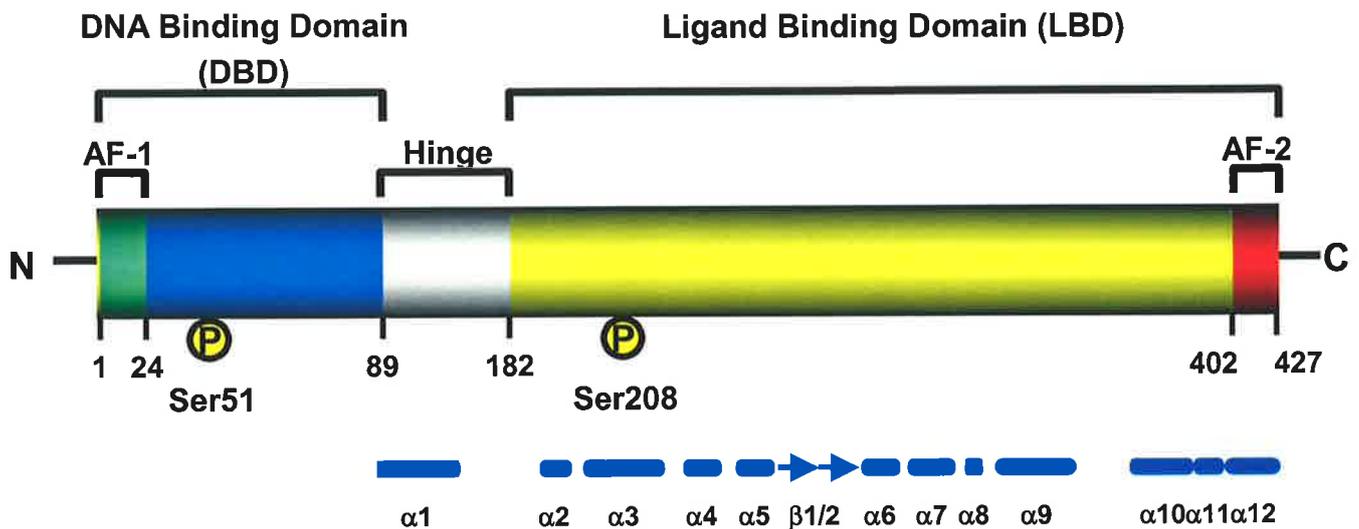
four broad classes based upon their dimerisation and DNA binding properties (Mangelsdorf *et al.*, 1995). The first class includes the well known steroid hormone receptors such as glucocorticoid (GR), mineralcorticoid (MR), progesterone (PR) and androgen receptors (AR) that are ligand induced homodimers which recognise inverted palindromic 5'-AGAACA-3' half-sites (Glass, 1994; Zilliacus *et al.*, 1995). The second class of nuclear receptors consists of receptors that bind the promiscuous partner protein, 9-*cis* retinoic acid receptor, RXR, and include vitamin D<sub>3</sub> receptor (VDR), retinoic acid receptor (RAR) and thyroid hormone receptor (TR) (Mangelsdorf and Evans, 1995). These receptors bind to imperfect direct repeats of the consensus 5' AGGTCA 3'-like sequences with varying spacings separating the half-sites. Specificity of NR interaction with VDR, RAR and TR for example is achieved through the spacing separating the direct repeats. VDR/RXR binds to direct repeats separated by 3 bp (DR3), TR/RXR binds to DR4 sequences and RAR/RXR binds to DR5 sequences (Mangelsdorf and Evans, 1995). The third class of the NR superfamily consists of receptors that homodimerise on direct repeats, these include RXR, and the orphan receptors constitutive androstane receptor (CAR), Nerve growth factor inducible (NGFI-B) and Chicken ovalbumin upstream promoter transcription factor (COUP-TF). The fourth class belongs to the monomeric orphan receptors. Orphan receptors are termed 'orphan' receptors because the identification of a ligand binding to these receptors has not yet been characterised. Table-1 summarises the DNA binding sequence, tissue expression, genes regulated and cofactor interaction of the well-known monomeric orphan receptors. As shown in Table-1, an important aspect of monomeric orphan receptors is their ability to bind DNA specifically as a monomer to 5'-AGGTCA-3'-like half-site sequences. The mechanism differentiating which orphan receptor member binds to a particular half-site sequence is dictated by the sequence of nucleotides immediately flanking the half-site (Enmark and Gustafsson, 1996).

The NR superfamily members share a common functional and structural architecture of their domains (Mangelsdorf *et al.*, 1995; Aranda and Pascual, 2001). As shown in Figure 1.3, a cartoon of the VDR will be used as an example to describe these domains. NR's comprise an amino-terminal A/B domain followed by a DNA binding domain (DBD) termed the C domain. The DBD is composed of two highly conserved zinc fingers (which is the characteristic hallmark of the NR superfamily) and also a constitutively active activation function domain (AF-1) that contributes to the transactivation function localised in the A/B domain

Monomeric Orphan receptor	DNA binding sequence	Cellular expression	Genes regulated	References
SF-1 (Steroidogenic Factor-1)	5'-C/TCAAGGTCA-3' 5'-GAAGGTCA-3' 5'-CCAAGGCG-3'	Hypothalamus, pituitary, gonads, placenta, adrenal glands	P450c17, P450c21, P450c11 3 $\beta$ -hydroxysteroid dehydrogenase follicle stimulating hormone steroidogenic acute regulatory protein	Heckert, 2001 Crawford <i>et al.</i> , 1998 Sadovsky and Crawford, 1998 Ito <i>et al.</i> , 2000b Jacobs <i>et al.</i> , 2003 Zimmerman <i>et al.</i> , 1998
ROR $\alpha$ (RAR-related orphan receptor) ROR $\alpha$ 1, $\alpha$ 2, $\alpha$ 3, ROR $\beta$ , ROR $\gamma$	5'-A/G/T, A/T, A/T, C/A, A/TAGGTCA-3'	ROR $\alpha$ , ROR $\gamma$ ubiquitously expressed ROR $\beta$ : CNS, brain, retina pineal gland	Apolipoprotein A-I $\gamma$ F-crystallin Prosaposin Rev-erb $\alpha$	Giguere <i>et al.</i> , 1994 Schaeren-Weimers <i>et al.</i> , 1997 Dassault and Giguere, 1997 Dassault <i>et al.</i> , 1998 Vu-Dac <i>et al.</i> , 1997 Tini <i>et al.</i> , 1995 Delerive <i>et al.</i> , 2002
ERR (Estrogen-related receptor) ERR $\alpha$ , ERR $\beta$ , ERR $\gamma$ or ERR1, ERR2, ERR3, respectively	5'-TCAAGGTCA-3'	ERR $\alpha$ , ERR $\gamma$ : ubiquitously expressed intestine, heart, bone, muscle ROR $\beta$ : restricted to early development low levels in liver, stomach, kidney, heart	Osteopontin Lactoferrin Medium-Chain Acyl Coenzyme A- Dehydrogenase	Giguere, 2002 Bonnelye and Aubin, 2002 Vanacker <i>et al.</i> , 1998 Giguere, 1999 Yang <i>et al.</i> , 1996 Sladek <i>et al.</i> , 1997 Zhang and Teng, 2000
Rev-erb Rev-erb $\alpha$ , Rev-erb $\beta$	5'-A/T, A, A/T, N, T, A/GGGTCA-3'	Rev-erb $\alpha$ , Rev-erb $\gamma$ : widely expressed brain, liver, lung kidney, muscle	N-myc $\alpha$ -Fetoprotein MyoD Myogenin p21 Apolipoprotein-III Apolipoprotein-I Rev-erb $\alpha$	Retnakaran <i>et al.</i> , 1994 Harding and Lazar, 1993 Dassault and Giguere, 1997 Bois-Joyex <i>et al.</i> , 2000 Burke <i>et al.</i> , 1996 Raspe <i>et al.</i> , 2002 Vu-Dac <i>et al.</i> , 1998 Coste and Rodriguez, 2002
NGFI-B (Nerve growth factor-inducible) NGFI-B $\alpha$ , NGFI-B $\beta$ , NGFI-B $\gamma$ or nur 77, nurr 1 and nor 1, respectively	5'-AA AGGTCA-3'	Nur 77, nor 1: widely expressed muscle, lungs, osteoblasts, brain nurr 1 restricted to central nervous system	Steroid 21-hydroxylase Plasminogen activator inhibitor-I Pro-opiomelanocortin	Castillo <i>et al.</i> , 1998 Zetterstrom <i>et al.</i> , 1997 Wilson <i>et al.</i> , 1993 Gruber <i>et al.</i> , 2003 Maria <i>et al.</i> , 1999

**Table-1: Known monomeric orphan receptors and their transcriptional properties.**

A brief overview of the properties for each monomeric orphan receptor is depicted. The underlined sequence represent the consensus half-site to which all members bind. The 5' flanking sequence dictates which orphan receptor member binds to a particular half-site in target gene promoters.



**Figure 1.3 Structural organisation of domains of the human vitamin D<sub>3</sub> receptor and its phosphorylation sites.** Structural organisation of the vitamin D<sub>3</sub> receptor (VDR) depicting the DNA binding domain (DBD) and the ligand binding domain (LBD). The flexible hinge region connects the DBD and the LBD together. An activation function (AF-1) domain at the N-terminus is shown but no known function has been attributed to it. A potent ligand dependent AF-2 domain at the C-terminus of the VDR is necessary for ligand dependent interaction with coactivator proteins. Amino acids depict domain interfaces. Two phosphorylation sites, serine 51 localised to the DNA binding domain and serine 208 localised in the ligand binding domain are phosphorylated by protein kinase C (PKC) and casein kinase-II (CK-II), respectively. The number and location of  $\alpha$ -helices and B-sheets that constitute the structure LBD are shown. The important helices to not are a  $\alpha$ 3,  $\alpha$ 4 ,  $\alpha$ 5,  $\alpha$ 11, and  $\alpha$ 12 (AF-2) which form an important interface for interaction with the signature motif of coactivator proteins.

(Mangelsdorf *et al.*, 1995). The E/F domain comprises the ligand binding domain, a ligand dependent molecular switch, ensuring specificity and selectivity to the physiological response to cognate hormone (Mangelsdorf *et al.*, 1995). The LBD contains a ligand-dependent activation function domain (AF-2), consisting of number of  $\alpha$ -helices including helix 12, that is critical for ligand induced transcriptional activation of NR's. In addition, this domain forms a hydrophobic cleft to mediate interaction with an amphipathic  $\alpha$ -helix of the consensus LXXLL from different coactivator proteins. In the absence of ligand, helix 12 present as part of the AF-2 domain is projected away from the ligand-binding pocket. The surface of the LBD in the absence of ligand and helix 12 interacts with an extended amphipathic  $\alpha$ -helix (LXXXIXXX(I/L) from SMRT or N-CoR to mediate gene silencing through histone deacetylation (Hu and Lazar, 1999; Rosenfeld and Glass, 2001). Upon ligand binding, the conformational changes that take place reposition helix 12 over the top of the ligand binding cavity. This mechanism of helix 12 repositioning has been termed the mouse trap model. The D region serves as a flexible linker, linking the DBD and LBD and varies considerably in length and sequence between members of the NR superfamily.

Recently, a novel N-terminal variant of the VDR was identified in human kidney tissue, and also in intestinal, kidney and osteoblast cell lines (Sunn *et al.*, 2001). The novel VDR variant, VDRB1, arises from alternative promoter usage of the VDR gene. VDRB1 contains an extra 50 amino acids in its A/B region. Interestingly, VDRB1 is expressed at a one-third lower level compared with VDR. Transient transfection analysis using CYP24 promoter constructs revealed that VDRB1 activates transcription to a lower level than VDR (Sunn *et al.*, 2001) but the physiological significance of this finding is not clear as yet.

The crystal structure of VDR has been elucidated (Rochel *et al.*, 2000). The high-resolution structure of the VDR confirmed the  $\alpha$ -helical nature and organisation of the  $\alpha$ -helices making up the ligand binding domain. The binding of active 1,25D into the pocket of the LBD demonstrates that 1,25D makes several contacts with helices 3,6, 7, and 11 which induces the necessary conformational changes critical for the stabilisation of the activation helix (helix 12), critical for interaction with coactivators (Rochel *et al.*, 2000). The elucidation of the crystal structure for the VDR will now pave the way for the engineering of 1,25D analogues that separate

the antiproliferative and differentiative effects from the unwanted hypercalcemic side effects.

### **1.3.1 Regulation of the Vitamin D Receptor**

Modulation of VDR levels is clearly an important factor governing the expression of vitamin D<sub>3</sub> target genes by 1,25D. 1,25D has been shown to increase VDR protein in a number of cultured cell systems of both of normal and cancerous origin. These include, rat and human osteosarcoma cells, human breast cancer cells, mouse fibroblasts, mouse adipose cells, human HL-60 promyelocytic leukemia cells and human colon cancer cells (Krishnan and Feldman, 1997; Issa *et al.*, 1998). The gene promoters for the mouse VDR (Jehan and DeLuca, 2000), chicken VDR (Lu *et al.*, 2000) and human VDR (Miyamoto *et al.*, 1997) have been cloned. The mouse VDR promoter for example lacks a TATA box and the expression of VDR promoter-constructs in NIH-3T3 cells appears to be driven by multiple Sp1 sites localised in the promoter (Jehan and DeLuca, 1997). Furthermore, the activity of the promoter was enhanced 3-5-fold in response to forskolin, a PKA activator (Jehan and DeLuca, 1997). The chicken and human VDR promoter lack a TATA box and are mainly under the control of multiple Sp1 sites (Lu *et al.*, 2000; Miyamoto *et al.*, 1997). Reporter gene analysis of a 4.0kb fragment of the human VDR promoter in Caco-2 cells demonstrated that the homeodomain protein-related caudal (Cdx-2) positively regulated the expression of these constructs. Furthermore, mutagenesis of the Cdx-2 binding site and antibody EMSA experiments established that Cdx-2 binding to the VDR promoter was essential for its transcriptional activity (Yamamoto *et al.*, 1999). A kruppel-type transcription factor termed ZEB, has also been shown to bind to two sites in the promoter of the VDR gene (Lazarova *et al.*, 2001). Exogenous expression of ZEB into SW620 colon carcinoma cells up-regulated the expression of endogenous VDR (Lazarova *et al.*, 2001). The regulation of VDR promoter activity appears complex and as a result cell-, and a species-type difference in the regulation of VDR expression exists.

### **1.3.2 Phosphorylation of the Vitamin D Receptor**

The sites and the biological outcomes of phosphorylation of the VDR are controversial and at present are unclear. Evidence *in vitro* and *in vivo* indicates

that the VDR is phosphorylated at two sites in the VDR. These two sites are serine-51, located between the two zinc fingers and serine-208 in the C-terminal domain (Figure 1.3). Hsieh *et al.* (1993) identified ser-51 as a phosphorylation site in the human VDR (hVDR). *In vitro* kinase assays using VDR immunoprecipitated from ROS 17/2.8 cells demonstrated that PKC- $\beta$  phosphorylated VDR, an effect not further enhanced by the addition of 1,25D. Mutagenesis of ser-51 to glycine abolished the phosphorylation VDR by PKC- $\beta$  (Hsieh *et al.*, 1993). *In vivo*, exposure of 1,25D treated CV-1 cells to the phorbol ester PMA, a PKC activator, led to an increase in  $^{32}\text{P}$  incorporation into wild type VDR but not the ser-51-glycine VDR mutant. Interestingly, Hsieh *et al.* (1993) also noted that in intact cells, hVDR is phosphorylated in the absence of PMA indicating that VDR is a substrate for other endogenous signalling kinases. Functionally, mutagenesis of ser-51 abolished VDRE-driven reporter gene activity in transfected CV-1 cells and this suggests that PKC-dependent phosphorylation of ser-51 plays a role in the transcriptional activation mechanism(s) of the hVDR (Hsieh *et al.*, 1993; Desai *et al.*, 1995).

Selective Edman degradation and site directed mutagenesis identified ser-205 as another phosphorylation site in the hVDR (Hilliard *et al.*, 1994). Functional tests of the significance of ser-205 phosphorylation demonstrated that conversion of this serine to alanine had no effect on reporter gene activity driven by VDREs from the osteocalcin, or CYP24 gene or by a consensus VDRE (Hilliard *et al.*, 1994). Interestingly, neither phosphorylation of wild-type hVDR by CK-II or the alteration of ser-208 seemed to affect DNA binding, heterodimerisation with RXR, binding affinity to 1,25D or subcellular localisation of the VDR in COS-7 cells (Jurutka *et al.*, 1996). This led Jurutka *et al.* (1996) to conclude that ser-208 phosphorylation must be a positive modulatory event that maintains the hVDR in a favourable conformation for interaction with coactivators or components of the RNA polymerase machinery. In support of this, Barletta *et al.* (2002) demonstrated that treatment of COS-7 cells with okadaic acid (a phosphatase inhibitor) led to a 4-fold enhancement over 1,25D treatment alone, of the interaction of VDR with DRIP205, a subunit of the DRIP coactivator complex. Although the kinases involved or the sites of phosphorylation were not investigated in the study by Barletta *et al.* (2002), these results highlight the important role(s) that phosphorylation events play in the nuclear function of the vitamin D receptor. Recent evidence by Dwivedi *et al.*

(2002) in our laboratory has shown that 1,25D-activated ERK1/2 does not phosphorylate hVDR in an *in vitro* kinase assay. Therefore MAP kinase phosphorylation of hVDR may not appear to play a role in the genomic signalling mediated by liganded VDR.

In conclusion the phosphorylation status is both complex and controversial and any functional outcome following phosphorylation of the VDR would appear to depend largely upon the cell type, promoter in question and the coactivator profile within the cell being examined.

### 1.3.3 Vitamin D receptor knockout

VDR knockout mice demonstrate a similar phenotype to patients with Hereditary 1,25D dihydroxyvitamin D<sub>3</sub>-resistant rickets (HVDDR II) and the symptoms observed are hypocalcemia, hyperparathyroidism, rickets, osteomalacia and alopecia (Li *et al.*, 1997). The alopecia observed in VDR knockout mice was later shown to be due to the absence of VDR during hair follicle morphogenesis and not due to the secondary toxic levels of 1,25D (Sakai *et al.*, 2001). The inability to maintain normal calcium homeostasis in VDR knockout mice is attributed to decreased calcium absorption by the intestine. Expression of genes involved in calcium absorption in the intestine such as the calcium transport protein (CaT1) and Calbindin-D9K are decreased in VDR knockout mice (Van Cromhaut *et al.*, 2001; Song *et al.*, 2003). Despite the phenotypic consequences derived from abnormalities in calcium absorption, surprisingly, when VDR knockout mice are fed a high calcium diet the development of normal bone fragility is permitted and there is normalisation of serum calcium levels. Essentially, these mice appeared morphologically normal to wild-type mice (Amling *et al.*, 1999; Van Cromhaut *et al.*, 2001; Song *et al.*, 2003).

### 1.3.4 VDR/RXR heterodimers bind to vitamin D<sub>3</sub> response elements (VDREs)

Most VDREs are composed of direct repeats of the half-site consensus 5'-AGGTCA-3' sequence separated by three nucleotides designated as DR-3. A number of DR-3 type VDREs have been characterised in the promoter regions of numerous different genes including osteopontin, osteocalcin, bone sialoprotein,

carbonic anhydrase-II and the 24-hydroxylase (CYP24) (Hausler *et al.*, 1998). The CYP24 promoter is unique with respect to all other 1,25D-regulated genes because it is the only gene that contains two functional VDR/RXR binding VDREs in its gene promoter as mentioned earlier. In addition to the more prevalent DR-3 type VDRE, VDR/RXR binding and activation from a DR-4 type and DR-6 type VDRE (Drocourt *et al.*, 2002) and also from inverted repeats separated by nine base pairs (IP-9) have been reported (Schrader *et al.*, 1995).

Evidence suggests the polarity of VDR/RXR binding to a VDRE is such that RXR occupies the 5' half-site and VDR the 3' half-site. However the polarity of VDR and RXR binding to the carbonic anhydrase-II VDRE is such that VDR occupies the 5' half-site and RXR binds to the 3'- half-site (Quelo *et al.*, 1994). Interestingly, the carbonic anhydrase-II VDRE and VDRE-1 and VDRE-2 are coded on the anti-sense strand whilst all other characterised VDREs are coded on the sense strand of DNA. Whether this is responsible for the reversed polarity of binding is unclear. Switching the polarity of VDR and RXR binding to the carbonic anhydrase-II VDRE resulted in decreased 1,25D mediated transactivation from this VDRE (Quelo *et al.*, 1994). A similar decrease in 1,25D-mediated transactivation was observed when the polarity of VDR and RXR binding to the osteocalcin VDRE was reversed (Schrader *et al.*, 1995).

In the absence of ligand, the VDR/RXR heterodimer binds to VDRE and represses basal transcription by recruiting a corepressor complex such as Alien (Polly *et al.*, 2000) or the N-CoR splice variant, RIP13 $\Delta$ 1 (Dwivedi *et al.*, 1998). The repression of basal transcription is most likely due to the recruitment of proteins with histone deacetylase (HDAC) activity which serves to facilitate a closed 'repressive' chromatin structure (Aranda and Pascual, 2001; Omdahl *et al.*, 2002). 1,25D binding to the VDR, results in a conformational change of VDR so that there is dissociation of the corepressor complex and subsequent binding of the coactivator complex as now described.

### **1.3.5 Coactivator proteins in 1,25D signalling**

The emergence of coactivators, activators of transcription, and corepressors, inhibitors of transcription, has provided valuable insight into the mechanism of gene expression by nuclear receptors. Coactivators bridge liganded receptors to the transcriptional machinery allowing gene expression to occur. Upon ligand binding, the ligand binding domain (LBD) undergoes a conformational change allowing the  $\alpha$ -helical amphipathic motif (LXXLL) from the coactivator, to dock into the hydrophobic groove in the LBD.

VDR mediated activation of gene expression is not limited to its interaction with coactivator proteins. The VDR has been shown also to interact with TAF<sub>II</sub>55 (Lavigne *et al.*, 1999), TAF<sub>II</sub>135 (Mengus *et al.*, 1997), TFIIB (Masuyama *et al.*, 1997) and the RNA polymerase holoenzyme II (Chiba *et al.*, 2000). This association is thought to be important for the recruitment or stabilisation of the transcriptional machinery at 1,25D target promoters.

To date, three distinct but related 160 kDa family members of the large steroid receptor coactivator (SRC)/nuclear receptor coactivator (NCoA) family have been identified (Leo and Chen, 2000; McKenna *et al.*, 1999; Torchia *et al.*, 1998). These include SRC-1/NCoA-1, TIF2/GRIP1/NCoA-2 and p/CIP/ACTR/RAC3. Their transcriptional enhancement effects are mediated mostly through recruitment of factors containing histone acetyltransferase activity (HAT) such as CBP, p300 and P/CAF. These proteins acetylate histones and alter the chromatin structure to allow the accessibility of transcription factors to the cognate binding site in the promoter (Collingwood *et al.*, 1999). Alternatively, ATP-dependent remodelling complexes such as SNF/SWI containing DNA dependent ATPase activity can be recruited to the VDR and further disrupt the nucleosomes to enhance transcription factor binding (Kitagawa *et al.*, 2003; Kingston and Narlikar, 1999; Yudkovsky *et al.*, 1999). Proceeding ligand binding, the resultant conformational change allows the dissociation of the corepressor complex containing histone deacetylase activity and the association of liganded receptor with coactivator proteins from the SRC/p160 family. The SRC family of coactivators recruit to the promoter proteins with potent HAT activity (notably CBP/p300) and also more the recently identified proteins, CARM1 and PRMT, that possess methyltransferase activity (Koh *et al.*, 2001). This results in the loosening of the repressive chromatin structure at the

promoter and the concomitant recruitment and stabilisation of the RNA pol II transcriptional machinery.

Various coactivators have been shown to functionally interact with VDR in a ligand and AF-2 dependent manner. These are SRC-1 (Ding *et al.*, 1998; Gill *et al.*, 1998; Liu *et al.*, 2000), GRIP-1 (Ding *et al.*, 1998; Liu *et al.*, 2000), RAC-3 (Leo *et al.*, 2000), ACTR (Jimenez-Lara and Aranda, 1999) and CBP (Jimenez-Lara and Aranda, 1999). Nuclear receptor coactivator-62 (NcoA-62) was cloned recently and is structurally distinct from the p160 coactivator family. Interestingly, the ability of NcoA-62 to interact and enhance 1,25D mediated transcription of the VDR (Baudino *et al.*, 1998; MacDonald *et al.*, 2001) occurred in an AF-2 independent manner. The interaction of NcoA-62 was mediated with helix-10 residues of the VDR (Barry *et al.*, 2003). Together, GRIP-1 and NcoA-62 both bind to the VDR and synergistically enhance ligand dependent VDR transactivation (Barry *et al.*, 2003). MacDonald *et al.* (2001) also demonstrated using GST-pulldown experiments that VDR forms a ternary complex with NcoA-62 and GRIP-1 and with NcoA-62 and SRC-1.

A surprising event in the VDR/coactivator field was the identification a large multisubunit coactivator complex that has no intrinsic HAT activity, termed the vitamin D receptor interacting protein (DRIP) or thyroid hormone receptor activating complex (TRAP) (Rachez *et al.*, 1998). The DRIP complex is recruited to the LBD AF-2 in response to 1,25D binding and a single subunit, DRIP-205, interacts via its two LXXLL motifs, in a manner similar to p160 coactivators, with VDR and RXR (Rachez *et al.*, 1999; Rachez *et al.*, 2000). Furthermore 1,25D mediated transcriptional enhancement by DRIP-205 (and its subunits) is further enhanced in the presence of the phosphatase inhibitor, okadaic acid (Barletta *et al.*, 2002). Several of the DRIP subunits were identified as components of the mediator complex, a complex of proteins that associate with the RNA polymerase II (Rachez *et al.*, 1999). This suggests that the DRIP complex might function in part by targeting the RNA polymerase to promoters. Whether the p160 coactivator family of proteins act first followed by the recruitment of the DRIP complex, or whether these complexes act alone is under investigation. For example, chromatin immunoprecipitation experiments have demonstrated that activation of the estrogen receptor (ER) involves cyclic association of liganded ER with the p160

coactivator AIB1 and the DRIP equivalent, PBP (Shang *et al.*, 2000). Recently, a cyclic pattern of promoter occupancy by liganded VDR, SRC-1 and NCoA-62 was observed on endogenous 1,25D target genes in ROS 17/2.8 cells (Zhang *et al.*, 2003). These results support a combinatorial mechanism for the action of p160 coactivators and the DRIP/TRAP complex. By contrast, another study examining ER activation noted a cyclic association and dissociation of coactivator proteins. Burakov *et al.* (2002) observed that recruitment of p160 coactivators and DRIP occurred in opposite phases suggesting that a sequential model of coactivator complex assembly at target promoters also occurs.

The physiological significance for the role of coactivators in various different and discrete signalling systems is unclear. For example, mice with targeted ablation of the GRIP-1 gene display testicular defects whilst mice with targeted deletion of the SRC-1 gene have no testicular defects (Grehin *et al.*, 2002). Similarly, deletion of the DRIP-205/TRAP220 subunit resulted in an attenuated thyroid hormone receptor response but retinoic acid receptor responses were not affected (Ito *et al.*, 2000a). Therefore functional redundancy may exist in the absence of DRIP-205. What has been disappointing is that 1,25D responses in all coactivator knockout models have not yet been assessed. The relevance of SRC and DRIPs in VDR mediated transcription *in vivo* is currently unknown.

### **1.3.6 Non-genomic signalling by 1,25D**

Genomic activation of 1,25D target genes in the nucleus of cells occurs via the nuclear VDR as described earlier. The non-genomic signalling of 1,25D is rapid (within minutes) and occurs outside the nucleus and involves the activation of different signalling pathways (Fleet, 1999; Revelli *et al.*, 1998). Non-genomic signalling by 1,25D has been widely documented and is currently an area under extensive investigation and controversy. Non-genomic signalling by other members of the nuclear receptor superfamily has also been demonstrated (Razandi *et al.*, 1999; Revelli *et al.*, 1998). 1,25D has been demonstrated in various cell types to increase the activity of PKC and PKA (Larsson and Nemere, 2003), tyrosine kinases (c-Src) (Morelli *et al.*, 2000; Buitrago *et al.*, 2002) PI-3K

(Buitrago *et al.*, 2002) and MAPK (Dwivedi *et al.*, 2002; Buitrago *et al.*, 2001; Solomon *et al.*, 1999).

Nemere *et al.* (1998) presented evidence for a 66kDa membrane bound vitamin D receptor which binds 1,25D and results in a rapid increase in PKC activity in resting and growing endochondrial cells and chick intestinal cells (Nemere *et al.*, 2000). Antibody Ab 099, raised against the putative membrane VDR, when incubated with membrane fractions from chondrocytes and chick intestinal cells, ablated 1,25D mediated PKC activation (Nemere *et al.*, 1998; Nemere *et al.*, 2000). However, antibody 9A7, directed against the nuclear VDR had no effect on the 1,25D induced increase in PKC activity. So far, these authors have not published the procedures for the generation of these antibodies and have not yet cloned or identified the amino acid sequence of the apparently novel receptor. However, these results do suggest that two distinct 1,25D receptors may exist. This is further supported by the fact that 6-s-cis locked analogues of 1,25D are preferred ligands for the putative membrane VDR rather than the nuclear VDR (Norman *et al.*, 2001 and references within). VDR knockout mice were thought to provide the pivotal clues as to whether the gene for the nuclear VDR also encoded the putative membrane VDR. These studies are also controversial. Erben *et al.* (2002) generated VDR knockout mice by deletion of the first zinc finger of the DNA binding domain of the VDR. Non-genomic responses such as an increase in intracellular calcium levels were abrogated in osteoblast cells derived from these knockout mice (Erben *et al.*, 2002). Interestingly, osteoblasts prepared from mice in which the second zinc finger of the DNA binding domain was deleted (Li *et al.*, 1997) displayed non-genomic activities in response to 1,25D (Wali *et al.*, 2003).

Recent studies with the ER have provided evidence that ER is recruited to the plasma membrane through interaction with caveolin proteins that serve as scaffolds (Razandi *et al.*, 2003; Razandi *et al.*, 2002). G-proteins, src and raf, for example, are recruited to the plasma membrane via these scaffold proteins so that ER in response to estrogen can rapidly modulate a variety of signalling pathways. This appears to be consistent with the non-genomic actions reported for 1,25D. Recent work by Dwivedi *et al.* (2002) has shown that 1,25D activates ERK1/2 and ERK5 kinase activity in COS-1 cells. The non-genomic activation of ERK1/2 and ERK5 leads to the phosphorylation of nuclear RXR and Ets-1, respectively, and

consequently the enhancement of CYP24 gene expression (Dwivedi *et al.*, 2002). Therefore, 1,25D activated non-genomic signalling acts to increase 1,25D mediated genomic signalling.

#### 1.4 Physiological role of CYP27B1

As mentioned, CYP27B1 is an hydroxylase that catalyses the rate limiting step in 1,25D synthesis by converting 25(OH)D<sub>3</sub> to 1,25D (Brenza *et al.*, 1998; Horst and Reinhardt, 1997). CYP27B1 activity is localised to the mitochondrial inner membrane. CYP27B1 forms part of a mini electron transport chain consisting of Ferredoxin-Reductase and Ferredoxin (an iron-sulphur containing protein) that uses electrons derived from NADPH to reduce molecular oxygen to water and to the hydroxyl group to be incorporated into carbon-1 (C-1) of 25(OH)D<sub>3</sub> or 24,25(OH)<sub>2</sub>D<sub>3</sub> (Henry, 1997; Omdahl *et al.*, 2002; Sawada *et al.*, 1999). The rate of formation of biologically active 1,25D from 25(OH)D<sub>3</sub> is significantly greater than the formation of 1,24,25(OH)<sub>3</sub>D<sub>3</sub> despite the fact that the CYP27B1 preferred substrate is the metabolite 24,25(OH)<sub>2</sub>D<sub>3</sub> (Sawada *et al.*, 1999; Omdahl *et al.*, 2002). This is due to the fact that 25(OH)D<sub>3</sub> is present at approximately 10-fold higher concentrations compared with 24,25(OH)<sub>2</sub>D<sub>3</sub> (Omdahl *et al.*, 2002).

Initially, CYP27B1 expression was thought to be predominantly localised to renal tubular cells of the kidney. However extra-renal expression of CYP27B1 has been detected in many different cell types including placental decidual cells, bone cells, keratinocytes, blood cells, lymph nodes, colon, pancreas, brain and adrenal medulla (Kato *et al.*, 1998; Adams and Ren, 1996; Zehnder *et al.*, 2001; Hewison *et al.*, 2000). The physiological relevance of extra-renal expression is not clear but it is generally agreed that the local production of 1,25D by CYP27B1 acts in an autocrine and/or paracrine manner.

cDNA clones for mouse (Takeyama *et al.*, 1997), rat (St-Arnaud *et al.*, 1997), and human (Shinki *et al.*, 1997) CYP27B1 enzyme have been isolated. This has led to the characterisation of CYP27B1 genes (Monkawa *et al.*, 1997; Kong *et al.*, 1999b) and the promoters driving the expression of the human and mouse

CYP27B1 genes are currently under extensive investigation and this is described in the next section.

As discussed, CYP27B1 is the rate-limiting enzyme involved in the synthesis of 1,25D. The pleiotropic actions of circulating 1,25D are evident from a mouse model deficient in the CYP27B1 gene (Panda *et al.*, 2001; Dardenne *et al.*, 2001). In CYP27B1 knockout mice, no serum 1,25D was detected. These mice developed secondary hyperparathyroidism and hypocalcemia with skeletal abnormalities characteristic of rickets. There was also associated growth retardation, infertility in female mice and immune dysfunction. These abnormalities are similar to humans with the genetic disorder vitamin D dependent rickets type I (VDDR-I) or otherwise known as pseudovitamin D-deficiency rickets type I (Glorieux, 1997; Thomas *et al.*, 2000). Through linkage analysis, VDRR-1 was correlated with mutations in the CYP27B1 gene since both the VDRR-1 disease locus and the CYP1 gene were mapped to chromosome 12q13.1-q13.3 (St-Arnaud *et al.*, 1997). This form of the disease can be treated with a daily administration of 1,25D or synthetic  $1\alpha(\text{OH})\text{D}_3$  (Kato *et al.*, 1998). As discussed by St-Arnaud (1999) and Omdahl *et al.* (2002), it will be necessary to examine tissue-specific inactivation of the CYP1 gene to determine precisely the contribution of CYP27B1 enzymatic expression in 1,25D autocrine/paracrine signalling.

### **1.5 CYP27B1 regulation by Parathyroid Hormone, 1,25D and calcitonin**

This next section will briefly discuss the regulation of CYP27B1 expression by parathyroid hormone (PTH), 1,25D and calcitonin (CT).

#### **1.5.1 PTH**

Numerous studies have demonstrated that PTH is the principle regulator of CYP27B1 expression. Treatment of rats with PTH induces CYP27B1 expression in the kidney (Murayama *et al.*, 1999; Jones *et al.*, 1998). Parathyroidectomy decreases and PTH administration enhances the *in vivo* production of 1,25D (Henry and Luntao, 1989). Furthermore, PTH mediated up-regulation of CYP27B1 mRNA expression is mediated through a cAMP-dependent PKA signalling

pathway that requires new protein synthesis (Murayama *et al.*, 1999; Brenza and DeLuca, 2000; Omdahl *et al.*, 2000). PTH mediated increase in CYP27B1 mRNA expression is ablated in the presence of the PKA inhibitor, H-89 (Murayama *et al.*, 1999) or stimulated by the adenylate cyclase activator forskolin (Armbrecht *et al.*, 2003; Murayama *et al.*, 1999; Brenza and DeLuca, 2000).

Studies have been initiated into the mechanism of PTH-mediated up-regulation of CYP27B1 expression. There is evidence that the 1.4kb promoter of the CYP27B1 gene (Omdahl *et al.*, 2000) can respond to PTH and cAMP. A 1.4kb mouse CYP27B1 promoter-luciferase construct transfected into AOK-B50 cells is induced 17-fold by PTH treatment and 3-fold with forskolin (Brenza *et al.*, 1998). A similar trend was also observed in AOK-B50 cells in another study (Kong *et al.*, 1999b). Reporter gene analysis of the human CYP27B1 promoter in AOK-B50 cells showed also that PTH and forskolin enhanced expression (Brenza and DeLuca, 2000). Potential involvement of transcription factor binding sites in PTH-mediated CYP27B1 promoter activation was not examined in any of these studies. However, recent studies by Gao *et al.* (2002) identified numerous putative transcription factor binding sites in the first 305 bp of the human CYP27B1 gene promoter. Mutagenesis and reporter gene analysis in transfected AOK-B50 cells demonstrated that an Ets site, Sp1 site and a CCAAT box site were important for basal expression of the CYP27B1 promoter. Furthermore, PTH induction of CYP27B1 reporter gene constructs was reduced upon mutagenesis of the CCAAT box site (Gao *et al.*, 2002). PTH mediated induction of CYP27B1 promoter constructs, as reported previously, was dependent on a functional PKA signalling pathway but not by PKC or MAPK in AOK-B50 cells (Gao *et al.*, 2002). These results demonstrate for the first time the identity of a transcription factor involved in PTH mediated CYP27B1 promoter expression in AOK-B50 cells. Armbrecht *et al.* (2003) demonstrated that a dominant negative form of CREB abolished forskolin-mediated induction of CYP27B1 promoter constructs in AOK-B50 cells. However, Armbrecht *et al.* (2003) did not investigate the involvement of potential CREB response element binding sites (CRE) in the CYP27B1 promoter.

### **1.5.2 CYP27B1 control by 1,25D**

Regulation of CYP27B1 expression by 1,25D has been shown both *in vivo* and *in vitro*. *In vitro*, 1,25D treatment of cultured kidney cells was shown to inhibit both PTH stimulated and non-PTH stimulated CYP27B1 gene expression (Murayama *et al.*, 1999; Armbrecht *et al.*, 2003; Kong *et al.*, 1999b; Brenza and DeLuca, 2000). The expression of CYP27B1 mRNA by 1,25D is repressed in wild-type and heterozygous VDR mice but not in VDR knockout mice (Takeyama *et al.*, 1997; Murayama *et al.*, 1999). Administration of 1,25D inhibited CYP27B1 expression in the kidney of wild-type VDR mice but not in VDR knockout mice (Takeyama *et al.*, 1997) These results imply that the negative feedback regulation of CYP27B1 expression in response to 1,25D requires the liganded VDR.

A negative VDRE (nVDRE) of the DR-3 type has been identified in the PTH and PTH related peptide gene promoters (Nishishita *et al.*, 1998; Koszewski *et al.*, 2000; Demay *et al.*, 1992) This VDRE deviates slightly in sequence to positively acting VDRE's (Koszewski *et al.*, 2000). The region of the CYP27B1 promoter mediating 1,25D dependent repression has been noted (Kong *et al.*, 1999b). Recent work has proposed a novel mechanism of repression in which VDIR bound to the CYP27B1 promoter facilitates the recruitment of a corepressor in the presence of 1,25D (Murayama *et al.*, 2001). In keratinocytes, an alternative mechanism of 1,25D repression exists. It appears that 1,25D mediated repression of CYP27B1 enzyme activity does not affect CYP27B1 mRNA or protein levels (Xie *et al.*, 2002). Rather it inhibition of CYP27B1 activity is mediated through a lack of substrate 25(OH)D<sub>3</sub> through catabolism by induced CYP24. In this regard, 1,25D mediated repression of CYP27B1 activity can be reversed upon selective inhibition of CYP24 enzyme activity by VID 400 (Xie *et al.*, 2002).

### 1.5.3 CYP27B1 control by Calcitonin

Calcitonin is a 34-amino acid peptide secreted by the C-cells of the thyroid gland and is important for calcium homeostasis (Jones *et al.*, 1998). Calcitonin has hypocalcemic actions so that when plasma calcium levels rise, calcitonin is secreted and binds to osteoclasts and osteoblasts to prevent the mobilisation of calcium from bone (DeLuca and Cantorna, 2001; Jones *et al.*, 1998). However, *in*

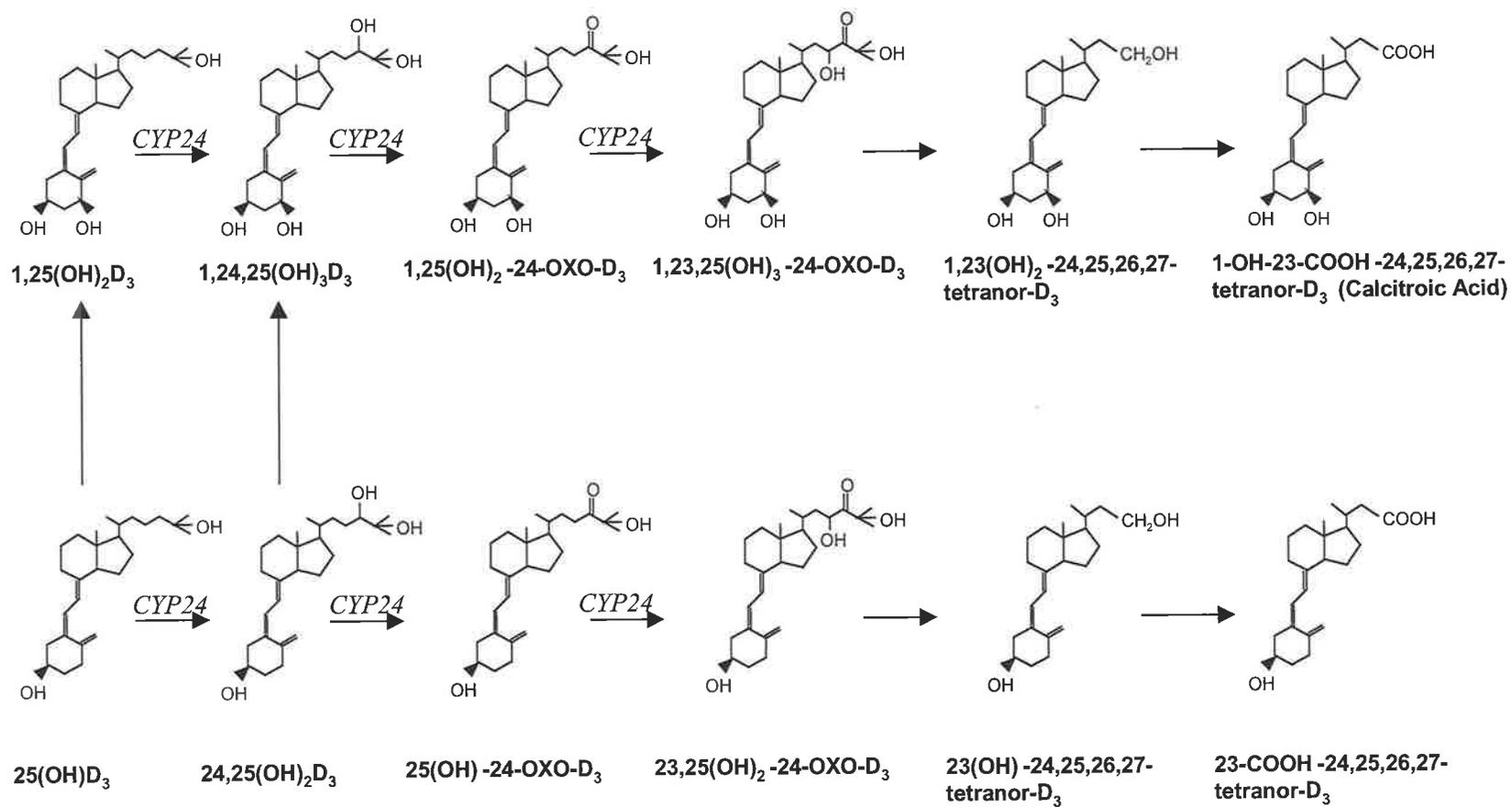
*in vivo*, calcitonin up-regulates the expression of CYP27B1 mRNA levels in mouse kidneys (Murayama *et al.*, 1999) and in normocalcemic and thyroparathyroidectomised rats (Shinki *et al.*, 1999). Furthermore, expression of CYP27B1 in mouse proximal tubule cells and porcine kidney cells was increased upon calcitonin treatment (Murayama *et al.*, 1999; Yoshida *et al.*, 1999). The mechanism and physiological relevance by which calcitonin up-regulates CYP27B1 mRNA synthesis is unclear.

An alternative mechanism for the hypocalcemic effect of calcitonin has been recently described. Gao *et al.* (2003, submitted) demonstrated that calcitonin acts to decrease serum calcium levels through up-regulating CYP24 expression in kidney cells. The regions underlying calcitonin responsiveness of the CYP24 were also identified (Gao *et al.*, 2003, submitted) and are described later.

## **1.6 Function and properties of CYP24**

### **1.6.1 Catabolism of 1,25D**

As discussed, CYP24 directs the synthesis of  $24,25(\text{OH})_2\text{D}_3$  and  $1,24,25(\text{OH})_3\text{D}_3$  from  $25(\text{OH})\text{D}_3$  and  $1,25(\text{OH})_2\text{D}_3$ , respectively. Both of these metabolites represent initial reactants of the C-23/C24 oxidation pathway that is catalysed by CYP24 and leads to the metabolic inactivation of 1,25D. The acquisition of a hydroxylated C-24 is critical for the entry and commitment of either of the above metabolites to the oxidation pathway. Oxidation of 1,25D by the C-24 oxidation pathway is the preferential activity observed in the rat kidney whilst in humans and guinea pigs, the C-23 pathway is predominantly expressed (Omdahl *et al.*, 2001; Omdahl and May, 1997; Siu-Caldera *et al.*, 1999). As shown in Figure 1.4 the initial step of 1,25D C-24 hydroxylation results in the production of  $1,24,25(\text{OH})_3\text{D}_3$ , which probably has no biological activity. This is followed by oxidation of the hydroxyl group to a keto (OXO). Subsequent steps involve the 23-hydroxylation of the 24-OXO metabolites and oxidative cleavage between C-23 and C-24 followed by oxidation to 23-COOH-24, 25,26,27-tetranor- $\text{D}_3$  or more commonly calcitroic acid (Omdahl *et al.*, 2001; Omdahl and May, 1997; St-Arnaud, 1999). Calcitroic acid is filtered and excreted from the kidney.



**Figure 1.4 The C-24 oxidation pathway catalysed by 25-hydroxyvitaminD<sub>3</sub> 24-hydroxylase (CYP24).**

Two pathways C-24/C-23 showing the metabolism of 1,25(OH)<sub>2</sub>D<sub>3</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub> to side chain cleaved 23-COOH water soluble calcitroic acid. The acquisition of a hydroxylated C-24 metabolite is crucial for entry into the C-24 oxidation pathway.

An alternative pathway for the metabolism of active 1,25D has been discovered in different cell types (Siu-Caldera *et al.*, 1999; Bischof *et al.*, 1998; Brown *et al.*, 1999). It appears that the C-3 epimerisation pathway may be an alternate pathway to the C-23/C-24 oxidation pathway to inactivate biologically active 1,25D including analogues of 1,25D (Reddy *et al.*, 2000; Kamao *et al.*, 2003). This may be supported by the fact that 50% of CYP24 null mice were unaffected by a deficiency in metabolism of active 1,25D (St-Arnaud *et al.*, 2000). The C-3 epimerisation pathway involves the epimerisation of the hydroxyl group at C-3 of the A-ring of 1,25D leading to the formation of the product  $1\alpha,25(\text{OH})_2\text{-3-epi-D}_3$  (Siu-Caldera *et al.*, 1999).  $1\alpha,25(\text{OH})_2\text{-3-epi-D}_3$  has a much lower affinity for the VDR and consequently reduced biological activity compared with 1,25D (Kamao *et al.*, 2003). It appears that neither CYP24 nor other P450 enzymes are involved in this process because the production of  $1,25(\text{OH})_2\text{-3-epi-D}_3$  is not blocked by cytochrome P450 inhibitors (Astecker *et al.*, 2000). The identity of the enzyme(s) involved in this process remains unclear.

### **1.6.2 Tissue distribution of CYP24**

The cDNA clones for CYP24 from the rat (Ohyama *et al.*, 1991), human (Chen *et al.*, 1993), pig (Zierold *et al.*, 2000), chicken (Jehan *et al.*, 1998) and mouse (Akeno *et al.*, 1997) have been reported. The human CYP24 gene maps to chromosome 20 q13.2-q13.3 (Hahn *et al.*, 1993). Interestingly, no known diseases have been attributed to this gene or its chromosomal locus. Quantitative measurement of DNA copy number across amplified regions using array comparative genomic hybridisation led to the identification of CYP24 as a potential oncogene in breast cancer cells (Albertson *et al.*, 2000). CYP24 over-expression in cancerous cell types could abrogate growth control mediated by 1,25D. The major site of CYP24 expression is the kidney (Akeno *et al.*, 1997). CYP24 expression is not limited to the kidney and is expressed in probably all cell types including intestine, skin, thymus, bone, spleen, pancreas and heart (Akeno *et al.*, 1997; Omdahl *et al.*, 2002; Yang *et al.*, 1999). The ubiquitous expression of CYP24 coupled to the broad expression of VDR (Omdahl and May, 1997), suggests an important and general role for both serum and cellular 1,25D levels in cellular physiology.

The *in vivo* role for CYP24 activity was highlighted in CYP24 null mice (St-Arnaud *et al.*, 2000). Genotyping of homozygous mutant mice demonstrated that 50% died before three weeks of age. Surviving homozygous mice had impaired 1,25D catabolism and exhibited high circulating levels of 1,25D. 1,25D target genes were also elevated 2-4-fold above wild-type and heterozygous mice. These results highlight the importance of CYP24 in regulating 1,25D levels. CYP24 null mice have impaired intramembraneous bone formation indicating a role for CYP24 in bone formation and homeostasis. This also raised the possibility that a deficiency of a CYP24 oxidation product, 24, 25(OH)<sub>2</sub>D<sub>3</sub> may be responsible for this defect (Kato *et al.*, 1998; Boyan *et al.*, 1997). The abnormal bone phenotype could be rescued by crossing CYP24 null mice with VDR null mice (St-Arnaud *et al.*, 2000). This demonstrated that elevated 1,25D levels signalling through the VDR and not a deficiency in 24,25(OH)<sub>2</sub>D<sub>3</sub>, was the cause of the aberrant bone phenotype. Although the cellular proliferation or differentiation capability of cells from CYP24 null mice was not examined, collectively, the results from the CYP24 null mice suggest that catabolism of active 1,25D is the major role of CYP24.

Recently, transgenic rats constitutively expressing the CYP24 gene have been generated (Kasuga *et al.*, 2002). Surprisingly, these rats had low circulating levels of 24,25(OH)<sub>2</sub>D<sub>3</sub> and normal levels of 1,25D compared with wildtype rats. These results are difficult to interpret without a more detailed investigation of biochemical serum parameters such as 25(OH)D<sub>3</sub> and PTH levels for example. These rats also developed albuminuria and hyperlipidemia with an increase in atherosclerotic lesions (Kasuga *et al.*, 2002). The constitutive expression of CYP24 activity in rats appears to highlight new functions for CYP24 other than the regulation of 1,25D catabolism.

### **1.6.3 Regulation of CYP24 by Parathyroid Hormone (PTH)**

1,25D and PTH, acting in a reciprocal manner, are the key physiological regulators of CYP24 expression (Jones *et al.*, 1998; Omdahl and May, 1997). During elevated PTH levels which favour 1,25D synthesis (hence CYP27B1 activation),

CYP24 activity in the kidney is inhibited. It has been shown that PTH inhibits 1,25D induced CYP24 mRNA expression in the porcine kidney cell line AOK-B50 (Zierold *et al.*, 2000). PTH mediated 1,25D inhibition of CYP24 activity is absent in intestinal cells because they lack the expression of PTH receptors (Shinki *et al.*, 1992). This action can be mimicked by forskolin or cAMP (Shinki *et al.*, 1992) but the inhibitory effect is not due to a decrease in VDR levels. Furthermore, 1.4 kb CYP24 promoter constructs were insensitive to the inhibitory effects by PTH suggesting that the PTH may be acting post-transcriptionally (Zierold *et al.*, 2000).

The analysis of CYP24 mRNA in AOK-B50 cells demonstrated that PTH down-regulated 1,25D induced CYP24 mRNA through altering mRNA stability (Zierold *et al.*, 2001). The mechanism and the regions underlying the inhibitory effect by PTH remain unclear. In contrast to kidney cells, PTH and 1,25D act synergistically to enhance CYP24 gene expression. PTH treatment alone of UMR-106 cells has no effect on CYP24 activity (Armbrecht *et al.*, 1998). PTH in a PKA dependent manner together with 1,25D, synergistically up-regulates CYP24 promoter activity, mRNA and protein levels. A similar effect is observed with PTH and 1,25D cotreatment (Armbrecht *et al.*, 1998). The physiological relevance of this mode of regulation in osteoblast cells is not clear, but the recent demonstration for the importance of CYP24 metabolising activity in the bones of CYP24 null mice has been established. Further work is required to fully clarify the reciprocal mechanisms by which PTH and 1,25D act to regulate CYP24 expression.

#### **1.6.4 Regulation of CYP24 by calcitonin**

As previously discussed, the hypocalcemic action of calcitonin together with its ability to up-regulate CYP27B1 activity is perplexing and difficult to answer in a physiological sense. A mechanism has been recently proposed for the first time that suggests calcitonin acts to lower serum calcium levels through up-regulating CYP24 expression and therefore limiting the action of 1,25D.

Studies by Gao *et al.* (2003, submitted) have shown that the expression of CYP24 promoter constructs transiently and stably transfected into 293T cells is enhanced in response to calcitonin. A GC box (-114/-101) and a CCAAT box (-62/-51)

binding Sp1 and NF-Y respectively, were shown to underlie both basal and calcitonin induced expression of CYP24 promoter constructs. Over-expression of a dominant negative clone of NF-Y strongly reduced both basal and calcitonin induced CYP24 promoter activity. Interestingly, calcitonin induction of CYP24 promoter constructs is dependent on PKA and PKC activities with the inhibitors H89 and calphostin C lowering calcitonin induced activity to 50-60% (Gao *et al.*, 2003, submitted). As previously discussed, calcitonin up-regulates CYP27B1 activity (Murayama *et al.*, 1999; Yoshida *et al.*, 1999) and also CYP24, a situation which appears paradoxical. However the studies by Gao *et al.* (2003) also showed that calcitonin together with 1,25D results in a large synergistic activation of CYP24 promoter constructs. This synergy may *in vivo* be responsible for ensuring that 1,25D levels are kept low in response to calcitonin production. Studies such as these are pivotal towards understanding the mechanistic of how calcitonin and 1,25D interact to regulate serum calcium levels.

### **1.6.5 Regulation of CYP24 by dexamethasone**

Chronic glucocorticoid therapy causes rapid bone loss and clinical osteoporosis. Studies were undertaken to examine the effect of the potent glucocorticoid, dexamethasone, on the expression of CYP24 in mice fed a normal calcium diet and mice on a vitamin D deficient (low calcium diet). Both CYP24 mRNA and enzyme activity were increased in the kidney of dexamethasone-treated mice fed normal calcium diets or low calcium diets (Akeno *et al.*, 2000). Similarly, dexamethasone (together with 1,25D) increased CYP24 mRNA and enzyme activity in AOK-B50 cells and in UMR-106 cells and this can be inhibited by pre-treatment of UMR-106 cells with the protein synthesis inhibitor cycloheximide (Kurahashi *et al.*, 2003). Dexamethasone treatment was concurrently shown to increase c-Fos and c-Jun mRNA, an effect mimicked by PMA and abolished by the PKC inhibitor, RO31-8220 (Kurahashi *et al.*, 2003). Therefore dexamethasone in the presence of 1,25D enhances the expression of CYP24 in a mechanism that may appear to require the activation of the AP-1 transcription factor. Whether AP-1 activates the CYP24 promoter or other genes involved in the 1,25D inductive process is not known. Furthermore, the physiological relevance of such an interaction between 1,25D and dexamethasone is unclear.

### **1.6.6 Regulation of CYP24 by Dietary Phosphate and Genistein**

Dietary phosphate deprivation induces CYP27B1 mRNA and protein levels as well as serum 1,25D levels (Yoshida *et al.*, 2001) but the expression of CYP24 mRNA levels are reduced (Wu *et al.*, 1996) through alteration of VDR levels. The mechanism by which dietary phosphate alters VDR expression remains to be established.

A recent study has demonstrated that the isoflavonoid, genistein (a tyrosine kinase inhibitor) potently inhibits CYP24 activity in the prostate cancer cells DU-145 and PC-3 (Farhan *et al.*, 2003). Interestingly, genistein inhibited 1,25D dependent transcriptional activation of not only CYP24 but also CYP1 in DU-145 and PC-3 cells (Farhan *et al.*, 2003). Furthermore, genistein mediated transcriptional inhibition of CYP27B1 activity involves histone deacetylase activity (HDAC) because TSA reversed the inhibitory effect of genistein treatment. In contrast, it was difficult to determine if HDAC activity was involved in CYP24 mediated inhibition since TSA alone appeared to repress CYP24 activity but together synergised to inhibit CYP24 activity in prostate cancer cells (Farhan *et al.*, 2003). Therefore, the synergistic inhibition of CYP24 activity with TSA and genistein coupled with the TSA mediated rescue of genistein mediated inhibition of CYP27B1 activity could potentially increase the responsiveness of prostate cancer cells during therapy to the antiproliferative effects of 1,25D.

Selective inhibitors of CYP24 activity are proving to be useful therapeutic tools. The recently synthesisedazole inhibitors, VID400 and SDZ 89-443, specifically and effectively antagonise CYP24 activity in keratinocytes leaving CYP27B1 activity unaffected (Schuster *et al.*, 2001). As suggested by Schuster *et al.* (2001) these powerful CYP24 inhibitors may become successful therapeutic agents especially for the topical treatment of skin disorders such as psoriasis (DiSepio *et al.*, 1999). Topical application of the CYP24 inhibitor would lead to a local increase in 1,25D levels and activity thereby regulating keratinocyte growth and differentiation.

### **1.6.7 Control of CYP24 by 1,25D**

An overwhelming amount of evidence exists that demonstrates both *in vitro* and *in vivo* that 1,25D is a most potent regulator of CYP24 gene expression. The mechanism by which 1,25D activates the CYP24 promoter is under extensive investigation. It is clear that up-regulation of the CYP24 gene in response to high 1,25D levels requires the VDR since VDR null mice lack CYP24 expression (Takeyama *et al.*, 1997). Two vitamin D response elements (VDRE's), termed VDRE-1 and VDRE-2, have been characterised in the human and rat CYP24 gene promoters (Chen and DeLuca, 1995; Ohyama *et al.*, 1996; Zierold *et al.*, 1995; Hahn *et al.*, 1994; Kerry *et al.*, 1996) and are under investigation in our laboratory. As shown in Figure 1.5, these VDREs consist of a direct repeat of the half-site consensus 5'-AGGTCA-3' separated by three nucleotides. The proximal VDRE-1 at -136/-150 (5'-AGGTGAgtgAGGGCG-3') and the distal VDRE-2 at -244/-258 (5'-GGTTCAcgcGGTGCG-3') are separated by approximately 100 bp. The presence of two functional VDRE's is unique to the CYP24 promoter and as far as we are aware, no other 1,25D target gene contains two VDRE's in its promoter. Together these two VDRE's synergistically activate the CYP24 gene promoter in response to 1,25D. Electrophoretic mobility shift assays (EMSA) employing antibodies demonstrated that VDR/RXR bind to VDRE-1 and VDRE-2 (Kerry *et al.*, 1996). Interestingly, oligonucleotides encompassing VDRE-1 bound VDR/RXR with significant less affinity compared with an oligonucleotide encompassing VDRE-2 (Kerry *et al.*, 1996). Mutagenesis and reporter gene analysis demonstrated that VDRE-1 contributes significantly greater to the 1,25D inductive response in COS-1, 293T and JTC-12 cells than VDRE-2 (Kerry *et al.*, 1996). This suggested that VDRE-1 co-operating with surrounding transcription factors may underlie the greater transcriptional enhancement compared with VDRE-2.

An Ets-1 binding site (EBS) (-119/-128) located on the anti-sense strand was identified downstream of VDRE-1. The EBS was required for maximal activation of CYP24 promoter constructs in COS-1 cells (Dwivedi *et al.*, 2000; Dwivedi *et al.*, 2002). Promoter studies demonstrated that the EBS co-operated with VDRE-1 and not the more distant, VDRE-2 and that mutagenesis of the EBS does not affect the fold-level of synergy between VDRE-1 and VDRE-2 (Dwivedi *et al.*, 2000). Ets-1 interacted directly with VDR in mammalian two-hybrid assays and the binding of Ets-1, VDR and RXR to an oligonucleotide encompassing VDRE-1 and the EBS leads to the formation of a ternary complex (Dwivedi *et al.*, 2000). The EBS does



**Figure 1.5 Localisation of the proximal promoter elements in the -298 bp CYP24 promoter.**

Shown is the -298 bp region of the proximal promoter region of the rat CYP24 promoter. Highlighted also is the -186bp promoter region used in transient transfection analysis. Localised in the -298bp promoter is the proximal vitamin D<sub>3</sub> response element (VDRE-1) at position -150/-136 and VDRE-2 at position -258/-244 with respect to the transcriptional start site. Arrow indicates the VDRE hexameric half-site and their orientation. Downstream of VDRE-1 at position -128/-119 lies an Ets-1 binding site (EBS) with additional flanking sequences highlighted. An arrow below the EBS indicates its orientation. Upstream of VDRE-1, a novel binding sequence at position -171/-168 termed the CYP24 hydroxylase enhancing protein (CHEP) binding site (CBS) has been identified. Also shown is a putative GC box and a CCAAT box currently under investigation in our laboratory.

not contribute to basal expression of CYP24 promoter constructs in the absence of 1,25D. The identification of a corepressor complex bound to unliganded VDR/RXR (Dwivedi *et al.*, 1998; Polly *et al.*, 2000) may sterically hinder the binding of Ets-1.

The following sequence of events on the CYP24 promoter is proposed. Upon 1,25D binding VDR, the resultant conformational change releases the corepressor complex from VDR/RXR allowing the binding of a coactivator complex. The coactivator complex with its associated histone acetyltransferase activity (HAT) remodels the local chromatin environment. Ets-1 may interact with the coactivator complex as well as with VDR/RXR bound at VDRE-1. 1,25D treatment of COS-1 leads to the non-genomic activation of the MAPK kinases, ERK2 and ERK5 (Dwivedi *et al.*, 2002). The activation of ERK5 in response to 1,25D stimulated the phosphorylation of Ets-1 at threonine 38 (T38) which was required for maximal activation of CYP24 promoter constructs. Mutagenesis of T38 abolishes ERK5 mediated phosphorylation and 1,25D dependent stimulation of CYP24 promoter activity by Ets-1. Similarly, 1,25D activated ERK2 activity led to the phosphorylation of serine 260 of RXR. Mutagenesis of serine 260 to alanine inhibited 1,25D activation of CYP24 expression in COS-1 cells (Dwivedi *et al.*, 2002). The precise role for the phosphorylated serine 260 in the 1,25D inductive response is currently under investigation.

The search for upstream sequences capable of enhancing VDRE-1 activity led to the identification of the half-site (5'-CGGTCA-3') that when mutated, substantially lowered 1,25D induction from this VDRE (Hahn *et al.*, 1994; Ohyama *et al.*, 1996). Subsequently, an initial characterisation of this sequence was commenced during my Honours project.

Collectively the data generated from my Honours year are as follows. The sequence identified as the CBS (5'-TGTCGGTCA-3') was shown to be critical for maximal induction of CYP24 promoter constructs containing VDRE-1 in response to 1,25D. The mutagenesis of the CBS resulted in CYP24 promoter constructs almost being unable to respond to exogenous hormone. The dependency on CBS appears to be greater in ROS 17/2.8 cells than 293T cells. Mutagenesis of the sequence 5'-TGTCGGCTA-3' correlated with the loss of protein binding to this sequence using nuclear extracts prepared from ROS 17/2.8 cells and 293T cells.

Database searches revealed that the identified CBS sequence has no resemblance to any cloned transcription factor sequence and therefore appears to be a novel sequence. Closer inspection of this sequence demonstrated that the CBS appears to resemble a monomeric orphan receptor binding site. As summarised in, monomeric orphan receptors bind to half-site consensus sequences 5'-AGGTCA Table-1-3' preceded by a 5'-extension of nucleotides that differs in both length and sequence for each member of this family. A comparison with these known monomeric orphan receptor binding sequences demonstrated that the CBS is similar but not identical, suggesting that the CBS could be a possible novel monomeric orphan receptor.

## 1.7 Aims

To summarise briefly, the regulation of the CYP24 gene promoter is as follows. At VDRE-1, VDR and RXR are already bound in a repressed complex with RIP13Δ1 (Dwivedi *et al.*, 1998). Upon binding 1,25D, a conformational change in VDR is thought to take place which releases the corepressor and exposes surfaces in the ligand binding domain that favour the interaction with a co-activator(s) complex containing histone acetyltransferase activity. Interaction through protein-protein interactions with this complex is thought to result in changes of the local chromatin environment and enhancement of transcription. Ets-1 binding downstream of VDRE-1 participates in this process through interacting with liganded VDR/RXR bound at VDRE-1 and possibly through interacting with the coactivator complex. Once the coactivator complex is recruited and stabilised, transcriptional synergy arises between VDRE-1 and VDRE-2. Upstream of VDRE-1, the binding of the factor to the CBS may also participate in the stabilisation of the liganded VDR/RXR heterodimeric complex and possibly the coactivator complex. To what degree the CBS contributes to VDRE-1 activity compared with the EBS and the role of the CBS in the transcriptional synergism evident between VDRE-1 and VDRE-2 is not known.

The major aim of my PhD project is to further define and characterise the possible identity of the proteins binding to the apparently novel CBS that appears critical for

maximal 1,25D dependent transcriptional regulation of CYP24 promoter expression. This was investigated mainly using transfected ROS 17/2.8 cells with some work utilising 293T cells. This aim will be divided into three major studies. The first study will further refine the sequences necessary for function of the CBS using both native and heterologous promoter constructs. Secondly, the over-expression of various monomeric orphan receptor clones, antibody supershift EMSA experiments using and pharmacological modulators of orphan receptor function will be analysed. Lastly, the contribution of the CBS to the transcriptional control mechanism will be investigated. This will include the design of native CYP24 promoter constructs evaluating the role of the CBS in the activity of VDRE-1 S, and importantly, in the transcriptional synergy mediated by both VDRE-1 and VDRE-2. The role of the CBS in 1,25D mediated coactivator signalling was also investigated.

*CHAPTER 2*

**Materials and Methods**

## **2. MATERIALS AND METHODS**

### **2.1 MATERIALS**

#### **2.1.1 Drugs, Chemicals and Reagents**

The following reagents were used in the course of experimental procedures and their source.

Adenosine triphosphate (ATP), Sigma; 1,25 (OH)<sub>2</sub>D<sub>3</sub>, Tetrionics, Madison, USA; Accugel (19:1), National Diagnostics, Atlanta, Georgia; N, N, N, N - tetramethylethylenediamine (TEMED), Sigma; Ammonium Persulphate (APS), Sigma; Cesium Chloride, Boehringer Mannheim, Germany; Lysozyme, Sigma; Bradfords Reagent, BIO-RAD, California, USA; DOTAP Liposomal Transfection Reagent, Boehringer Mannheim; Lipofectamine PLUS™ Reagent, GibcoBRL Life Technologies; Agarose, Promega, Madison, USA; Bovine Serum Albumin (BSA) (10mg/ml), New England Biolabs; PolydI-dC, Pharmacia Biotech; 4-Hydroxytamoxifen (4-OHT), Sigma; Diethylstilbestrol (DES), Sigma; Phenylmethylsulfonyl fluoride (PMSF), Sigma; Ampicillin, Sigma; Sodium dodecyl sulphate (SDS), Sigma.

All other chemicals and reagents used in experiments were of analytical grade.

#### **2.1.2 Antibodies**

All antibodies were purchased from Santa Cruz unless otherwise stated.

Supershiftting c-Jun (sc-45), Jun B (sc-46), Jun D (sc-74), c-Fos (sc-52), Fos B (sc-48), Fra-1 (sc183), Fra-2 (sc-171), RORα1 (sc-6062).

RXRα supershiftting (4RX1D12- kind gift from Dr. Pierre Chambon, France).

VDR neutralising (IgG2b) (Affinity Bioreagents, NJ).

#### **2.1.3 Radiochemical**

[α-<sup>32</sup>P]-dCTP (10μCi/μl) was purchased from GeneWorks.

#### **2.1.4 Enzymes.**

Restriction enzymes were purchased from Pharmacia or New England Biolabs.

Other enzymes were purchased from the following sources:

E. coli. DNA polymerase I (Klenow fragment I): Geneworks.

Ribonuclease A (Rnase A): Sigma.

Pfu turbo polymerase (Pyrococcus furiosus): Stratagene, USA.

T4 DNA ligase and T4 polynucleotide kinase: Geneworks.

### **2.1.5 Buffers**

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

**TE:** 10mM Tris-HCl (pH 7.5), 0.1 mM EDTA.

**TfB1** (Transformation buffer I): 30mM KAc, 100mM RbCl, 10mM CaCl<sub>2</sub>(2H<sub>2</sub>O),

50mM MnCl<sub>2</sub> (4H<sub>2</sub>O) and 15% Glycerol.

**Tfb2** (Transformation Buffer II): MOPS (acid), 10mM RbCl, 10mM CaCl<sub>2</sub>(H<sub>2</sub>O)

and 15% Glycerol.

**Nuclear extract Lysis Buffer:** 10mM Tris-HCl (pH 7.9), 10mM KCl, 1mM

Dithiothreitol, 1.5mM MgCl<sub>2</sub> and 0.5% Nonidet NP40.

**Buffer I:** 10mM HEPES-KOH (pH 7.9), 1.5mM MgCl<sub>2</sub>, 10mM KCl, 0.5mM

Dithiothreitol and 0.2mM PMSF (added fresh).

**Buffer II:** 20mM HEPES-KOH (pH7.9), 25% Glycerol, 420mM NaCl, 1.5mM

MgCl<sub>2</sub>, 0.2mM EDTA, 0.5mM Dithiothreitol, 0.2mM PMSF (added fresh).

**TM-1 Buffer:** 25mM Tris-HCl (pH 7.6), 100mM KCl, 0.5mM Dithiothreitol, 5mM

MgCl<sub>2</sub>, 0.5mM EDTA, 10% Glycerol.

**One-Phor-All Buffer** (10x): 100mM Tris-acetate (pH 7.5), 100mM Magnesium

Acetate, 500mM Potassium Acetate.

**GTE Buffer**: 50mM Glucose, 25mM Tris-HCL (pH 8.0), 10mM EDTA.

All buffers were sterilised by autoclaving where appropriate.

**Klenow Buffer** (10x): 70mM Tris-HCL (pH 7.5), 70mM MgCl<sub>2</sub>, H<sub>2</sub>O, final volume

1ml.

**Oligonucleotide Annealing buffer** (5x): 200mM Tris-HCL, pH 7.5, 100mM MgCl<sub>2</sub>, 250mM NaCl

### 2.1.6 Vectors

pGL3 (basic) vector was purchased from Promega and contained -186bp (encompassing VDRE-1) or -298bp (encompassing VDRE-1 & 2) together with 74bp of 5' untranslated region of the native CYP24 promoter driving expression of the Luciferase Reporter gene cloned in by Dave Kerry (1996).

pRc/RSV-CBP: kind gift from David Tremethick, Australian National University, Canberra.

Human vitamin D receptor (hVDR) cDNA was a kind gift from Nigel Morrison, Gavin Institute and was cloned into the pRSV vector by Chris Hahn to generate pRSV-hVDR.

pCR 3.1-SRC-1a: kind Gift from Bert O'Malley, Bayer College of Medicine, Texas

pSG5-GRIP-1: kind gift from Michael Stallcup, University of Southern California, LA

pSG5-ROR $\alpha$  and pSG5-Rev-erb $\alpha$ : kind gift from George Muscat, Queensland;  
pCMV-SF-1: kind gift from Keith L Parker, USA; pCMX-ERR $\alpha$ , kind gift from  
Vincent Giguere, Canada; pCB6-NGFI-B, kind gift from Jeffery Milbrandt, USA.  
pDR8-TK-Luc: containing two ROREs separated by 8bp spacer upstream of  
minimal TK promoter driving expression of the firefly luciferase reporter, kind gift  
from Brigitte Fournier, Pharma Novartis, Switzerland.  
pEF-BOS-c-Jun and pEF-BOS-c-Fos: kind gift from Ismail Kola, Monash  
University, Melbourne, Australia.

### **2.1.7 Synthetic Oligonucleotides**

Oligonucleotides for QuikChange site-directed mutagenesis and gel shift assays  
were obtained from GeneWorks or Sigma.

The oligonucleotide sequences are listed below.

### **2.1.8 Sequencing Primers**

EA2 primer (binds in +74 5'-UTR of -186 & -298bp CYP24 promoter constructs:  
5'-CTC GAG CCT AAA GCC ACG GGG GAA TCA-3'

T7 primer: 5'-GATATCACTCAGCATAA-3'

pSG5 reverse: 5'-GTGATGCTATTGCTTTATTTGTAACC-3'

Rvprimer3 sequencing of pGL3-basic: 5'-CTAGCAAATAGGCTGTCCC-3'

pGL2-TK sequencing primer: 5'-TGTATCTTATGGTACTGTAAGT-3'

### **2.1.9 Site-Directed mutagenesis oligonucleotides**

Introduced sequences are underlined.

#### **Mutagenesis of VDRE-1 into VDRE-2:**

Mutagenesis of 3' half-site of VDRE-1 into VDRE-2

mV1-1: 5'-GCG CCC TCA CTG AAC CCG CTG ACT CCA TC-3'  
3'-CGC GGG AGT GAC TTG GGC GAC TGA GGT AG-5'

Mutagenesis of the 5' half-site of VDRE-1 into VDRE-2

mV1-2: 5'-GCC CCG GCG CAC CCG CTG AAC CCG CTG-3'  
3'-CGG GGC CGC GTG GGC GAC TTG GGC GAC-5'

Mutagenesis of C to A in TGTCGGTCA

CΔA: 5'-CCA GCA GCG TGT AGG TCA CCG AGG CC-3'  
3'-GGT CGT CGC ACA TCC AGT GGC TCC GG-5'

### 2.1.10 Gel Shift oligonucleotides

Sequences of binding sites are underlined.

WT-CBS: 5'-G GCG TGT CGG TCA CCG C-3'  
3'-C CGC ACA GCC AGT GGC G-5'

M1-CBS: 5'-G GCG ATG CGG TCA CCG C-3'  
3'-C CGC TAC GCC AGT GGC G-5'

M1a-CBS: 5'-GGC GAG TCG GTC ACC GC-3'  
3'-CCG CTC AGC CAG TGG CG-5'

M1b-CBS: 5'-GGC GTT TCG GTC ACC GC-3'  
3'-CCG CAA AGC CAG TGG CG-5'

M1c-CBS: 5'-GGC GTG GCG GTC ACC GC-3'  
3'-CCG CAC CGC CAG TGG GC-5'

M2-CBS: 5'-G GCG TGT AAG CTT CCG C-3'  
3'-C CGC ACA TTC GAA GGC G-5'

SV40 AP-1: 5'-TCG ACG CTT GAT GAC TCA GCC GGA ACT GCA-3'  
3'-AGC TGC GAA CTA CTG AGT CGG CCT TGA GCT-5'

WT-CBSext: 5'-GCA GCA GCG TGT CGG TCA CCG AGG CCG-3'  
3'-CGT CGT CGC ACA GCC AGT GGC TCC GGG-5'

WT C $\Delta$ A: 5'-GGC GTG TAG GTC ACC GC-3'  
3'-CCG CAC ATC CAG TGG CG-5'

RE-3: 5'-GAA TAG TGA CCC ACA GGG ACA GTC TTA C-3'  
3'-CTT ATC ACT GGG TGT CCC TGT CAG AAT G-5'

RORE: 5'-GTC GAC TCG TAT AAC TAG GTC AAG CGC TG-3'  
3'-AGC TGA GCA TAT TGA TCC AGT TCG CGA CG-5'

SFRE: 5'-GAG CAG TGG CGA TTT GTC AAG GTC ACA CAG T-3'  
3'-TCG TCA CCG CTA AAC AGT TCC AGT GTG TCA G-3'

NBRE: 5'-GTC GAG TTT TAA AAG GTC ATG CTC AAT TTG-3'  
3'-AGC TCA AAA TTT TCC AGT ACG AGT TAA ACG-5'

RevRE: 5'-GTC GAG AGT GAT AAT GTA GGT CAC GGC TG-3'  
3'-AGC TCT CAC TAT TAC ATC CAG TGC CGA CG-5'

### 2.1.11 Bacterial Strain

The following *E. coli* bacterial strain was used as a host for recombinant plasmid DNA propagation and procedures.

*E. coli*. DH5 $\alpha$ : supE44  $\Delta$ lacU169 (p80 lacZ $\Delta$ M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 host for recombinant plasmids, obtained from the *E. coli* Genetic Stock Centre, Yale University, New Haven.

### 2.1.12 Bacterial Growth media

Growth media was prepared in double distilled water and sterilised by autoclaving, and addition of liable chemicals and antibiotics were added after the solution cooled to 50°C.

**Luria (L) broth:** contained 1% (w/v) Bacto-tryptone (Difco), 0.5% (w/v) yeast extract (Difco), 1% (w/v) NaCl, adjusted to pH 7.0 with NaOH. Agar plates were prepared by adding 1.5% (w/v) Bacto-agar (Difco) to the L-broth.

**Fantastic Terrific Broth (FTB):** contained 1% Bacto-tryptone (Difco), 0.5% (w/v) yeast extract (Difco).

**Psi ( $\Psi$ ) Broth:** 2% Bacto-tryptone, 0.5% yeast extract, 0.5% MgSO<sub>4</sub> adjusted to pH 7.6 with 0.1M KOH.

Ampicillin (100µg/ml) was added where appropriate for growth of transformed bacteria to maintain selection pressure for the plasmid.

### **2.1.13 Miscellaneous.**

X-ray film: FUJI medical X-ray Film, Tokyo, Japan and KODAK Biomax Diagnostic Film, USA, was used.

Dual Luciferase™ Reporter Assay System (Promega): Madison, USA.

## **2.2 Methods**

### **2.2.1 DNA Preparation and Manipulation**

#### **General DNA Methods**

The following methods were performed as outlined in “Molecular Cloning: A Laboratory Manual” Sambrook *et al.* (1989). Growth, maintenance and preservation of bacteria: quantification of DNA: autoradiography: agarose and polyacrylamide gel electrophoresis and precipitation of DNA.

All manipulations involving viable organisms, which contained recombinant DNA, were carried out in accordance with the regulations and approval of the Australian

Academy of Science Committee on Recombinant DNA and the University Council of the University of Adelaide.

### **2.2.2 Small Scale Miniprep Plasmid Preparation**

A modification of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981) procedure utilising the alkaline-lysis method or the Ultra Clean Mini Plasmid Prep kit (MoBio Laboratories) was used to isolate plasmid DNA routinely obtained from 2ml overnight bacterial culture.

### **2.2.3 Large Scale Plasmid Preparation- Cesium Chloride method**

The generation of  $\mu\text{g}$  quantities of purified, supercoiled plasmid DNA for transient transfection analysis or preparative digests was performed using a superficially modified version by Maniatis *et al.* (1989). Briefly, 500 $\mu\text{l}$  of desired clone was inoculated into 250mls of FTB + Ampicillin and incubated overnight at 37°C with shaking. The bacterial cell suspension pelleted using a SLA-3000 rotor at 6K, at 4°C for 5 minutes. 10mls of GTE buffer and lysozyme (2mg/ml) was added to lyse the cells and incubated on ice. 20mls of freshly prepared solution II (0.2M NaOH, 1% SDS) was added and the rotor tubes inverted gently and left on ice. Then 15mls of NaAc (3M, pH 4.6) was added, mixed gently by inverting the rotor tubes and centrifuged at 6K at 4°C for 5 minutes. The supernatant was collected and passed through cloth (band-aid) and the volume recorded. Plasmid DNA was precipitated through the addition of 0.6 volumes of isopropanol and incubated on ice for 10 minutes. The precipitated plasmid DNA was pelleted at 9K, 4°C for 20 minutes and the supernatant discarded. The pelleted plasmid DNA was allowed to air dry and resuspended in 4mls of 1xTE. To separate the plasmid DNA from RNA and genomic DNA, a CsCl<sub>2</sub> density gradient was utilised. To achieve this, 1.05g of CsCl<sub>2</sub> was weighed out per ml of 1xTE and mixed thoroughly with the dissolved plasmid DNA. Following CsCl<sub>2</sub> addition, 100 $\mu\text{l}$  of ethidium bromide (10mg/ml) was mixed into the solution and placed into 2ml Beckman centrifuge tubes balanced to within 10 $\mu\text{g}$  of each other. Centrifugation was performed in a Beckman TL-100 in a TLA 100.2 rotor at 80K, 20°C, overnight. Plasmid DNA (seen as a red central band) was removed using a syringe and the ethidium bromide extracted using H<sub>2</sub>O saturated Butanol. Following ethanol precipitation, the plasmid DNA was

resuspended in 1xTE and quantified spectrometrically and authenticity reconfirmed through appropriate restriction enzyme digestion and analysis on 1% agarose gels to confirm concentration and supercoiling.

#### **2.2.4 Restriction Enzyme Digestions of DNA**

Restriction digests contained 1-4 $\mu$ l of miniprep DNA or 0.2-0.5 $\mu$ g of CsCl<sub>2</sub> DNA and were digested with 6U/ $\mu$ l of appropriate enzyme. One Phor-All Buffer concentration was used as specified by the manufacturer. All digests were performed at 37°C for 1-2 hours and termination of the reaction accomplished by the addition of 3x Loading Buffer to a final concentration of 1x. Separation of digested DNA was performed on 1% or 2% Agarose gels in 1xTBE, visualised by staining with 1 $\mu$ g/ml ethidium bromide and photographed.

#### **2.2.5 Preparation of cloning vectors**

Plasmids were linearised with the appropriate restriction enzyme(s). To prevent self-ligation of the vector, 5' terminal phosphate groups were removed by incubation in 50mM Tris-HCL, pH 9.0, 1mM ZnCl<sub>2</sub>, with 1 unit of calf intestinal phosphatase (CIP), in a final volume of 50 $\mu$ l for 15 minutes at 37°C followed by 15 minutes at 56°C. Another 1 unit of CIP was added and the incubations repeated. The vector DNA was electrophoresed on a 1% agarose TBE gel and the gel stained with ethidium bromide. The linearised vector DNA was visualised under UV light, excised and purified using the QIAEX II gel extraction kit (QIAGEN) according to the manufacturers instructions.

#### **2.2.6 Preparation of DNA restriction fragments**

DNA was incubated with appropriate restriction enzyme(s) as described in section 2.2.4, and restriction fragments were isolated using 1%-2% agarose 1xTBE gel, depending on the restriction fragment size. Bands representing the correct restriction fragment were visualised under UV light following staining with ethidium bromide and the appropriate fragment(s) excised from the gel. DNA fragments were isolated from the agarose gels using the QIAEX II gel extraction kit (QIAGEN) according to the manufacturers instructions.

### **2.2.7. Ligation of DNA**

A 10 $\mu$ l reaction contained 10ng or 20ng of prepared vector, a 3 molar excess of the insert DNA, 50mM Tris-HCL, pH 7.6, 10mM MgCl<sub>2</sub>, 1mM DTT, 5% (w/v) PEG<sub>6000</sub>, 1mM ATP, and 1 unit of T4 DNA ligase. The reactions were incubated overnight at 16°C. A control ligation with vector only was set up and included in the subsequent transformation to determine the background levels of undigested or recircularised vector DNA.

### **2.2.8 Site-Directed Mutagenesis**

Mutagenesis reactions were performed employing the method by Stratagene's Quikchange™ Site-Directed Mutagenesis Kit with oligonucleotide primer design and temperature cycling performed as recommended by manufacturer. Briefly, the Quikchange site-directed mutagenesis method employs the use of two complementary oligonucleotide primers containing the desired mutation. The oligonucleotide (125ng), each complementary to opposite strands of the vector to be mutated, are extended upon by the action of Pfu polymerase (2.5U/ $\mu$ l). Cycling parameters are 95°C for 30 seconds, 55°C for 1 minute and 68°C for 11 minutes (2 minutes/kb of plasmid length) with the reaction cycling 18 times. Incorporation of the oligonucleotides results in a nicked vector free from methylation. The end product is then digested with Dpn I (10U/ $\mu$ l), an endonuclease specific for methylated and hemimethylated DNA and digests any parental (non-mutated) vector. Following digestion, vector sequences containing desired mutation were transformed into competent DH5 $\alpha$  cells. The recovered transformants were subjected to small-scale plasmid purification to recover propagated plasmid DNA. Purified DNA was sequenced to confirm incorporation of the correct mutation.

### **2.2.9 Dye-Terminator Sequencing of PCR Products**

DNA sequencing was performed using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). In PCR tubes, 8 $\mu$ l of Big Dye-terminator mix (version 3), 1 $\mu$ l (100ng) of sequencing primer and 0.2-0.5 $\mu$ g template was made up to a final volume of 20 $\mu$ l with water and overlaid with 25 $\mu$ l

of mineral oil (Sigma). Cycling parameters used were 96°C for 30 seconds, 50°C for 15 seconds, 60°C for 4 minutes cycled 25 times in a PTC-100™ (MJ Research, Inc.). Following cycling, the product was precipitated with isopropanol. Briefly, extension products were aliquoted into a fresh eppendorf tube. To this 80µl of fresh 75% isopropanol was added and vortex briefly. Samples were left at room temperature for 15 minutes to precipitate the extension products. Following precipitation, samples were centrifuged in a microcentrifuge for 20 minutes at maximum speed. Supernatants were aspirated and 250µl of 75% isopropanol added. Tubes were vortexed again briefly and centrifuged in a microcentrifuge for 5 minutes at 13K rpm. Supernatants were aspirated and the pellets dried in a 37°C heating block. The dehydrated pellet was taken to the DNA sequencing facility at the Institute of Medical and Veterinary Science where it was analysed on an ABI Prism 3700 DNA Analyser and the sequence provided as an electropherogram.

#### **2.2.10 Preparation of Electro-competent Cells**

Electro-competent DH5α cells were made by using a modified version of Sharama and Schimke (1996) protocol that utilises the growth and preparation of competent cells in a salt-free medium reducing the probability of arcing. Briefly, 5mls of overnight culture grown in YENB, was inoculated into 500mls of YENB and grown at 37°C-shaking to an O.D<sub>600</sub> reading of 0.6. The cells were chilled on ice and then centrifuged at 4K for 10mins at 4°C. The supernatant was discarded and the pellet washed twice in 100mls of cold water and centrifuged as before. The pellet was then resuspended in 20mls of cold 10% glycerol and centrifuged as before. Finally, the pellet was resuspended in 3mls of cold 10% glycerol and aliquoted as 500µl aliquots into sterile screw-capped tubes and stored at -80°C. Electro-transformation of the cells using the PCR products was performed essentially as by Sharama and Schimke (1996) with the exception being that TB or 2YT was added to cells immediately following electroporation. Briefly, to a 40µl aliquot of electrocompetent cells, 1µl of the PCR product was added to cells and incubated on ice for 5mins. Following incubation the cells were placed into cold 0.2cm electroporation cuvettes (Bio-Rad) and electroporated (960µF, 1.8kV, 200Ω) in a Bio-Rad Gene Pulser. Immediately following electroporation, 1ml of TB or 2YT

was used to resuspend the transformed cells. The cells were then transferred to eppendorf tubes where they were incubated at 37°C for 1hr at shaking. The cells were centrifuged, 850µl of supernatant discarded and the remaining 150µl plated onto LB + Amp100 plates.

### **2.2.11 Preparation of Competent DH5α**

E.coli cells were made competent using a RbCl<sub>2</sub> and CaCl<sub>2</sub> method. Briefly, a single E.coli colony was picked and inoculated into 10mls of LB and grown overnight at 37°C shaking. 5mls was subcultured into warm Psi (Ψ) broth and grown to an O.D<sub>600</sub> of 0.6. The cells were pelleted at 4K rpm at 4°C for 5mins, supernatant discarded and resuspended in 0.4 volumes (40mls) of Tfb1 (30mM KAc, 100mM RbCl, 10mM CaCl<sub>2</sub>(2H<sub>2</sub>O), 50mM MnCl<sub>2</sub> (4H<sub>2</sub>O) and 15%Glycerol) and left on ice for 5 minutes. The cells were centrifuged again, supernatant discarded and resuspended in 0.04 volumes (4mls) of Tfb2 (MOPS (acid), 10mM RbCl<sub>2</sub>, 10mM CaCl<sub>2</sub>(H<sub>2</sub>O) and 15% Glycerol ) and left on ice for 5 minutes. The competent cells were stored as 200µl aliquots at -80°C.

### **2.2.12 Transformation of Competent Bacteria**

An aliquot, 40µl-100µl of the cells suspension prepared using the rubidium chloride method was mixed with 5µl of the ligation mix (section 2.2.7), or 1-6µl of other plasmid DNA, and left on ice for 20 minutes. The cells were heat shocked at 42°C for 3 minutes, placed on ice for 2 minutes and 500µl of sterile LB medium added. The cells were then incubated at 37°C for 20 minutes, centrifuged for 30 seconds and all but 100µl of supernatant was removed. The transformed cells were resuspended in the remaining 100µl of medium and then plated onto warmed L-agar containing 100µg/ml of ampicillin by spreading with a wire spreader. The agar plates were routinely incubated overnight at 37°C.

## **2.3 Methods for Maintenance and Transient Expression of Constructs in Tissue Culture Cell Lines**

### **2.3.1 Tissue Culture media**

**Phosphate Buffered Saline (PBS):** 136mM NaCl, 2.6mM KCl, 1.5mM  $\text{KH}_2\text{PO}_4$  and 8mM  $\text{Na}_2\text{HPO}_4$  (pH 7.4), was sterilised by autoclaving (20psi for 25minutes at 140°C).

**Trypsin/EDTA solution:** 0.1 % trypsin (Difco) and 1x EDTA Versene buffer solution (CSL), was sterilised by filtration through a 0.2 $\mu\text{m}$  filter (Whatman).

**Dulbecco's Modified Eagle's medium (DMEM)** (Gibco): 28mmol/L  $\text{NaHCO}_3$ , 19mmol/L glucose, and 20mmol/L Hepes (pH 7.3), was supplemented with 50,000 Units/L of Gentamycin (Gibco), and filter sterilised as described before.

**Ham's F12** (Gibco): was supplemented with L-Glutamine (4500mg/ml) and 28mmol/L  $\text{NaHCO}_3$  (pH 7.4), was supplemented with 50,000 Units/L of Gentamycin (Gibco), and filter sterilised as described before.

**RPMI 1640** (Gibco): with 28mmol/L  $\text{NaHCO}_3$  (pH 7.4), was supplemented with 50,000 Units/L of Gentamycin (Gibco), and filter sterilised.

**Foetal Calf Serum (FCS):** heat inactivated and batch tested (Gibco).

### **2.3.2 Tissue Culture Cell Lines**

COS-1, an adherent Green African monkey kidney cell line was obtained from the American Type Culture Collection (ATCC).

293T, an adherent human embryonic kidney cell line was obtained from Suzanne Cory (WEHF).

ROS 17/2.8, an adherent Rat osteosarcoma cell line was obtained from the Garvan Institute, Sydney.

UMR-106, an adherent rat osteosarcoma cell line was obtained from T.J. Martin, St. Vincent's Institute of Medical Research, University of Melbourne.

### **2.3.3 Maintenance of cells**

293T, COS-1, were maintained in DMEM + 10% FCS.

ROS 17/2.8 cells were maintained in DMEM + 50% Ham's F12 + 10% FCS.

Growth of all cells and cell lines was performed at 37°C with 5% CO<sub>2</sub> and humidified air.

#### **2.3.4 Passaging Cells**

Passaging of the cell lines was routinely performed upon cells reaching 90-100% confluence. All cells were washed generously with PBS to remove old media and cell debris. To dislodge cells, 1ml (75cm<sup>2</sup> flask) or 2mls (175cm<sup>2</sup> flask) of trypsin was added to the flask. Flasks containing ROS 17/2.8 cells and COS-1 cells were placed in the incubator for 1-5 minutes to allow quicker dislodgment of cells. 293T cells were incubated at room temperature for 1-2 minutes. Following incubation, flasks were tapped gently to dislodge remaining adhered cells. 10 mls of appropriate media was added to the flask to dilute trypsin enzyme activity. The 10ml cell suspension was centrifuged for 2 minutes at 1500 rpm. The supernatant was aspirated and the cell pellet resuspended in 10 mls of appropriate fresh media and aliquoted accordingly.

#### **2.3.5 Preparation of charcoal stripped foetal calf serum**

Routinely, 50mls of stripping solution (125mg Dextran, 1.2g activated charcoal (Sigma), 50mls sterile water) was added to 200mls of foetal-calf serum. This solution was stirred continuously for 1hour at 50-60°C. The solution was chilled and left overnight at 4°C. The activated charcoal was pelleted in a HB-4 rotor at 10,000rpm for 10 minutes. The supernatant was placed into a fresh rotor tube and centrifuged again to remove residual activated charcoal. The stripped foetal calf serum was filter sterilised and divided up into equal aliquots and stored at -20°C.

#### **2.3.6 Transient Transfection of 293T, COS-1 and ROS 17/2.8 cell lines**

On the morning of the transient transfection experiment, cells were dislodged from the flask (section 2.3.4) and counted using a haemocytometer. 293T cells at 8x10<sup>5</sup> cells/well, 4x10<sup>5</sup> COS-1, ROS 17/2.8 and UMR-106 cells/well were seeded into a 24-well tray in the presence of appropriate serum containing media and allowed to attach for 2-6 hours.

### 2.3.7 Preparation of DNA/DOTAP complexes

Transfections were carried out according to manufacturers instructions. The following dilution of DNA is prepared for the transfection of 6 wells (1 row) of a 24-well tray. Aseptic techniques were routinely used for the preparation of DNA and DOTAP solutions such that contamination of mammalian tissue cell cultures was prevented. A total of 1.2µg (200ng/well) of the appropriate luciferase reporter constructs and 0.3µg (50ng/well) of pRL-TK (or pRL-null) was diluted with HEPES buffer to a final volume of 35µl. In a separate tube, a total of 9µg (1.5ug/well) of DOTAP was mixed with HEPES buffer to final volume of 35µl. The DOTAP mixture was added to the DNA solution using a swirling motion to ensure even dispersion of the DOTAP mixture. The mixture was incubated at room temperature for 15 minutes to allow the formation of DNA/DOTAP complexes. For transfection, 10µl aliquots were added to each well and allowed to incubate overnight to maximise transfection efficiency. The following day, the media was aspirated and replaced with 400ul of fresh RPMI + 10% stripped FCS. Two hours later, 1,25D at a concentration of  $10^{-7}$ M was added to designated wells and vehicle to untreated wells. Cells were left to incubate for 24 hours.

### 2.3.8 Measurement of Dual Luciferase Activity (DLR)

Following the incubation period, the media was carefully aspirated from each well. To each well, 100µl of 1x Passive lysis buffer (1x PLB) was added and left to incubate at 37°C shaking (optional) for 15 minutes and DLR activity assayed. At this point, if Dual luciferase activity were to be measured at a later time, 24-well trays were wrapped in cling film and stored at -20°C until further use. For measurement of Dual luciferase activities, 10µl aliquot of the cell lysate was taken directly from a well from the 24-well tray and placed into an eppendorf tube. To measure the luminescence of the firefly luciferase, 25µl of LAR II substrate was added to the eppendorf tube and the luminescence recorded using a TD-20/20 Luminometer. The reaction was quenched by the addition of 25µl of Stop & Glo™, which concomitantly activates the control reporter (*Renilla* luciferase) allowing the measurement of the second luminescence reaction. Relative luciferase activity is a measure of the ratio of the first luminescence with the second luminescence (which represents the corrected value of luciferase activity).

## **2.4 Electrophoretic mobility shift assays (EMSA)**

### **2.4.1 Nuclear Extract Preparation**

A modified version by Andrews and Faller (1991) procedure (derived from Dignam *et al.*, 1983) utilises hypotonic lysis followed by high salt extraction to obtain DNA binding proteins. Briefly,  $2 \times 10^6$  cells (i.e. 293T or ROS 17/2.8 cells) were washed in cold PBS and pelleted for 15 seconds at room temperature. The cells were then resuspended by flicking the tube in 400 $\mu$ l of cold Buffer I and placed on ice for 15 minutes. The sample vortexed for 15 seconds and then centrifuged at 4°C for 1 minute. The supernatant fraction discarded, the pellet was resuspended in 100 $\mu$ l of cold Buffer II and placed on ice for 30 minutes (high salt extraction). Cellular debris removed by centrifugation for 5 minutes at 4°C and the supernatant fraction (containing DNA binding proteins) placed into a clean, labeled tube. Quantification of the protein sample was performed using a Bradford's Assay (see section 2.4.2).

### **2.4.2 Bradfords Assay**

The protein content of the nuclear extracts was determined using 2 $\mu$ l of nuclear extract in the Bio-Rad protein microassay procedure according to the manufacturer's instructions. Bovine Serum Albumin (BSA) at 1mg/ml was used as the standard. All extracts were stored at -80°C as small aliquot's to avoid frequent freeze/thawing events.

### **2.4.3 Annealing Single Stranded Oligonucleotides**

2.5 $\mu$ g of sense and anti-sense oligonucleotides were added to 4 $\mu$ l of (5x) annealing buffer and the reaction made up to a final volume of 20 $\mu$ l. The reaction contents were placed into a 95°C heating block for 2 minutes followed by incubation in a 65°C block to cool in at room temperature. Annealed double-stranded oligonucleotides were appropriately labeled and stored at -20°C.

### **2.4.4 End-Filling of Oligonucleotides**

Oligonucleotides shown in section 2.1.10 were end-filled with [ $\alpha^{32}\text{P}$ ]-dCTP using Klenow fragment. Briefly, 1 $\mu\text{l}$  of annealed double stranded oligonucleotide, 1 $\mu\text{l}$  Klenow fragment (2U/ $\mu\text{l}$ ), 1.5 $\mu\text{l}$  (10x) Klenow buffer, 9 $\mu\text{l}$  H<sub>2</sub>O, and 2.5 $\mu\text{l}$  ADC-2 ([ $\alpha^{32}\text{P}$ ]-dCTP) was incubated at room temperature for 30 minutes. Following incubation, labeled oligonucleotides were precipitated with 40 $\mu\text{l}$  of 100% ethanol, 2 $\mu\text{l}$  NaAc (pH 4.6) and 1 $\mu\text{g}$  of poly dl-dC at -80°C for 15 minutes and centrifuged at 4°C for 20 minutes. The precipitated oligonucleotides were then washed with 40 $\mu\text{l}$  of 70% ethanol, centrifuged, supernatant removed and pellet air dried. The oligonucleotides were resuspended in 20 $\mu\text{l}$  of 1x TE and 1 $\mu\text{l}$  counted in a scintillation counter.

#### **2.4.5 Binding Reaction**

Binding reactions typically contained 6 $\mu\text{g}$ -12 $\mu\text{g}$  of nuclear extracts, 1 $\mu\text{l}$  poly dl-dC, 6 $\mu\text{l}$  of TM-1 binding buffer in a final volume of 11 $\mu\text{l}$  and incubated on ice for 15-20 minutes. Radiolabelled oligonucleotides (100,000 cpm) were added and the reaction incubated further on ice for 20 minutes. Retarded protein-DNA complexes were resolved on a 4% non-denaturing polyacrylamide gel (pre-electrophoresed for 90mins at 110 Volts in pre-cooled 0.5x TBE at 4°C) and electrophoresed for exactly 90mins at 250 volts at 4°C. The gel was dried and exposed to FUJI medical X-ray film or KODAK Biomax diagnostic film in an intensifying screen at -80°C.

#### **2.4.6 Competition EMSA**

Gel shift competition assays were performed with unlabelled competitor at molar excess concentrations of 10x, 40x and 50x in the binding reaction prior to the addition of [ $^{32}\text{P}$ ]-labeled oligonucleotide. Retarded DNA/protein complexes were resolved as described in section 2.4.5.

#### **2.4.7 Antibody supershift assays**

For the detection of supershift assays, 1-4 $\mu\text{l}$  of supershifting antibody (specified in Figure) was added to the binding reaction and incubated for 20 minutes on ice. Following incubation, labeled probe was added and the reaction incubated for a

further 20 minutes. The reactions were electrophoresed as described in section 2.4.5.

#### **2.4.8 UV-cross linking of nuclear extracts to oligonucleotide probes**

Binding reactions were performed similar to section 2.4.5. Briefly, binding reactions containing nuclear extract incubated with [<sup>32</sup>P]-labeled oligonucleotide were scaled up 2-fold. For competition assays, a 10-fold or 40-fold molar excess of unlabeled competitor oligonucleotide was added to the binding reaction prior to the addition of probe. The tops of the screw capped tubes were covered in plastic wrap and placed 5 cm from UV light source and reactions UV cross-linked (7000μJ/cm<sup>2</sup>x100: Stratalinker, Stratagene). Following irradiation, samples were cooled on ice, 4μl of 6x SDS sample buffer added and boiled at 100°C for 5 minutes. Supernatants were fractionated using a 4% stacking and 8% running SDS-polyacrylamide gel and fractionated products compared with a recombinant full range rainbow molecular weight marker (Amersham Life Science). Following electrophoresis, the gels were dried, and exposed to film with an intensifying screen at -80°C.

**Characterisation of the protein(s) binding  
to an apparently novel transcription  
factor binding site in the 24-Hydroxylase  
(CYP24) gene promoter**

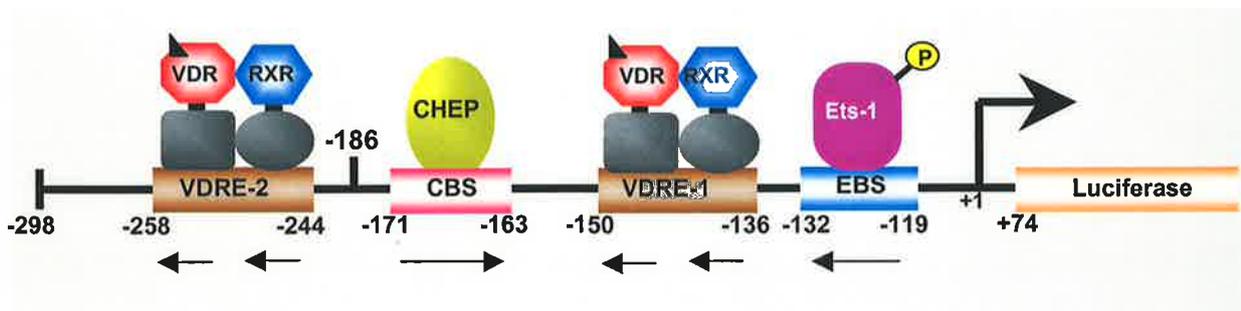
### **3. Characterisation of the protein(s) binding to an apparently novel transcription factor binding site in the 24-Hydroxylase (CYP24) gene promoter**

#### **3.1 Introduction**

The tight control of serum and cellular 1,25D levels is a prerequisite for the maintenance of correct calcium and skeletal homeostasis, cellular proliferation and differentiation, and modulation of immune function as discussed in chapter 1. A feedback mechanism whereby 1,25D bound to the VDR activates the expression of the CYP24 gene contributes to this tight control (Hahn *et al.*, 1994; Kerry *et al.*, 1996; Omdahl *et al.*, 2000). The enzymatic inactivation of 1,25D via a series of hydroxylation reactions catalysed by CYP24 ensures that the biologically active hormone is inactivated to calcitroic acid and 23-hydroxylated metabolites.

As already discussed, the rat CYP24 gene promoter is unique because it contains two vitamin D<sub>3</sub> response elements (VDREs) (Kerry *et al.*, 1996; Ohyama *et al.*, 1996; Zierold *et al.*, 1995). These two VDREs termed VDRE-1 (proximal) and VDRE-2 (distal) bind liganded VDR/RXR heterodimers, which act to drive transcription of the 24-hydroxylase gene in response to 1,25D. Transactivation and DNA binding assays have firmly established that in the presence of 1,25D, VDRE-1 is transcriptionally more potent than VDRE-2 and interestingly these two VDREs together synergise in response to 1,25D hormone (Kerry *et al.*, 1996). Strikingly the binding affinity of liganded VDR heterodimerised with RXR is approximately 5-times greater for VDRE-2 in contrast to VDRE-1 (Kerry *et al.*, 1996). This anomaly led our laboratory to hypothesize that transcription factors binding in close proximity to VDRE-1 could contribute to the 1,25D inductive process.

This led to the identification of an Ets-1 binding site on the anti-sense strand immediately 3' and adjacent to VDRE-1 (Dwivedi *et al.*, 2000) (Figure 3.1) that was shown to bind Ets-1. In a 1,25D dependent fashion a cooperative interaction between VDR/RXR and Ets-1 occurs resulting in the formation of a ternary complex that mediates the activation of the CYP24 promoter in response to 1,25D (Dwivedi *et al.*, 2000). Furthermore, non-genomic activation of MAP kinases, more



**Figure 3.1: Localisation of the various transcription factor binding sites in the rat CYP24 promoter.**

Shown is the first -298bp of the rat CYP24 proximal promoter region. Relative positions of the vitamin D<sub>3</sub> response elements (VDRE-1 and VDRE-2), Ets-1 binding site (EBS) and the CHEP binding site (CBS) are depicted. VDRE-1 and VDRE-2 both bind liganded VDR/RXR heterodimeric complexes. Ets-1 is known to be phosphorylated on threonine 38 in response to 1,25D. Also indicated is the shorter -186bp construct encompassing only the proximal VDRE, VDRE-1, used in transient transfection experiments. *Arrows* indicate the orientation of the transcription factor binding sites. The transcriptional start site is denoted by +1. All CYP24 luciferase reporter constructs contained 74bp of CYP24 5'-UTR.

specifically ERK-5, by 1,25D, is required for the inductive process by Ets-1 (Dwivedi *et al.*, 2002). During the search for potential VDREs in the CYP24 promoter, the sequence 5'-TGTCGGTCA-3' was identified at position -171/-163bp that when mutated, lowered 1,25D induction. Preliminary investigations into this apparently novel transcription factor binding site termed CYP24 enhancing protein binding site (CBS) and the protein binding to it termed CYP24-hydroxylase enhancing protein (CHEP), was undertaken during my B.Sc. Honours year.

Detailed mutagenesis of native -186bp CYP24-promoter constructs depicted in Figure 3.2A, transiently transfected into ROS 17/2.8 osteoblast cells and kidney 293T cells, further characterised the importance of the sequence 5'-TGTCGGTCA-3'. ROS 17/2.8 cells and 293T cells were chosen as model cell lines because they represent two major target organs for the action of 1,25D. Data summarising the mutagenesis are shown in Figure 3.2B. It can be seen from Figure 3.2B that mutagenesis of the nucleotides 5'-TGT-3' in the construct pCYP24(M1-CBS)-Luc significantly reduced 1,25D fold induction in both transfected ROS 17/2.8 cells and 293T cells. Mutagenesis of the entire sequence 5'-CGGTCA-3' in the construct pCYP24(M2-CBS)-Luc reduced 1,25D induction to a similar level observed with mutagenesis of 5'-TGT-3' sequence in ROS 17/2.8 and 293T cells. Similarly, mutagenesis of the sequence 5'-TCA-3' only resulted in a similar decrease in fold 1,25D induction to that seen with mutated 5'-TGT-3' and 5'-CGGTCA-3' both in transfected ROS 17/2.8 cells and 293T cells. Mutagenesis of sequences around 5'-TGTCGGTCA-3' had no effect on 1,25D induction (Figure 3.2B). Experiments also showed that mutagenesis of 5'-TGTCGGTCA-3' within the CYP24 promoter did not affect basal expression. This sequence contributes significantly more to the 1,25D inductive process in ROS 17/2.8 cells than in 293T cells. Based on these results, the 5'-CGGTCA-3' sequence will be termed the 'core' sequence and the 5'-TGT-3' sequence the '5'-flanking sequence'. Overall it was concluded that the CBS located at position -171/-163 bp (upstream of VDRE-1) on the sense strand was crucial for maximal activity of VDRE-1 in the CYP24 promoter but more so in ROS 17/2.8 cells than 293T cells in response to 1,25D (Figure 3.2A). Additionally, the inactivation of the CBS did not affect basal expression of mutant CYP24 constructs.

**A****B**

		ROS 17/2.8	293T
pCYP24(WT-186)-Luc	GCGTGTTCGGTCACCGAGG	12.7 ± 0.95	7.9 ± 1.8
pCYP24(M1-CBS)-Luc	---ATG-----	2.17 ± 0.47	4.48 ± 0.83
pCYP24(M2-CBS)-Luc	-----AAGCTT-----	2.83 ± 0.63	3.85 ± 0.23
pCYP24(M3-CBS)-Luc	-----TTA	13.7 ± 2.2	7.74 ± 2
pCYP24(M4-CBS)-Luc	CAC-----	12.24 ± 1.35	7.5 ± 0.51
pCYP24(M5-CBS)-Luc	-----TTA---	10.96 ± 1.3	6.7 ± 0.3
pCYP24(M6-CBS)-Luc	-----CTT-----	2.73 ± 0.36	3.17 ± 0.63
pCYP24(mVDRE-1)-Luc		1.03 ± 0.28	1.2 ± 0.46

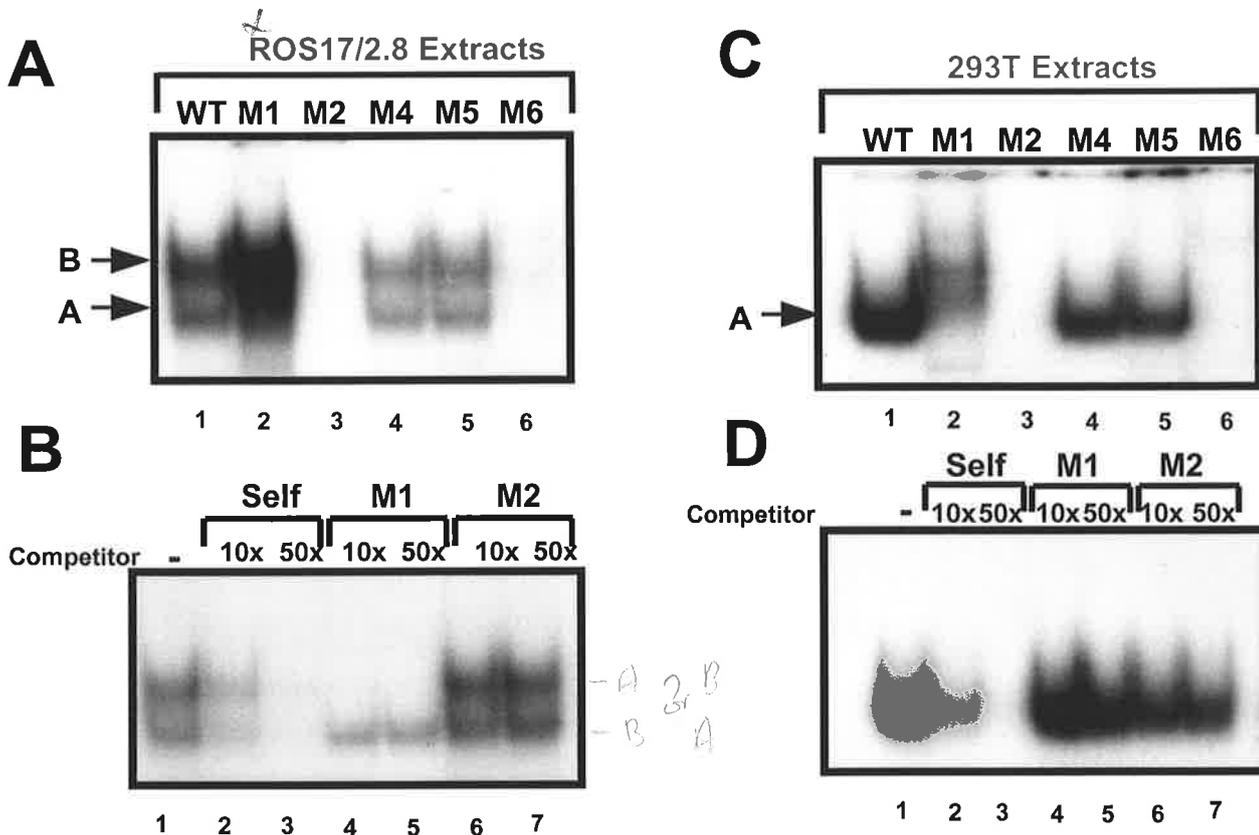
**Figure 3.2: Summary of the mutagenesis data (obtained during my Honours year) characterising the CBS in ROS 17/2.8 and 293T cells.**

(A), pCYP24(WT-186)-Luc constructs contain -186bp of native CYP24 promoter driving the expression of the Luciferase gene were used routinely in transient transfection studies. Contained in the -186bp promoter is VDRE-1 only, the EBS and the CBS (underlined). Position of the CBS relative to the transcriptional start site (+1) is also depicted.

(B), Using site directed mutagenesis, the CBS (underlined in (A)) in pCYP24((WT-186)-Luc constructs was mutated and transfected into ROS 17/2.8 cells and 293T cells. 293T cells were also cotransfected with the pRSV-hVDR expression vector to compensate for the low endogenous levels of VDR. Functionality of wildtype and mutant CBS (pCYP24(mCBS)-Luc constructs was examined following treatment of ROS 17/2.8 cells or 293T cells with  $10^{-7}$ M 1,25(OH)<sub>2</sub>D<sub>3</sub> for 24 hours. The levels of induction (Fold 1,25D induction) are calculated from the ratio of luciferase activity from 1,25(OH)<sub>2</sub>D<sub>3</sub> treated cells to that from untreated (ethanol) cells. Data presented in experiment are the means ± SD of triplicate samples from a representative experiment. The experiment was performed four times.

In other experiments during my Honours year, the binding of proteins from nuclear extracts (prepared from ROS 17/2.8 and 293T cells) to wild-type and mutant double-stranded oligonucleotide probes in EMSA experiments was investigated. These results are in agreement with the results observed from transactivation assays in ROS 17/2.8 cells and 293T cells as shown in Figure 3.3A & B, respectively.

Using nuclear extracts from ROS 17/2.8 cells, two protein complexes A and B were identified which bound to the WT-oligonucleotide. Although difficult to see, mutagenesis of the 5'-TGT-3' sequence abolished the binding of complex B but concomitantly increased the binding of complex A. The binding of complex A to mutated 5'-TGT-3' oligonucleotide probes consistently produced a smeared band (Figure 3.3A, lane 2). Mutagenesis of the 5'-CGGTCA-3' core abolishes the retardation of both complexes A and B (lane 3). Similarly, mutagenesis of 5'-TCA-3' within the core of CBS abolished the binding of both complexes. Mutagenesis of sequences surrounding 5'-TGTCGGTCA-3' did not affect the binding of either complex A or B (lanes 4 and 5). Cold competition assays using a 10-fold and 50-fold molar excess of self (lane 2 & 3), mutated 5'-TGT-3' (lanes 4 & 5) and mutated 5'-CGGTCA-3' (lanes 6 & 7) were performed to determine which complex consisted of CHEP. As shown in Figure 3.3B, self competitor oligonucleotides competed effectively for the binding of complexes A and B to the wild-type oligonucleotide. Mutagenesis of 5'-TGT-3' and competition with a 10-fold or 50-fold molar excess prevented the formation of complex A leaving complex B (Figure 3.3B, lanes 4 & 5). Mutagenesis of 5'-CGGTCA-3' and competition with these oligonucleotides did not affect the binding of complexes A or B to wild-type oligonucleotides (Figure 3.3B, lanes 6 & 7). As shown in Figure 3.3C, lane 1, a single protein/DNA complex denoted complex A bound the WT oligonucleotide using 293T nuclear extracts. Although not depicted in Figure 3.3, complex A from 293T cell nuclear extracts has equal mobility to complex A binding to the WT oligonucleotides using ROS 17/2.8 cell nuclear extracts and this is shown later in Figure 3.7. Mutagenesis of the CBS sequence (5'-TGTCGGCTA-3') mimicked the profile of protein binding to the mutant oligonucleotides observed using ROS 17/2.8 cell nuclear extracts. The data in Figure 3.3D for cold competition assays using oligonucleotide probes with 5'-TGT-3' mutated (lanes 4 & 5) or mutated 5'-CGGTCA-3' (lanes 6 & 7), confirmed that complex A binds specifically to this



**Figure 3.3: CHEP binds to the CBS (5'-TGTCGGTCA-3')** using ROS 17/2.8 and 293T nuclear extracts. (data obtained during my Honours year).

(A),  $^{32}$ P-Labeled double stranded oligonucleotide probes encompassing WT-CBS or mutant M1, M2, M4, M5 and M6 sequences from transient transfection assays (figure 3.2B) were analysed for the binding of CHEP in EMSA experiments using nuclear extracts prepared from ROS 17/2.8 cells (lanes 1-6). Although difficult to see in the gel, the mutant M1 oligonucleotide abolishes the formation of *complex A* whilst enhancing *complex B* binding (A, *bottom gel*) (B), The specificity of CHEP binding to the WT-CBS was analysed using cold competition assays. A 10-fold and 50-fold molar excess of self (TGTCGGTCA) (lanes 2 & 3), M1 (ATGCGGTCA) (lanes 4 & 5) and M2 (TGTAAGCTT) (lanes 6 & 7) was used to determine the specificity of proteins binding to the CBS. *Complex A* is the specific CHEP/CBS complex. The identity of *complex B* is unknown and is present only in ROS 17/2.8 nuclear extracts.

(C), as for (A) except that 293T nuclear extracts were used as the source of nuclear proteins. Only a single protein complex (*complex A*) is formed on WT-CBS oligonucleotide probes which has mobility equal to *complex A* observed in ROS 17/2.8 nuclear extracts (for example see Figure 3.7). Similarly, the mutant M1 oligonucleotide (B, *top gel*) appears to create a smeared band of altered mobility to *complex A* which represents the binding of CHEP to the CBS.

(D), As for (B) except 293T nuclear extracts were used as the source of nuclear proteins.

sequence. These results support the binding data observed with complex A using ROS 17/2.8 cell nuclear extract. These results therefore demonstrated that the specific binding of CHEP to the sequence 5'-TGTCGGTCA-3' correlates with its ability to enhance 1,25D mediated transactivation of pCYP24(WT-186)-Luc constructs. It should be noted that these studies using CYP24 promoter-luciferase constructs cannot eliminate the possibility that other sequences within the -186bp CYP24 promoter may also be required for the functioning of the CBS. For example, the sequence 5'-CGGTCA-3' could be part of a half-site from a DR-3 type VDRE with the other half-site of this potential VDRE being separated by three nucleotides. As shown in Figure 3.2A, a potential half-site of the sequence 5'-AGGCCC-3' is located downstream of the CBS. Furthermore this potential half-site sequence is separated from the CBS core 5'-CGGTCA-3' by three nucleotides making this appear more like a potential VDRE. This is clearly not a possibility because these nucleotide sequences have been shown in the mutagenesis study presented in Figure 3.2 to have no effect on CYP24 promoter activity. EMSA experiments with the double-stranded WT-CBS oligonucleotide (5'-GGCGTGTCGGTCACCGC-3') in combination with neutralising VDR and supershifting RXR antibodies failed to detect the binding of these proteins to the CBS in nuclear extracts prepared from 293T and ROS 17/2.8 cells (data not shown). This rules out an alternate possibility that the CBS could represent a novel VDRE. The identification of other non-classical VDRE's (i.e. non direct repeats separated by three nucleotides [DR-3 type]), VDRE's such as an everted repeat (ER-6) separated by six nucleotides (Thompson *et al.*, 2002) or an inverted palindrome (IP-9-type) VDRE separated by nine nucleotides (Herdick *et al.*, 2000; Quack and Carlberg, 1999; Schrader *et al.*, 1997) must also be considered. The CBS core 5'-CGGTCA-3' may be part of a VDRE such as the IP-9-type or ER-6 type. Examination of promoter sequences surrounding the 5'-CGGTCA-3' for potential half-sites separated by 6 nucleotides or more from the CBS core did not reveal the identity of such half-sites. Furthermore the number of nucleotides inactivated in each mutant CYP24 promoter-luciferase construct was three or more base pairs as shown in Figure 3.2B. This potentially allows for further refinement of the sequences necessary for binding and transactivation of protein(s) from the CBS.

All nuclear receptors bind to repeats having resemblance to the consensus half-site sequence 5'-AGGTCA-3' (Mangelsdorf *et al.*, 1995; McKenna *et al.*, 1999). Searches of known transcription factor binding site databases using TRANSFAC (Wingender *et al.*, 2000) MatInspector (Quandt *et al.*, 1995) and Tfsite (Heinemeyer *et al.*, 1998) programs demonstrated that the CBS did not resemble any known transcription factor binding site. It does however exhibit some similarity towards a nuclear receptor half-site with 5'-CGGTCA-3' in the CBS making up the core half-site. Mutagenesis of 5'-TGT-3' immediately 5' to this core abrogates the binding and transactivation of CHEP binding to the CBS (figure 3.2B and 3.3). The only family of nuclear receptors known to bind sequences with 5'-flanking sequences preceding a core half-site are monomeric orphan receptors (Enmark and Gustafsson, 1996; Giguere, 1999). As already discussed monomeric orphan receptors bind to extended half-site sequences with the length and sequence of the 5'-extension governing specificity of binding of a particular orphan receptor. Comparison of the CBS with known monomeric orphan receptor DNA binding sites is shown in Figure 3.4. The core of the CBS sequence, 5'-CGGTCA-3', resembles the consensus 5'-AGGTCA-3' half-site with "C" replacing the usual "A" located at position +1 of the consensus half-site (figure 3.4). There is some similarity of the proposed 5'-extension 5'-TGT-3' sequence with the binding site for ROR $\alpha$ 2 and also the binding sites for ERR $\alpha$  and SF-1 which have 5'-flanking sequences similar in length but not sequence compared with the 5'-flanking sequence of the CBS. ROR $\alpha$ 1 has been shown to bind to 5'-extensions containing TGT in its 5'-extension (Dassault and Giguere, 1997; Giguere *et al.*, 1994).

Sequencing of the human genome established that all the genes for monomeric orphan receptors have been cloned (Robinson-Rechavi *et al.*, 2001) ruling out the possibility that the CBS represents a binding site for a new monomeric orphan receptor. Instead the CBS could represent a binding site for an already known monomeric orphan receptor or an alternate form of a known orphan receptor. Conversely, the CBS could bind a non-nuclear receptor transcription factor that may be novel.

Due to the apparent novelty of the CBS sequence, its importance in CYP24 promoter expression and the unknown identity of the protein(s) binding to this site, the aims of this chapter are two-fold. The first aim of this chapter is to further

	5'-Flanking sequence	Core
NGFI-B	AA	AGGTCA
SF-1	TCA	AGGTCA
ROR $\alpha$ 1	ATATCA GATA T T	AGGTCA
ROR $\alpha$ 2	ATAAGT TA TC	AGGTCA
ERR $\alpha,\beta,\gamma$	TCA	AGGTCA
Rev-erb $\alpha$	AAANT T T	AGGTCA
CHEP	TGT	CGGTCA

**Figure 3.4: A Comparison of known monomeric orphan receptor binding sites with the CBS.**

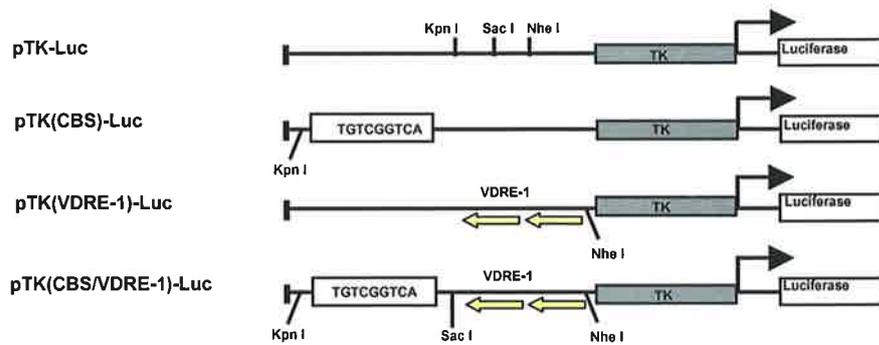
Comparison of the DNA binding sequences of the known monomeric orphan receptors. Shown are the 5'-flanking sequences of Rev-erb $\alpha$ , Estrogen related receptor (ERR), Retinoid related receptor (ROR $\alpha$ ), Nerve growth factor inducible (NGFI-B) and Steroidogenic factor-1 (SF-1). Each member binds to a core half-site of the consensus AGGTCA. Highlighted in red is the 5'-flanking sequences dictating specific binding by each member of this family. Note the length and sequence differences of the 5'-flanking sequences. The CBS shares similar characteristics with TGT (highlighted in pink) constituting the 5'-flanking nucleotides and CGGTCA making up the core of the CBS. ROR $\alpha$ 1 has also been shown to bind the same 5'-flanking sequences bound by ROR $\alpha$ 2.

define the CBS sequence characterised during my honours project using native and heterologous promoter-luciferase constructs transfected into kidney 293T and ROS 17/2.8 osteoblast cells. The second aim was to investigate if the CBS represents a binding site for an orphan receptor by overexpressing a cDNA clone for each known monomeric orphan receptor. The first section of this chapter will focus on the characterisation of the CBS and the second section will examine whether the CBS represents a known transcription factor binding site.

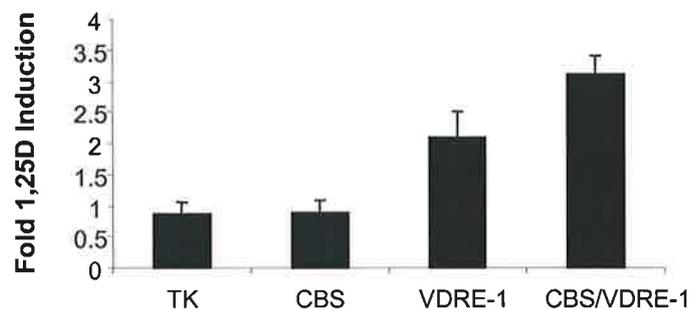
## RESULTS

### **3.2 The CBS (5'-TGTCGGTCA-3') is capable of enhancing 1,25D dependent activity from VDRE-1 in heterologous promoter constructs**

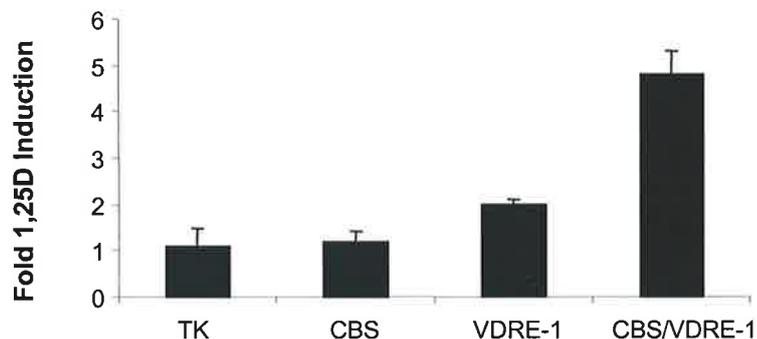
Mutagenesis experiments using native CYP24 promoter constructs and transient transfection analysis in 293T and ROS 17/2.8 cells delineated the sequence of the CBS to be 5'-TGTCGGTCA-3'. As stated earlier, the sequence 5'-TGT-3' could be the 5'-flanking nucleotides and 5'-CGGTCA-3' the core of the CBS (Figure 3.2B). As mentioned this work does not address the question as to whether in the native CYP24 promoter other sequences contribute towards the function of the CBS. Specifically the CBS could represent part of an ER-6 or IP-9-type VDRE, as discussed earlier. To answer these questions, a heterologous reporter construct was synthesised that examined the ability of the CBS sequence (5'-TGTCGGTCA-3') to enhance 1,25D-mediated activity from VDRE-1. The method used to synthesize the heterologous promoter construct is described below. As shown at the top of Figure 3.5, a Kpn I and Nhe I fragment containing the CBS, 5'-GCGTTGTCGGTCACCG-3' (additional native promoter sequences are underlined) upstream of VDRE-1 was cloned into the Kpn I and Nhe I site of the pGL2-TK-Luc vector. An additional 3bp of native CYP24 promoter sequence flanking the CBS was used to maintain identical spacing between the CBS and VDRE-1 as that in the native CYP24 promoter. A Sac I site separating the CBS and VDRE-1 in this fragment was used to generate the following constructs. A Kpn I and Sac I digest excised the CBS generating pTK(VDRE-1)-Luc while a Sac I and Nhe I double digest excised VDRE-1 generating pTK(CBS)-Luc. These constructs were verified



**A**



**B**



**Figure 3.5: Functionality of the CBS in heterologous TK promoter constructs transfected into 293T and ROS 17/2.8 cells.**

Heterologous promoter constructs driven by a minimal 160 bp fragment of the thymidine kinase (TK) promoter (shown above (A)), were used to examine if the CBS could enhance 1,25D dependent activity from VDRE-1. A Kpn I and Nhe I fragment (containing the CBS cloned 25 bp upstream of VDRE-1) was cloned into the Kpn I and Nhe I digested site of the pGL2-TK-Luc vector. To generate individual binding sites for CBS or VDRE-1, the pTK(CBS/VDRE-1)-Luc construct was double digested with Sac I and Nhe I to release the VDRE-1 fragment. To generate pTK(CBS)-Luc, pTK(CBS/VDRE-1)-Luc was double digested with Kpn I and Sac I restriction enzymes. Digested vectors were blunted and re-ligated and sequenced to verify the removal of appropriate binding site. (A), Transcriptional activation of each of these constructs in response to 1,25D treatment (24hrs) was evaluated in 293T cells and (B) ROS 17/2.8 cells. 200ng of pRSV-hVDR was co-transfected into 293T cells to compensate for the low endogenous levels of expressed VDR. Data presented in experiment are the means  $\pm$  SD of triplicate samples from a representative experiment. The experiment was performed three times with the same trend obtained.

by sequencing prior to transient transfection analysis. The heterologous promoter constructs were transfected into ROS 17/2.8 cells and 293T cells and luciferase activity measured in cell lysates using the dual luciferase assay, following exposure of the cells to  $10^{-7}$ M 1,25D for 24 hours.

Briefly, transient transfection experiments were set up as follows. Cells were plated at a confluency of 60-80% and allowed to attach in DMEM + 10% fetal calf serum. Cells were transfected overnight with the DNA/DOTAP mixtures in the same media. The next day following transfection, the media was removed and replaced with RPMI + 10% charcoal stripped fetal calf serum to remove any endogenous hormones that are present in the serum. The cells were left in this media for 2hrs prior to treatment with 1,25D. 1,25D at a concentration of  $10^{-7}$ M, dissolved in ethanol was routinely used in all experiments. Cells not treated with 1,25D hormone were instead treated with ethanol and this represented basal conditions of the assay. In these experiments, the pRSV-hVDR expression vector was co-transfected into 293T cells to compensate for the low endogenous levels of VDR protein in these cells (Kerry *et al.*, 1996).

As expected pTK-Luc was unresponsive to 1,25D in both 293T and ROS 17/2.8 cells since the TK promoter does not contain a VDRE (Figure 3.5A & B, respectively). Similarly, pTK(CBS)-Luc was unresponsive to 1,25D exposure demonstrating that the CBS is not a VDRE. This finding agrees with EMSA experiments in which neutralizing VDR and supershifting RXR antibodies failed to detect the association of these proteins with the CBS using 293T and ROS 17/2.8 cell nuclear extracts (data not shown). Furthermore cloning of the CBS into the heterologous TK promoter did not significantly affect basal luciferase expression in the absence of added 1,25D in either cell type (compare relative luciferase activities for pTK-Luc and pTK(CBS)-Luc in 293T cells of  $0.38 \pm 0.03$  and  $0.42 \pm 0.03$  respectively and in ROS 17/2.8 cells of  $0.33 \pm 0.10$  and  $0.30 \pm 0.02$  respectively). Cloning of VDRE-1 upstream of the TK promoter resulted in a  $2.0 \pm 0.1$ -fold increase in 1,25D activity of pTK(VDRE-1)-Luc in ROS 17/2.8 cells and a  $2.1 \pm 0.4$ -fold increase in 293T cells. The CBS enhanced the 1,25D inducibility of the pTK(CBS/VDRE-1)-Luc construct compared with pTK(VDRE-1)-Luc in both cell types ( $3.1 \pm 0.3$ -fold compared to  $2.1 \pm 0.4$ -fold respectively for 293T cells and  $4.8 \pm 0.5$ -fold compared to  $2 \pm 0.2$ -fold respectively for ROS 17/2.8 cells). The

enhancement of 1,25D activity by the CBS was observed routinely to be stronger in ROS 17/2.8 cells than in 293T cells.

These results demonstrate that the CBS alone could enhance 1,25D activity through cooperation with VDRE-1 and in the context of a heterologous promoter is greater in ROS 17/2.8 cells than 293T cells. The CBS does not alter basal activity of heterologous TK promoter, and is itself unresponsive to added 1,25D.

### 3.2.1 Role of the first C in CGGTCA

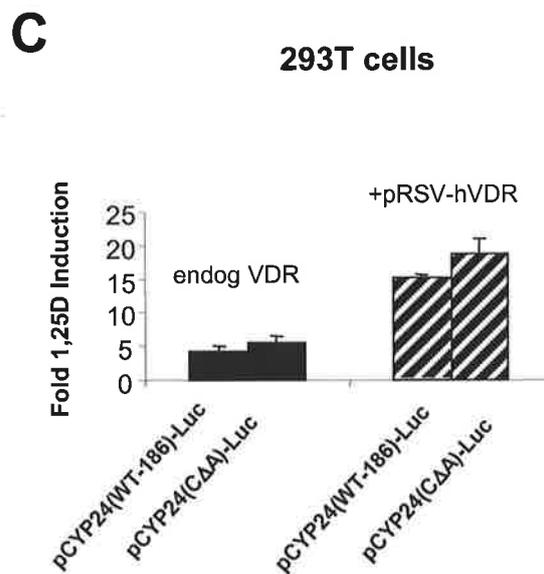
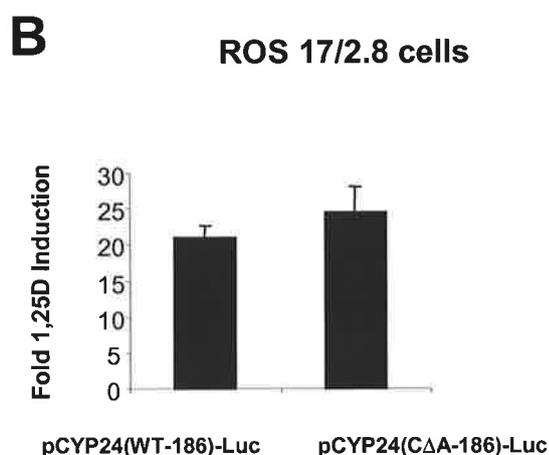
As discussed earlier, nuclear receptors bind to composite sequences composed of half-sites of the consensus sequence 5'-AGGTCA-3' as inverted, everted or direct repeats separated by 1-4bp. The CBS contains a half-site strongly resembling a nuclear receptor half-site with the notable exception that a cytosine (pyrimidine) at position +1 of the half-site is present rather than the more prevalent adenine (purine) as shown in Figure 3.4.

To ascertain if this cytosine was important for both DNA binding and functionality of CHEP, C was replaced within the context of the native WT-186 CYP24 promoter-luc construct with A using site-directed mutagenesis (Figure 3.6A). The functionality of this new construct designated pCYP24(CΔA-186)-Luc was examined by transfecting ROS 17/2.8 and 293T cells and treating these cells with 1,25D. The -186bp CYP24 promoter construct that contains only VDRE-1 was utilized for these studies. It can be seen in Figure 3.6B, that mutagenesis of cytosine to adenine in the proposed half-site of CBS resulted in a similar level of expression of pCYP24(CΔA-186)-Luc in ROS 17/2.8 cells compared with the wild-type promoter ( $20.9 \pm 1.65$ -fold compared with  $24.5 \pm 3.63$ -fold, respectively).

293T cells express low levels of endogenous VDR. Therefore the response to 1,25D of pCYP24(CΔA-186)-Luc was evaluated in 293T cells expressing endogenous levels of VDR as well as in 293T cells co-transfected with an expression vector for the human VDR. A  $4.36 \pm 0.63$ -fold level of induction from the wild-type CYP24 promoter was observed in 293T cells expressing endogenous levels of VDR. Similarly, pCYP24(CΔA-186)-Luc gave rise to a  $5.57 \pm 0.6$ -fold level

**A**

WT (CBS)	5'-TGTCGGTCA-3'
CΔA (CBS)	5'-TGTAGGTCA-3'



**Figure 3.6: Functionality of CYP24 promoter constructs when the first C in the core of the CBS 5'-CGGTCA-3' is replaced with A to generate a perfect consensus nuclear receptor half-site.**

(A), Shown is the sequence of the CBS. Using site directed mutagenesis, C at position +1 of the putative CBS core was replaced with A (highlighted in orange) to give the sequence 5'-AGGTCA-3' and to generate pCYP24(CΔA-186)-Luc. The inducibility of VDRE-1 in the pCYP24(CΔA-186)-Luc constructs was examined in transfected ROS 17/2.8 cells (B) and 293T cells (C) treated with  $10^{-7}M$   $1,25(OH)_2D_3$ . The functionality of pCYP24(CΔA-186)-Luc constructs was examined in 293T cells also transfected with the pRSV-hVDR expression vector depicted by hatched bars in (C). Data presented in experiment are the means  $\pm$  SD of triplicate samples from a representative experiment. The experiment was performed three times with the same trend obtained.

of induction that is similar to that observed with wild-type promoter. Co-transfection of 200ng of pRSV-hVDR (Figure 3.6C) resulted in a significant enhancement of wild-type promoter activity increasing induction from  $4.36 \pm 0.63$  -fold to  $15.06 \pm 0.97$ -fold thus reflecting the low level of VDR present in these cells. Mutagenesis of C to A of the CBS core in pCYP24(C $\Delta$ A-186)-Luc did not alter 1,25D responsiveness since an  $18.63 \pm 2.37$ -fold level of induction was observed with this construct (figure 3.6C). No significant effect on basal expression of wild-type and mutant CYP24 promoter constructs was observed confirming a role of the CBS only in the 1,25D inductive process (data not shown).

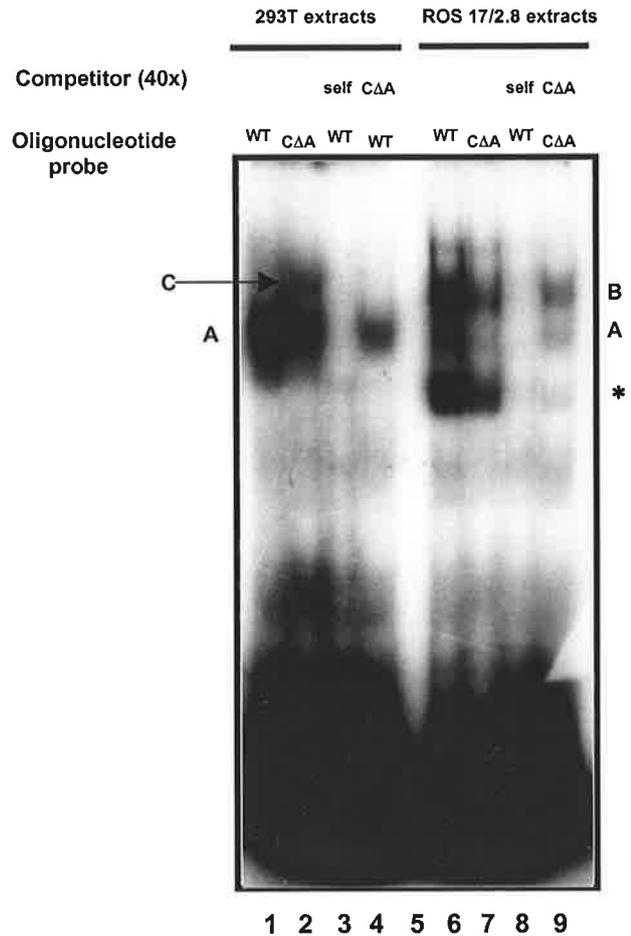
These results clearly demonstrate that conversion of C to A (hence conversion of the CBS core into a perfect consensus nuclear receptor half-site) has no significant effect on 1,25D driven inducibility and hence the transactivation potential from the CBS of -186bp CYP24 promoter constructs in both ROS 17/2.8 cells and 293T cells. Furthermore this effect appears to be independent of the expression levels of VDR protein.

In separate experiments, a double-stranded oligonucleotide probe equal in length to the WT-CBS oligonucleotide probe except with the C $\Delta$ A mutation incorporated into the CBS as shown in Figure 3.7A, was used in EMSA experiments with nuclear extracts prepared from ROS 17/2.8 cells and 293T cells. This was performed to ascertain if the binding of nuclear proteins from ROS 17/2.8 or 293T cells was altered.

With 293T nuclear extracts, a single retarded DNA/protein complex (complex A) is retarded by the WT-CBS oligonucleotide probe (Figure 3.7B, lane 1). This complex appears somewhat smeared in the figure but is also shown elsewhere (Figure 3.3 & 3.8). A complex of similar mobility to complex A but with slightly reduced intensity is also retarded on the mutant C $\Delta$ A oligonucleotide probe (Figure 3.7B, lane 2 versus lane 1). Interestingly, the C $\Delta$ A oligonucleotide probe appears to have retarded another faint complex of higher mobility (denoted by arrow labeled C). The identity of this complex is unknown. A 40-fold molar excess of cold unlabelled self competitor completely prevented the formation of the CBS complex binding to the WT-CBS oligonucleotide probe (Figure 3.7B, lane 3). A 40-fold

**A**

WT-CBS      5'-GGCGTGTCGGTCACCGC-3'  
 CΔA (CBS)    5'-GGCGTGTAGGTCACCGC-3'

**B**

**Figure 3.7: Analysis of CHEP binding to the CBS when C in the putative core 5'-CGGTCA-3' is replaced with A.**

(A), Sequence of the oligonucleotides used in EMSA analysis. C at position +1 of the WT-CBS core was mutated to A (highlighted in red) to generate CΔA oligonucleotide.

(B), <sup>32</sup>P-Labeled double stranded oligonucleotide probes encompassing WT-CBS or mutant CΔA-CBS (shown in A) was analysed for the binding of CHEP (*complex A*) in EMSA experiments using nuclear extracts prepared from 293T cells (lanes 1-4) and ROS 17/2.8 cells (lanes 6-9). The ability of the CΔA-CBS oligonucleotide to compete for the binding of CHEP to the WT-CBS oligonucleotide probe was examined by including a 40-fold molar excess of unlabeled competitor to the binding reaction (lanes 4 and 8). *Complex B* is a non-specific complex of unknown identity binding to the WT-CBS only in ROS 17/2.8 cell nuclear extract. *Complex C* (black arrow) is a non-specific complex having a slightly lower mobility compared to complex B. *Asterisk* denotes degradation product of the nuclear extracts.

molar excess of unlabelled C $\Delta$ A-CBS oligonucleotide probe competed with the formation of the protein/DNA complex binding to the WT-CBS oligonucleotide probe (Figure 3.7D, lane 4) but not to the same level as wild-type oligonucleotide (compare lane 4 with lane 3).

As highlighted previously in Figure 3.3, two protein/DNA complexes (labeled A and B) are retarded using the WT-CBS oligonucleotide probe with ROS 17/2.8 cell nuclear extract and these are observed in Figure 3.7B, lane 6. The asterisk denotes an apparent degradation product since this band is only present on occasions following multiple freeze/thawing of the nuclear extracts. For example, this is not seen in Figure 3.3 where the extracts have been frozen/thawed on three occasions. Furthermore this apparent degradation product is independent of the source of extracts and is observed on some occasions in 293T nuclear extracts but importantly is not present following fresh extract preparation. Previous EMSA analysis (Figure 3.3B) has demonstrated that the slower migrating protein/DNA complex (complex B) is a non-specific complex of unknown identity. The faster migrating protein/DNA complex (complex A) having mobility equal to the single complex in 293T nuclear extracts is CHEP. The pattern of protein binding to the C $\Delta$ A-CBS oligonucleotide probe was similar to that observed with the WT-CBS oligonucleotide probe using ROS 17/2.8 cell nuclear extracts (Figure 3.7B, compare lanes 6 and 7). As seen with 293T nuclear extracts, the intensity of complex A retarded with the C $\Delta$ A oligonucleotide was weaker compared to the WT-CBS oligonucleotide. A 40-fold molar excess of unlabelled self (WT-CBS) oligonucleotide probe prevented the binding of both complex A and B to the WT-CBS oligonucleotide using ROS 17/2.8 cell nuclear extracts (Figure 3.7B, lane 8). Cold competition with a 40-fold molar excess of C $\Delta$ A-CBS oligonucleotide probe competed weakly with the binding of protein complexes A and B to the WT-CBS probe (Figure 3.7B, lane 9). As shown in Figure 3.3 also, complex B (of unknown identity) has some partial requirement/affinity for sequences contained in the core of the CBS but not the 5'-flanking sequence 5'-TGT-3'. Therefore replacing C with A would compete for the binding of complex B to the WT-oligonucleotide.

In conclusion, the results demonstrate that mutagenesis of the cytosine to adenine in the CBS does not affect the inducibility of the CYP24 promoter constructs in response to 1,25D in transfected 293T cells or ROS 17/2.8 cells. This result is

furthermore independent on the relative levels of VDR protein being expressed in 293T cells. Conversion of the CBS core sequence into a perfect nuclear receptor half-site reduces but does not abolish the binding of CHEP complex to the C $\Delta$ A-CBS oligonucleotide.

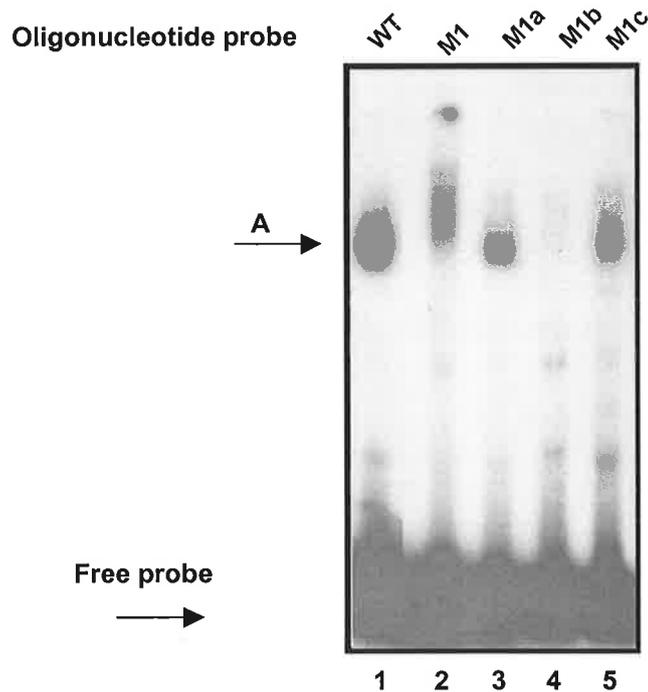
### **3.2.2 The 5'-flanking sequence TGT of the CBS is critical for the specific binding of CHEP**

Mutagenesis experiments using both native (Figure 3.2) and heterologous promoter constructs (Figure 3.5) and EMSA experiments (Figure 3.3) have defined the CBS as the sequence 5'-TGTCGGTCA-3' critical for 1,25D induced expression of the CYP24 promoter. All three 5'-flanking nucleotides (5'-TGT-3') may be essential for binding and transcriptional functioning of CHEP since mutagenesis of all three of these nucleotides abolishes 1,25D inducibility and protein binding. Further experiments were undertaken to determine whether each nucleotide present in the 5'-flanking sequence (5'-TGT-3') is required for the binding of CHEP. Oligonucleotide probes harboring single nucleotide substitutions of the TGT sequence shown in Figure 3.8A were evaluated for their effect on the binding of CHEP using 293T nuclear extracts.

As shown in Figure 3.8B, mutagenesis of all three 5'-flanking nucleotides in M1-CBS oligonucleotides abolishes the formation of the CHEP complex and produces a smear of higher mobility (Figure 3.8B, lane 2). This result has been consistently observed upon inactivating all three nucleotides. Mutagenesis of the first nucleotide (5'-TGT-3' to 5'-AGT-3') in M1a-CBS oligonucleotide reduced but did not abolish the binding of proteins to the CBS (lane 3) in comparison to the WT-CBS oligonucleotide (lane 1). Mutagenesis of the second nucleotide (5'-TGT-3' to 5'-TTT-3') in the M1b-CBS oligonucleotide completely abrogated formation of retarded complexes (lane 4). Mutagenesis of the third nucleotide in the 5'-flanking sequence (5'-TGT-3' to 5'-TGG-3') in the M1c-CBS oligonucleotide significantly reduced the binding of proteins to this oligonucleotide probe and to a greater extent than that observed with the M1a-CBS oligonucleotide (lane 3). These

**A**

WT-CBS	5' - <u>TGT</u> CGGTCA - 3'
	3' - ACA GCCAGT - 5'
M1-CBS	5' - <u>ATG</u> CGGTCA - 3'
	3' - <u>TAC</u> GCCAGT - 5'
M1a-CBS	5' - <u>AGT</u> CGGTCA - 3'
	3' - <u>TCA</u> GCCAGT - 5'
M1b-CBS	5' - <u>TTT</u> CGGTCA - 3'
	3' - <u>AAA</u> GCCAGT - 5'
M1c-CBS	5' - <u>TGG</u> CGGTCA - 3'
	3' - <u>ACC</u> GCCAGT - 5'

**B**

**Figure 3.8: Investigating the effect of CHEP binding to single nucleotide mutations in the 5'-flanking sequence of the CBS.**

(A), Sequence of the double stranded oligonucleotide probes used in the EMSA study. Single nucleotide mutations of the 5'-flanking sequence TGT are underlined in each oligonucleotide.

(B), <sup>32</sup>P-Labeled double stranded oligonucleotides encompassing the sequences depicted in (A) were analysed for the effect of binding CHEP (complex A) with nuclear extracts prepared from 293T cells. Between lane 1 and lane 2 in this gel, lanes were excised following scanning of the original gel to generate this picture. These lanes were excised because they were not relevant to the current study. It must be emphasized that all the binding reactions were performed at the same time and electrophoresed on the same gel.

results were routinely observed and identical trends were also shown using ROS 17/2.8 cell nuclear extracts (data not shown).

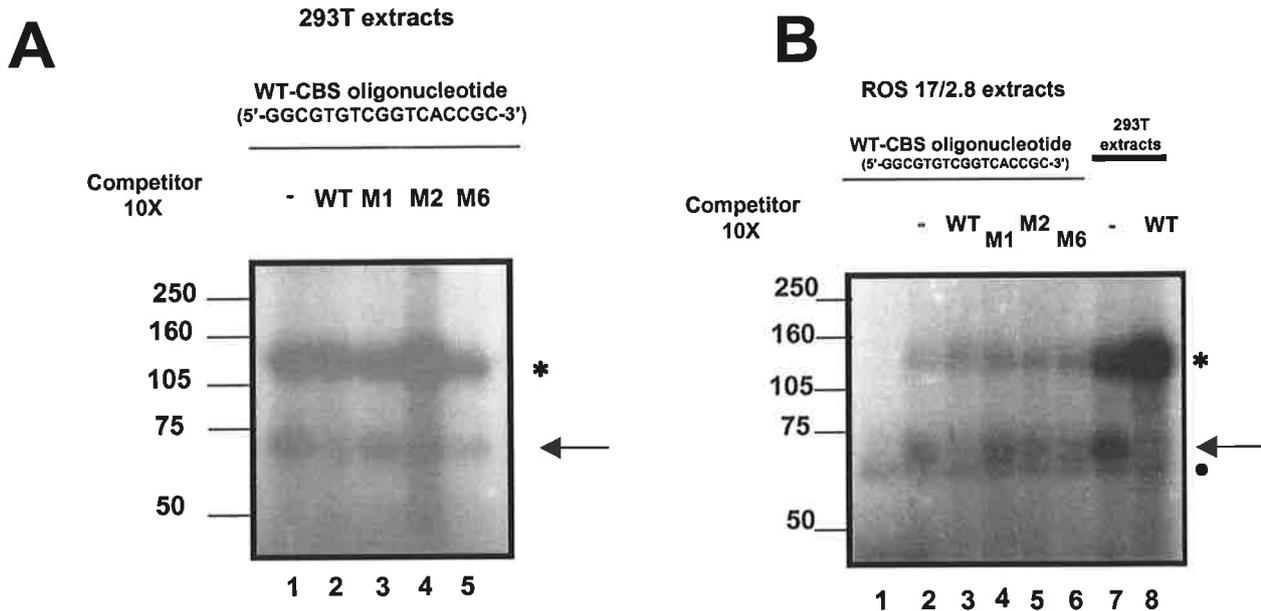
These results suggest that the central 'G' nucleotide of TGT is critical for the binding of CHEP. The 'T' at position 3 of the TGT sequence is also important although to a lesser degree than the central 'G' for the binding of CHEP. The 'T' at position 1 of the 5'-flanking sequence was not as important for the binding of CHEP to the CBS.

These results suggest that the entire 5'-flanking sequence 5'-TGT-3' of the CBS is required for maximal binding of CHEP. It will be important to determine if these single nucleotide substitutions affect the functionality of CYP24 promoter constructs in transfected cells, in a similar fashion to that observed with the binding of CHEP to the CBS. This was not carried out due to time constraints.

### **3.2.3 CHEP has a molecular weight of approximately 60kDa**

UV cross-linking experiments were performed to determine the molecular weight of CHEP. The procedure employed has been routinely used by other researchers (Wieczorek *et al.*, 2000; Krehan *et al.*, 2000; Yoo *et al.*, 2000). EMSA binding reactions containing the radiolabeled WT-CBS double-stranded oligonucleotide probe was incubated with 293T nuclear extracts. These nuclear extracts were initially chosen because a single specific DNA/protein complex is retarded with the WT-CBS oligonucleotide probe under non-denaturing conditions (Figure 3.9A). The tops of the tubes were placed 5cm from the UV light source and irradiated. Following UV-light irradiation reactions were cooled, boiled in SDS buffer and cross-linked products separated on an 8% denaturing polyacrylamide gel and exposed to autoradiographic film.

UV cross-linking of 293T extracts in the presence of the WT-CBS double-stranded oligonucleotide probe resulted in the formation of a large molecular weight protein/DNA complex of ~150 kDa (Figure 3.9A, lane 1) and a smaller cross-linked protein/DNA complex of ~75 kDa (lane 1, arrow). To determine which of these protein/DNA complexes was specific for binding to the CBS, a 10-fold molar



**Figure 3.9: UV cross-linking of proteins binding to the CBS.**

(A), DNA binding reactions of nuclear extracts prepared from 293T cells were incubated with the WT-CBS oligonucleotide probe (see materials & methods). Prior to UV irradiation, binding reactions were incubated with a 10-fold molar excess self or mutant (mutations underlined) of cold competitor oligonucleotide, self (WT), M1-CBS (ATGCGGTCA), M2-CBS (TGTAATCTT) or M6-CBS (TGTCGGCTT) to examine their ability to compete with the binding of CHEP to the CBS. Cross-linked products were visualised by autoradiography. *Asterisk* denotes a 150kDa cross-linked complex, *arrow* the specific CHEP complex of 75kDa.

(B), As in (A) except ROS 17/2.8 cell nuclear extracts (lanes 1-6) were used as a source of nuclear protein to bind to <sup>32</sup>P-labeled double stranded WT-CBS oligonucleotides. As a negative control, a binding reaction consisting of ROS 17/2.8 cell nuclear extract and WT-CBS oligonucleotide probe without exposure to UV irradiation was also included (lane 1). For comparison, binding of proteins using 293T nuclear extracts was also included on the same gel to compare the profile of cross-linked DNA/protein products. Numbers on the left represent the molecular weight markers. *Closed circle* below arrow denotes an unknown band observed upon electrophoresis of DNA binding reactions.

excess of self (WT-CBS), M1-CBS (5'-ATGCGGTCA-3'), M2-CBS (5'-TGTAAGCTT-3') and M6-CBS (5'-TGTCGGCTT-3') mutant unlabeled double stranded oligonucleotides were included in the binding reaction prior to UV irradiation. Inclusion of a 10-fold molar excess of self competitor abolished the formation of the smaller 75 kDa protein/DNA complex (Figure 3.9A, lane 2). Inclusion of either of the mutant-CBS oligonucleotide competitors M1, M2 or M6 which prevent binding and transcriptional activation of CYP24 promoter constructs from the CBS (Figure 3.3), did not affect any of the cross-linked products (Figure 3.6A, lanes 3,4 and 5, respectively). These results suggest that the 75 kDa protein/DNA complex contains the protein which binds specifically to the CBS. The identity of the large 150 kDa complex is unknown and appears to cross-link to the oligonucleotide probes non-specifically. The same experiment was performed using ROS 17/2.8 cell nuclear extracts (Figure 3.9B). As a comparison for the number and size differences of cross linked proteins that may arise between the two cell types, 293T extracts cross-linked to the WT-CBS oligonucleotide probe were electrophoresed on the same denaturing gel.

Similarly, ROS 17/2.8 cell nuclear extracts exhibited the same size and number of UV cross-linked protein/DNA complexes upon incubation with the WT-CBS oligonucleotide probe (Figure 3.9B, lane 2). The notable exception was the appearance of a band of smaller molecular weight than the CHEP/DNA complex denoted by a closed circle (Figure 3.6B, lane 2). This band appeared inconsistently throughout UV cross-linking experiments. A control binding reaction that was not subjected to UV cross-linking and electrophoresed under similar denaturing conditions as the other samples resulted in the formation of this band. This band was also present upon UV irradiation of 293T nuclear extracts with WT-CBS oligonucleotide (lane 7 and 8). Incubation of ROS 17/2.8 cell nuclear extracts with self competitor prevented the formation of the 75 kDa protein/DNA complex but had no effect on the 150 kDa complex (Figure 3.9B, lane 3). As shown in Figure 3.9B (lane 7), the 75 kDa complex formed with ROS 17/2.8 cell nuclear extract is equivalent in size to the protein/DNA complex produced upon UV irradiation of 293T cell nuclear extracts in the presence of the WT-CBS oligonucleotide probe. The Mutant oligonucleotide competitors M1, M2 or M6 did not affect either complex using ROS 17/2.8 cell nuclear extracts (Figure 3.9B, lanes 4,5 and 6, respectively). The molecular weight of the specific complex

observed in the SDS polyacrylamide gels is 75 kDa but this consists of a protein cross-linked to double-stranded DNA. Therefore subtracting the molecular weight of the double-stranded oligonucleotide probe (~15 kDa) results in the CHEP protein having a molecular weight of ~60 kDa. These results do not however distinguish whether CHEP represents a monomeric or dimeric transcription factor

### 3.3 Analysis of known transcription factors binding to the CBS

#### 3.3.1 Investigating the role of ROR $\alpha$ and other monomeric orphan receptors on the transcriptional regulation of CYP24 through the CBS

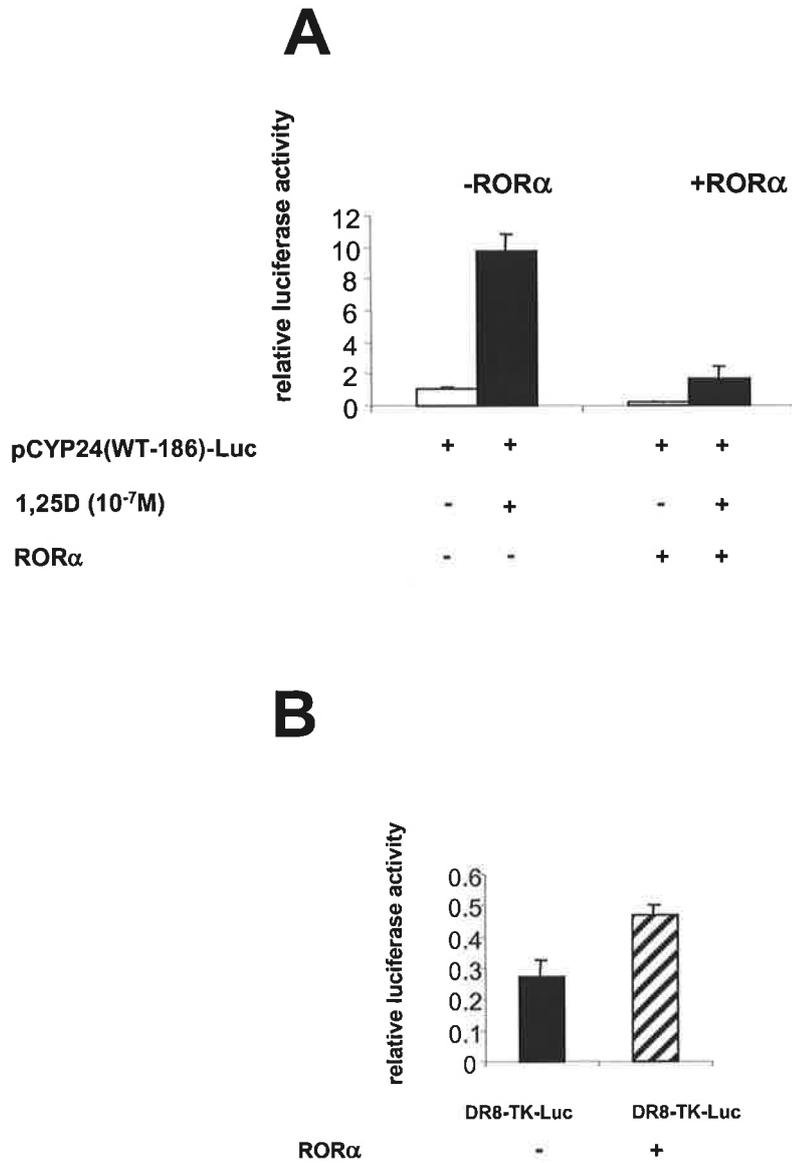
Experimental analysis using transient transfections, DNA binding studies and UV cross-linking have so far identified CHEP as a DNA binding protein of approximately 60kDa that appears to bind specifically to the CBS (5'-TGTCGGTCA-3'). As mentioned earlier, database searches using MatInspector (Quandt *et al.*, 1995), Tfsite (Heinemeyer *et al.*, 1998) and TRANSFAC (Wingender *et al.*, 2000) failed to identify this sequence as a binding site for any known transcription factor. As previously discussed, monomeric orphan receptors recognise the core half-site motif 5'-AGGTCA-3' or slight deviations of this but the feature that distinguishes which member will bind to a half-site is governed by the 5'-flanking sequence. The 5'-extension of the CBS (5'-TGT-3') has not been reported as an extension for any known orphan receptor. However, based upon the organisation of the CBS (i.e. a core half-site preceded by a 5'-extension) the CBS may represent a binding site for a monomeric orphan receptor.

As shown in Figure 3.4, of all the known monomeric orphan receptors, ROR $\alpha$ 1 appeared to be a potential candidate. ROR $\alpha$ 1 (including the ROR $\alpha$ 2 isoform) was an attractive candidate because it can binds the sequence 5'- TAT/ATNT AGGTCA-3', is expressed in osteoblasts and kidneys cells, is important for bone formation (Meyer *et al.*, 2000), modulates the transcriptional response of the osteocalcin gene in response to 1,25D (Meyer *et al.*, 2000) and has several isoforms all having subtle differences in binding site preferences (Giguere *et al.*, 1994). Therefore the over-expression of ROR $\alpha$ 1 (kind gift from Dr. Muscat,

University of Queensland) in 293T and ROS 17/2.8 cells was used to determine what role, if any, ROR $\alpha$ 1 plays in CYP24 gene expression in response to 1,25D. As a positive control, a DR-8 TK-luciferase vector containing two RORE $\alpha$ 1 binding sites separated by 8bp and driving the expression of the minimal TK promoter (DR8/TK-Luc, gift from Dr. Brigitte Fournier, Novartis Pharma, Switzerland) was also tested.

In ROS 17/2.8 cells transiently transfected with pCYP24(WT-186)-Luc, a  $9.13 \pm 1.05$ -fold level of induction was observed upon 1,25D treatment (Figure 3.10A). Cotransfecting 200ng of the expression vector pSG5-ROR $\alpha$ 1 resulted in a large decrease in both basal activity and 1,25D dependent promoter activity. This was not due to a general toxic effect from ROR $\alpha$ 1 over-expression because expression from the internal control vector, pRL-TK-Renilla, that corrects for transfection efficiency was not altered. A similar inhibition of 1,25D induced osteocalcin activity upon transfection with comparable doses of ROR $\alpha$ 1 in ROS 17/2.8 cells was also demonstrated by Meyer *et al.* (2000). Interestingly, the overall fold 1,25D-dependent induction of the CYP24 promoter was similar to the induction observed in the absence of cotransfecting pSG5-ROR $\alpha$ 1 ( $7.76 \pm 0.76$ -fold versus  $9.13 \pm 1.05$ -fold, respectively). Over-expression of ROR $\alpha$ 1 on the positive control vector, DR8-TK-Luc, weakly enhanced activity of the DR8-TK-Luc construct  $1.70 \pm 0.12$ -fold (Figure 3.10B). Similar results were observed in another osteoblast cell line UMR-106 (data not shown). The activity of DR8-TK-Luc construct upon ROR $\alpha$ 1 over-expression was not modulated by 1,25D in ROS 17/2.8, UMR-106 or 293T cells (data not shown).

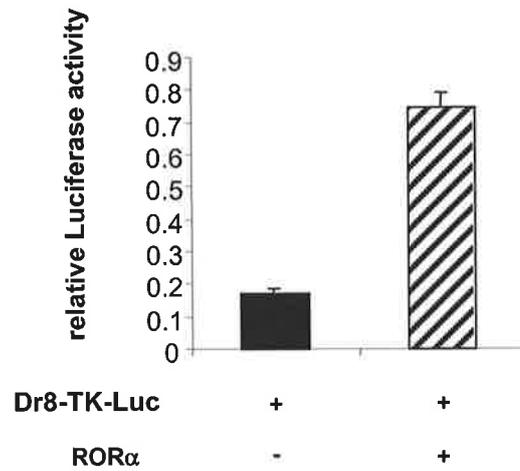
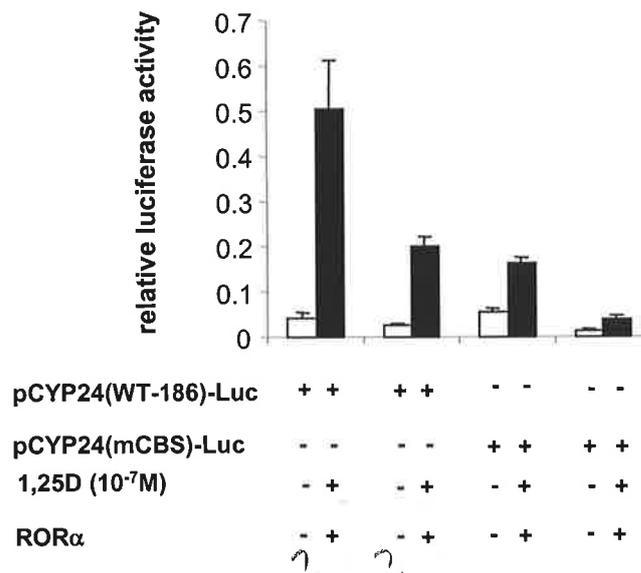
In 293T cells expressing endogenous VDR, the response of the DR8-TK-Luc construct to ROR $\alpha$ 1 over-expression was significantly higher than in ROS 17/2.8 cells with a  $4.2 \pm 0.2$ -fold level of induction observed upon over-expression of ROR $\alpha$ 1 (Figure 3.11A). In the next experiment, pRSV-hVDR was also cotransfected with ROR $\alpha$ 1 to mimic the higher levels of VDR expressed in ROS 17/2.8 cells. Similarly, over-expression of pSG5-ROR $\alpha$ 1 led to a significant decrease of 50% in both basal and 1,25D induced promoter activity in 293T cells (Figure 3.11B). A similar decrease in both basal and 1,25D activity was observed in 293T expressing endogenous VDR (data not shown). To ascertain if the



**Figure 3.10: Overexpression of ROR $\alpha$ 1 on pCYP24(WT-186)-Luc constructs in ROS 17/2.8 cells.**

(A), ROS 17/2.8 cells were transiently transfected with pCYP24(WT-186)-Luc constructs and treated with vehicle (open bars) and 10<sup>-7</sup>M 1,25(OH)<sub>2</sub>D<sub>3</sub> (shaded bars). The effect of ROR $\alpha$ 1 overexpression on wildtype promoter activity in the presence of hormone was evaluated by cotransfecting the pSG5-ROR $\alpha$ 1 expression vector into ROS 17/2.8 cells. Relative luciferase activity shown represents the means  $\pm$  SD of triplicate samples from a representative experiment. The experiment was performed four times.

(B), ROS 17/2.8 cells were transiently transfected with the DR8-TK-Luciferase vector. The ability of ROR $\alpha$  to transactivate the DR8-TK-luciferase reporter was investigated by coexpressing 200ng of pSG5-ROR $\alpha$ . Black bars are ROS 17/2.8 cells not cotransfected with pSG5-ROR $\alpha$  expression vector. Hatched bars represent ROS 17/2.8 cells transfected with 200ng of pSG5-ROR $\alpha$ . The relative luciferase activity shown represents the mean  $\pm$  SD of triplicate samples from a representative experiment. The experiment was performed four times with the same trend obtained.

**A****B**

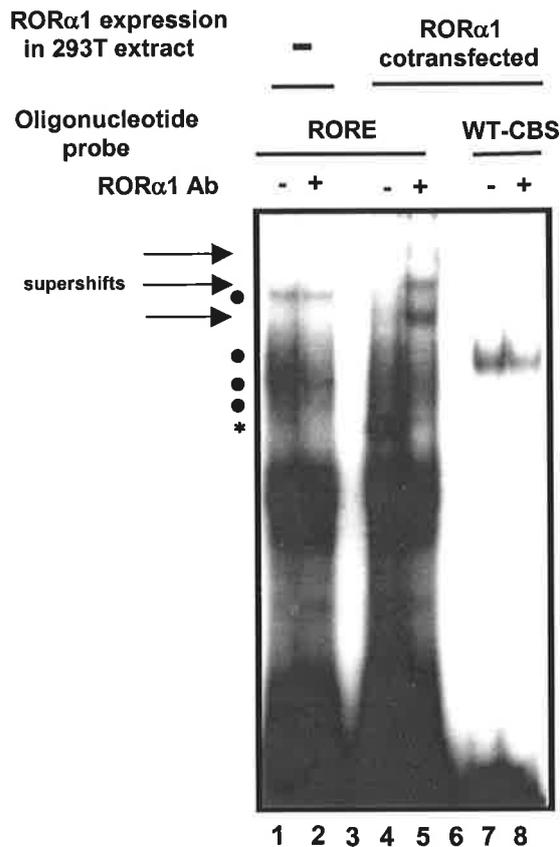
**Figure 3.11: Overexpression of RORα1 on pCYP24(WT-186)-Luc constructs in 293T cells.**

(A), 293T cells were transiently transfected with the DR8-TK-Luciferase vector. The ability of RORα1 to transactivate the DR8-TK-luciferase reporter was investigated by coexpressing 200ng of pSG5-RORα. The relative luciferase activity shown is the mean ± SD of triplicate samples from a representative experiment. This experiment was performed four times.

(B), 293T cells were transiently transfected with pCYP24(WT-186)-Luc and pCYP24(mCBS)-Luc constructs and an expression vector pRSV-hVDR. Cells were treated with vehicle (open bars) and 10<sup>-7</sup>M 1,25(OH)<sub>2</sub>D<sub>3</sub> (shaded bars). The effect of RORα1 overexpression on wildtype and mutant CBS promoter activity in the presence of hormone was evaluated by cotransfecting the pSG5-RORα expression vector. Relative luciferase activity shown is the mean ± SD of triplicate samples from a representative experiment. This experiment was performed four times.

repressive effect of ROR $\alpha$  over-expression was mediated through the CBS, ROR $\alpha$ 1 was over-expressed on the pCYP24(mCBS)-Luc construct and the effect examined in the presence and absence of 1,25D. As highlighted previously, mutagenesis of the CBS does not affect basal expression of the CYP24 promoter. Interestingly the repressive effect of ROR $\alpha$ 1 over-expression is still preserved in the absence of a functional CBS demonstrating that the repressive effect of ROR $\alpha$ 1 is occurring elsewhere on the CYP24 promoter. Basal and hormone induced repression of the CYP24 promoter upon ROR $\alpha$ 1 cotransfection appears to be mediated predominantly through VDRE-1 as inactivation of VDRE-1 almost abolishes all of the repressive effect (data not shown). These results clearly demonstrate that over-expression of ROR $\alpha$  represses CYP24 promoter activity and that the CBS plays no role in this repression in 293T and ROS 17/2.8 cells. Furthermore, over-expression of ROR $\alpha$  does not positively regulate 1,25D responsiveness of the CYP24 promoter through the CBS.

There are several isoforms of ROR $\alpha$  that result from alternate promoter usage or splicing of the ROR gene (Giguere *et al.*, 1994). Similarly, sequence and affinity of the 5'-flanking sequence of the RORE has been shown to differ amongst these isoforms (Figure 3.4). Therefore the  $\alpha$ 1-isoform tested in these experiments may not be the endogenous isoform acting on the CYP24 gene promoter. To test the possibility that an alternate isoform of ROR $\alpha$  could activate the CYP24 promoter through the CBS, EMSA experiments were employed. A supershifting antibody (sc-6062, Santa Cruz Biotechnology, CA) that recognises multiple isoforms of ROR $\alpha$  (ROR $\alpha$ 1-2) was utilised in EMSA experiments. For this work, 293T nuclear extracts were prepared from cells cotransfected with pSG5-ROR $\alpha$ 1 and cells not transfected with the ROR $\alpha$ 1 expression vector. The binding of proteins was examined using a double stranded RORE oligonucleotide probe known to bind ROR $\alpha$ 1 and ROR $\alpha$ 2 (Giguere *et al.*, 1994; Dassault and Giguere, 1997) and the WT-CBS oligonucleotide probe from the CYP24 promoter. Several protein/DNA complexes were retarded on the RORE oligonucleotide probe in 293T nuclear extracts endogenously expressing ROR proteins (Figure 3.12, lane-1). Of note is the disappearance of some protein/DNA complexes upon ROR $\alpha$  over-expression depicted by closed circles in Figure 3.12, lane 4. The reason for this is unknown. The protein /DNA complex marked with an asterisk in lane 1 is likely to consist of



**Figure 3.12: ROR $\alpha$ 1 supershifting antibody analysis of proteins binding to the CBS in 293T nuclear extracts.**

$^{32}$ P-Labeled double stranded RORE oligonucleotide probe (ROR $\alpha$ 1 Binding site underlined-TCGACTCGTATAACTAGGTCAGCGCTG) and the WT-CBS (GCGTGTCGGTCACCG) oligonucleotide were incubated with 293T nuclear extracts not transfected with pSG5- ROR $\alpha$  (lanes 1 and 2) or ectopically expressing ROR $\alpha$  (lanes 4-8). For supershift analysis, 2 $\mu$ ls of polyclonal ROR $\alpha$ 1 antibody (sc-6062) were included into the binding reaction prior to the addition of probe. Several protein/DNA complexes (closed circles) are retarded with the RORE oligonucleotide probe. The *asterisk* denotes ROR $\alpha$ 1 binding to the RORE. *Arrows* indicate supershifted complexes formed upon inclusion of ROR $\alpha$ 1 antibody into the binding reaction.

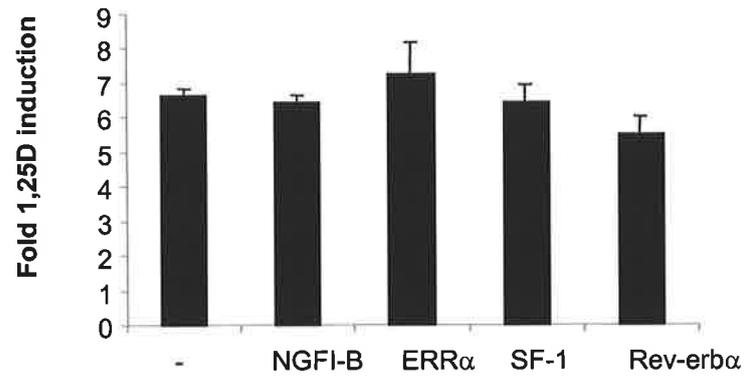
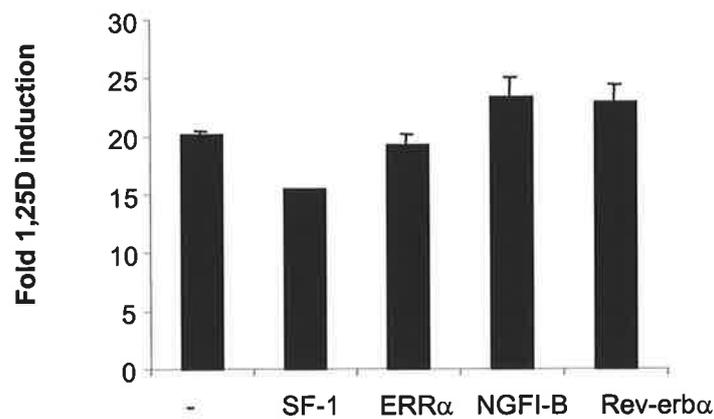
the ROR $\alpha$ 1/RORE complex as the intensity of this complex is largely increased in 293T nuclear extracts cotransfected with the pSG5-ROR $\alpha$ 1 expression vector (Figure 3.12, compare lane 4 and lane 1).

Inclusion of ROR $\alpha$ 1 supershifting antibody to the binding reaction consisting of the RORE oligonucleotide and 293T nuclear extracts from cells endogenously expressing ROR $\alpha$ 1, failed to produce any supershifted bands (lane 2). Although very difficult to see in Figure 3.12, the ROR $\alpha$  antibody did appear to very weakly decrease the intensity of the ROR $\alpha$ /RORE complex denoted by the asterisk (lane 2). In 293T extracts transfected with the pSG5-ROR $\alpha$  expression vector, as depicted by the three large arrows, inclusion of the supershifting ROR $\alpha$  antibody supershifted the entire ROR $\alpha$ /RORE complex (denoted by the asterisk) and concomitantly formed three supershifted complexes each with different mobilities and intensities (lane 5). The reason for three supershifted complexes is unknown. Using the WT-CBS oligonucleotide as the probe, a single protein/DNA complex was retarded in 293T cells transfected with pSG5-ROR $\alpha$ 1. It is interesting to note that the CHEP/CBS complex has a slower mobility through the gel compared to the ROR $\alpha$ /RORE complex (compare lanes 4 and 7). Inclusion of ROR $\alpha$  antibody failed to supershift the CHEP/CBS complex demonstrating that the CHEP/CBS complex is immunogenically unrelated to ROR $\alpha$  proteins (Figure 3.12, lane 8). The band in lane 8 of Figure 3.12 does appear to be slightly reduced upon inclusion of the antibody. This result is consistent with loading variation of protein samples. This result has been repeated several times with no effect observed with the single complex retarded using the WT-CBS oligonucleotide probe. This EMSA gel was used in Figure 3.12 because it gave the clearest representation of the supershifted complexes formed upon inclusion of ROR $\alpha$  antibodies with 293T nuclear extracts and ROR $\alpha$  oligonucleotide probes. Similarly, the binding of CHEP to the WT-CBS oligonucleotide probe using extracts from ROS 17/2.8 cells endogenously expressing ROR $\alpha$  was unaltered with the inclusion of ROR $\alpha$  supershifting antibody (data not shown). In conclusion, these results demonstrate that in nuclear extracts prepared from 293T and ROS 17/2.8 cells, the protein complex binding to the WT-CBS probe is immunologically distinct from ROR proteins.

### **3.3.2 The effect of over-expression of various other known monomeric orphan receptors on wild-type CYP24 promoter constructs**

To extend the possibility that the CBS may represent a known monomeric orphan receptor binding site, cDNA expression clones for SF-1 (gift from Dr. Keith Parker, University of Texas), NGFI-B (gift from Dr. Tim Fahrner, Washington University), Rev-erb $\alpha$  (gift from Dr. Muscat, University of Queensland), and ERR $\alpha$  (gift from Dr. Vincent Giguere, McGill University) were ectopically expressed in 293T and ROS 17/2.8 cells and the response of the pCYP24(WT-186)-Luc constructs examined in the presence and absence of 1,25D. There was no significant effect of any monomeric orphan receptor cDNA clone on 1,25D induced activation of pCYP24(WT-186)-Luc in either 293T (endogenously expressing VDR) (Figure 3.13A), ROS 17/2.8 cells (Figure 3.13B) or UMR-106 cells (data not shown). ROS 17/2.8 cells and 293T cells have been employed in other studies to establish a response to exogenous expression of these orphan receptors (Meyer *et al.*, 2000; Vu-Dac *et al.*, 1997; Harding and Lazar, 1995; Vanacker *et al.*, 1999a and 1999b; Sanyal *et al.*, 2002; Tremblay *et al.*, 2001; Jacob *et al.*, 2001). This would seem to rule out the observation that the lack of effect from over-expression of these receptors shown in Figure 3.13 is due to high endogenous levels that may mask the effect of the transfected receptor. EMSA experiments were conducted to determine if oligonucleotide probes known to bind each monomeric orphan receptor could compete for the binding of CHEP to the WT-CBS oligonucleotide. In repeated experiments, a 10-fold molar excess of unlabeled competitor NGFI-B (Cheng *et al.*, 1997), SF-1 and ERR $\alpha$  (Bonnelye *et al.*, 1997), Rev-erb $\alpha$  and ROR $\alpha$ 1 (Dussault and Giguere, 1997) and ROR $\alpha$ 2 isoform (Dussault and Giguere, 1997; Giguere *et al.*, 1994) oligonucleotide probes failed to compete with the binding of CHEP to the WT-CBS oligonucleotide using 293T and ROS 17/2.8 cell nuclear extracts (data not shown). It would also prove valuable to detect the specific binding of each monomeric orphan receptor to its cognate sequence using antibodies recognising each receptor used in this study.

### **3.3.3 Investigating the role of ERR isoforms in 1,25D dependent CYP24 promoter activity**

**A****B**

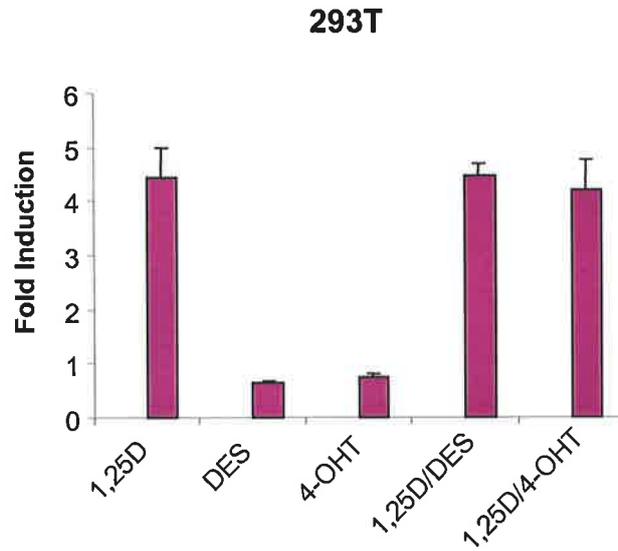
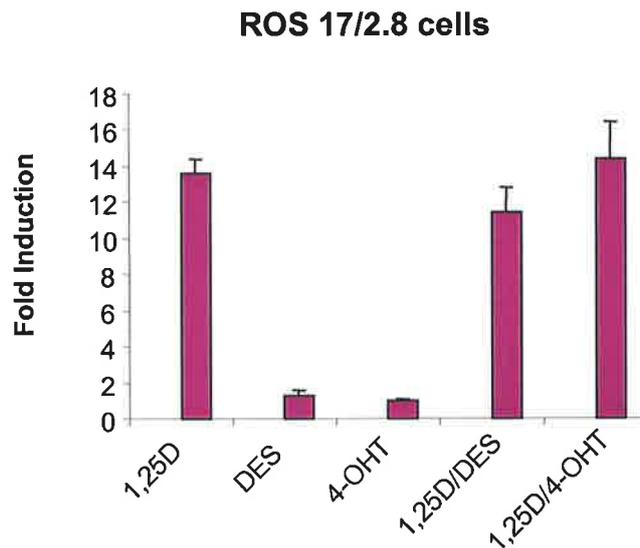
**Figure 3.13: Overexpression of known monomeric orphan receptors on pCYP24(WT-186)-Luc constructs.**

(A), 200ng of pCYP24(WT-186)-Luc together with 200ng of an expression vector for either Steroidogenic Factor-1 (pCMV5-SF-1), Nerve growth factor inducible-1 (pCB6-NGFI-B), Estrogen related receptor (pCMX-ERR $\alpha$ ) or pSG5-Rev-erb $\alpha$  was coexpressed in 293T cells treated with  $10^{-7}$ M  $1,25(\text{OH})_2\text{D}_3$ .

(B), As for (A) except that ROS 17/2.8 cells were used in transient transfection experiments. Data presented in experiment are the means  $\pm$  SD of triplicate samples from a representative experiment. The experiment was performed four times with the same trend obtained.

The coexpression of various monomeric orphan receptor clones did not induce 1,25D dependent activity of the CYP24 promoter in either 293T or ROS 17/2.8 cells. Some monomeric orphan receptors bind to 5'-flanking sequences similar in length but differing in sequence to the CBS (Figure 3.4). For example, ERR $\alpha$  and SF-1 bind to the 3bp 5'-flanking sequence 5'-TCA-3'. Their spatial expression prevents these two orphan receptors binding to the same sequence *in vivo* (Crawford *et al.*, 1998; Bonnelye *et al.*, 1997). High and overlapping expression of ERR $\alpha$  and ERR $\gamma$  isoforms has been detected in multiple sites including kidney and bone cells but ERR $\beta$  expression is predominantly restricted to embryonic development although 10-100x lower levels of ERR $\beta$  have been detected in tissues such as the kidney (Giguere *et al.*, 1988; Heard *et al.*, 2000; Hong *et al.*, 1999; Sladek *et al.*, 1997).

To further investigate if these subtypes of ERR can alter 1,25D dependent CYP24 promoter activity and further identify possible chemical regulators of CHEP function, pharmacological modulators of ERR function were analysed for their effect on the 1,25D inducibility of pCYP24(WT-186)-Luc constructs in 293T and ROS 17/2.8 cells. The synthetic estrogen 4-Hydroxytamoxifen (4-OHT) abolishes the constitutive activity of ERR $\beta$  and ERR $\gamma$  in transient transfection assays by preventing the interaction of coactivator SRC-1 with these receptors (Tremblay *et al.*, 2001). Similarly, another synthetic estrogen Diethylstilbestrol (DES) is an inhibitor of ERR $\alpha$  constitutive activity (Lu *et al.*, 2001; Coward *et al.*, 2001). Transfection of 293T and ROS 17/2.8 cells with pCYP24(WT-186)-Luc promoter constructs and treatment with either 4-OHT (2.5 $\mu$ M) or DES (10 $\mu$ M) alone did not affect promoter activity (Figure 3,14A & 14B, respectively). 4-OHT at a concentration of 2.5 $\mu$ M and DES at a concentration of 10 $\mu$ M was used in these studies because these concentrations have been used by others in transient transfection assays to antagonise ERR function. Similarly, cotreatment of these cells with these compounds together with 1,25D failed to modulate the activity of the CYP24 promoter in both cell types and therefore the effect of treating pCYP24(mCBS)-Luc constructs with these compounds was not further investigated. To validate that these compounds are effective in their ability to antagonise the constitutive activity of ERR subtypes, a reporter gene construct responsive to ERR over-expression should be investigated. These results suggest

**A****B**

**Figure 3.14: Pharmacological modulators of ERR receptor subtype and their effects on CYP24 promoter activity in 293T and ROS 17/2.8 cells.**

(A) 293T cells and (B), ROS 17/2.8 cells, were transfected with 200ng of pCYP24(WT-186)-Luc constructs. Transfected cells were treated with either 10 $\mu$ M Diethylstilbestrol (DES) or 2.5 $\mu$ M 4-hydroxytamoxifen (4-OHT) alone or in combination with 10<sup>-7</sup>M 1,25(OH)<sub>2</sub>D<sub>3</sub>. The levels of fold induction are calculated from the ratio of luciferase activity from hormone or inhibitor treated cells to that of untreated cells. Data presented in experiment are the means  $\pm$  SD of triplicate samples from a representative experiment. This experiment was performed three times with the same trend obtained.

that neither  $ERR\alpha$ ,  $ERR\beta$  nor  $ERR\gamma$  subtype appears to be functioning on the CYP24 promoter in response to 1,25D treatment and further supports the notion that over-expression of  $ERR\alpha$  did not affect CYP24 promoter activity in these cell types.

In conclusion, these results demonstrate that the over-expression of the known monomeric orphan receptor clones described in these studies does not act cooperatively or synergistically with 1,25D to enhance the activity of the CYP24 promoter. Coexpression of  $ROR\alpha1$  in 293T and ROS 17/2.8 cells repressed both basal and 1,25D induced CYP24 activity independent on the functional integrity of the CBS. Whether alternate isoforms of the members analysed in this study represent CHEP requires further investigation. Pharmacological modulators of ERR function did not significantly affect 1,25D inducibility of the CYP24 promoter. This data appears to rule out the involvement of ERR subtypes during the 1,25D inductive process. Therefore CHEP appears to be a transcription factor distinct from the monomeric orphan receptor clones analysed in this study.

### 3.3 Discussion

The transcriptional regulation of CYP24 gene expression by 1,25D is an important aspect of vitamin D metabolism. Up-regulation of CYP24 promoter activity is mediated by the presence of two VDREs, the proximal VDRE-1 being transcriptionally more active than the distal VDRE-2. In contrast to their activity, VDRE-2 binds liganded VDR/RXR heterodimers with greater affinity (Kerry *et al.*, 1996). This anomaly led to the proposal that nearby transcription factors may cooperate with VDRE-1. An Ets-1 binding site (EBS) located downstream of VDRE-1 was subsequently shown to be important for the 1,25D inductive response through VDRE-1 (Dwivedi *et al.*, 2002). The focus of this chapter has been the characterisation of an apparently novel binding site termed CBS (CYP24 Hydroxylase enhancing protein binding site) located approximately two turns upstream from VDRE-1. Previous transient transfection analysis (Figure 3.2) has established that the CBS is crucial for 1,25D responsiveness of pCYP24(WT-186)-Luc constructs, this activation being greater in ROS 17/2.8 cells than 293T cells.

Transient transfection of another osteoblast cell line termed UMR-106 cells and also COS-1 kidney cells and mutagenesis of the CBS, resulted in an approximate 90% and approximate 70% decrease respectively, of CYP24 promoter activity in response to 1,25D (data not shown). These results highlight that mutagenesis of the CBS in CYP24 promoter constructs is not restricted to ROS 17/2.8 cells or the 293T cells used in this study. Experiments using native and mutated-promoter constructs to define the CBS could not rule out the possibility that further sequence additional to 5'-TGTCGGTCA-3' could be required for the 1,25D-dependent transcriptional activation. For example, a non-classical VDRE consisting of two AGGTCA half-sites separated by 9bp instead of the usual 3bp has been shown to bind VDR/RXR complexes (Herdick *et al.*, 2000; Quack and Carlberg, 1999; Schrader *et al.*, 1997). Cloning the CBS (5'-GCGTGTCGGTCACCG-3') upstream of VDRE-1 in pTK(CBS/VDRE-1)-Luc led to the enhancement of VDRE-1 activity in response to 1,25D. This demonstrates that the sequence 5'-TGTCGGTCA-3' does not require any additional sequences from the native CYP24 promoter for 1,25D dependent enhanced activity.

The ability of the CBS to enhance pTK(VDRE-1)-Luc activity was greater in ROS 17/2.8 cells than in 293T cells (Figure 3.5). The reason for this is not known but the expression profile of coactivator proteins between these two cell types may be an important factor contributing to the level of transcriptional activation of the reporter constructs in response to 1,25D. It was noted that the activity of pTK(CBS/VDRE-1)-Luc in 293T and ROS 17/2.8 cells was low, with a 3-4-fold level of induction routinely observed, compared with the 12-20-fold induction observed with the CYP24 construct containing this VDRE. The low induction level directed by VDRE-1 when inserted in the TK promoter most likely reflects the absence of transcriptional cooperation with surrounding transcription factors that are present in the native CYP24 promoter.

The CBS alone did not confer 1,25D as shown by the lack of response of the pTK(CBS)-Luc construct to 1,25D. In support of this, VDR neutralising and RXR supershifting antibodies did not detect VDR or RXR binding to 5'-GCGTGTCGGTCACCG-3' in EMSA experiments with nuclear extracts from untreated and 1,25D-treated ROS 17/2.8, 293T and COS-1 cells (data not shown). This demonstrates that liganded heterodimers of VDR/RXR do not bind the CBS.

Additionally, inactivation of the VDREs within the native CYP24 promoter abolishes responsiveness to 1,25D.

The CBS did not contribute to basal expression of pTK(CBS)-Luc or basal expression of native CYP24 promoter constructs. In the absence of added 1,25D, the association of the corepressor RIP13Δ1, a variant of the corepressor N-CoR, has been shown to bind unliganded VDR and to weakly repress the activity of VDRE-1 (Dwivedi *et al.*, 1998). The corepressor bound at VDRE-1 could sterically prevent the binding of CHEP or alternatively the transactivation function of bound CHEP could be repressed directly by this corepressor. Whether CHEP is prevented from binding directly to the CBS *in vivo* will require the use of the CHIP (Chromatin immunoprecipitation) assay. Basal expression was not altered in heterologous constructs with the CBS cloned upstream of the TK promoter and in the absence of a VDRE supporting the proposal that CBS does not contribute to basal expression. It is possible that *in vivo* CHEP binds to the CBS in the native CYP24 promoter only in the presence of 1,25D. Alternatively, CHEP may need to interact at the protein-protein level with the VDR/RXR heterodimeric complex bound at the adjacent VDRE-1. This is speculation and requires further investigation.

As stated in section 3.2, the strategy employed resulted in the cloning of CBS upstream of VDRE-1 in TK constructs that led to the preservation of orientation and spacing of the CBS relative to VDRE-1 observed in the native promoter. As mentioned above, it is not known if CHEP can interact directly with the liganded VDR/RXR heterodimeric complex bound to VDRE-1. Furthermore the orientation of VDR/RXR binding to VDRE-1 is not known and therefore CHEP could be interacting directly with either VDR or RXR. Therefore it would be of interest to examine the effect of changing the spacing and orientation relative to VDRE-1 and re-examine the ability of the CBS to enhance 1,25D activity from this VDRE in both native and heterologous promoter constructs.

Computer based searches during this PhD. project using different transcription factor binding site programs did not identify the CBS as a binding site for any known transcription factor. The CBS containing a core 5'-CGGTCA-3' sequence and the 5'-flanking sequence 5'-TGT-3' appears to share the same characteristics

to binding sites for monomeric orphan receptors. These receptors bind as monomers to target DNA sequences consisting of a core half-site 5'-AGGTCA-3' preceded by 5'-flanking nucleotides of different length and sequence (Figure 3.4). The latter characteristic determines which monomeric orphan receptor binds to a particular site. Similarly, the CBS appears to share these characteristics. The CBS contains the core 5'-CGGTCA-3' almost identical to a consensus half-site 5'-AGGTCA-3' except for the 'C' at position 1 of the half-site. The 5'-flanking sequence 5'-TGT-3' is required also for the binding and transcriptional activity of CHEP binding to the CBS. Mutagenesis and expression analysis in ROS 17/2.8 and 293T cells demonstrated that conversion of the C in 5'-CGGTCA-3' to A generating the consensus nuclear receptor half-site 5'-AGGTCA-3', expressed equally as well as wild-type CBS constructs in response to 1,25D. The binding of CHEP to (5'-TGTAGGTCA-3') was slightly reduced in both cell types. Furthermore, the CBS sequence carrying a consensus nuclear receptor half-site (5'-TGTAGGTCA-3') had a significantly reduced ability to compete for the binding of CHEP to the wild-type CBS (5'-TGTCGGTCA-3'). The inability of 5'-TGTAGGTCA-3' sequences to compete for the binding of CHEP to the CBS despite the lack of effect on 1,25D induced CYP24 promoter activity is difficult to interpret. The reduced binding of CHEP to the TGTAGGTCA sequence may be compensated by CHEP interacting in a cooperative manner with surrounding transcription factors such as the liganded VDR/RXR heterodimeric complex binding at VDRE-1. Alternatively, the binding of CHEP could be stabilised through interactions with coactivators.

As shown in Figure 3.2, previous experimental analysis of the 5'-flanking nucleotides 5'-TGT-3' of the CBS demonstrated that mutagenesis of all three nucleotides abolishes both the DNA binding and transactivation function of CHEP. In the current study each nucleotide in the 5'-flanking sequence 5'-TGT-3' was mutated and examined for its ability to bind CHEP. Interestingly mutagenesis of 'G' in 5'-TGT-3' abrogated the binding of CHEP whilst mutagenesis of the third 'T' underlined in the sequence 5'-TGT-3' reduced but did not abolish the binding of CHEP. Mutagenesis of the first 'T' of 5'-TGT-3' marginally affected the binding of CHEP. These results strongly support the role of the 5'-flanking sequence in the binding of CHEP and furthermore leave the CBS with the characteristics of core binding site 5'-CGGTCA-3' and a 5'-flanking sequence 5'-TGT-3'. The functionality

of these mutations should be assessed using native-reporter constructs transfected into 293T cells. This would establish whether the loss of CHEP binding to these mutants correlates with a decrease in 1,25D activation of the CYP24 promoter. Due to time constraints this was not analysed in the current study.

UV cross-linking experiments demonstrated that CHEP is a DNA binding protein of approximately 60kDa. Interestingly, monomeric orphan receptors range between 50-70 kDa in size (Giguere, 1999). However, a limitation that the UV cross-linking technique carries is that it cannot differentiate whether CHEP represents a monomeric DNA binding or dimeric DNA binding protein.

To assess if the CBS represented a known monomeric orphan receptor binding site, cDNA expression clones for SF-1,  $ERR\alpha$ ,  $Rev-erb\alpha$ , NGFI-B and  $ROR\alpha1$  were over-expressed on WT-186bp CYP24 promoter constructs transfected into 293T and ROS 17/2.8 cells. None, except for  $ROR\alpha1$ , had any effect on 1,25D induction of wild-type CYP24 promoter constructs.  $ROR\alpha1$  over-expression led to a decrease in both basal and 1,25D induced activity of wild-type and mutant CBS promoter constructs. This was a surprising result.  $ROR\alpha1$  appeared to be a likely candidate factor binding to the CBS for several reasons. For example  $ROR\alpha$  is expressed in osteoblasts and  $ROR\alpha1$  knockout mice produce a “staggerer” phenotype that has also led to the identification that the bones of  $ROR\alpha$  mice are osteopenic (Meyer *et al.*, 2000). Furthermore  $ROR\alpha$  has multiple isoforms each with subtle binding differences in the recognition of 5'-extension sequences (Giguere *et al.*, 1994) and lastly  $ROR\alpha$  has been shown to modulate 1,25D induced expression of the osteocalcin gene (Meyer *et al.*, 2000). The observation that repression continued following the inactivation of the CBS suggested that the repressive effect of  $ROR\alpha1$  over-expression is occurring elsewhere on the promoter. This effect in part appears to be mediated through VDRE-1 as inactivation of this VDRE almost abolished the repressive effect of  $ROR\alpha1$  over-expression. The data presented in this chapter resembles that reported for the over-expression of  $ROR\alpha1$  on the osteocalcin gene (Meyer *et al.*, 2000) where the over-expression of  $ROR\alpha1$  led to suppression of 1,25D induced osteocalcin activity. The authors did not examine the molecular basis behind  $ROR\alpha1$  mediated suppression of 1,25D induced osteocalcin activity. The significance of CYP24

promoter repression or the molecular basis behind this repression is not known. Orphan receptors and ROR $\alpha$  have been shown to interact strongly with coactivator proteins (Atkins *et al.*, 1999). It is plausible that sequestering of a coactivator protein binding to the liganded VDR/RXR heterodimeric complex at VDRE-1 is occurring upon ROR $\alpha$  overexpression. This requires further investigation. In contrast, ROR $\alpha$  overexpression has been shown to augment thyroid hormone receptor (Koibuchi *et al.*, 1999) and peroxisome proliferator-activated receptor (Winrow *et al.*, 1998) mediated transcriptional activation. Therefore cross-talk between nuclear receptors and orphan receptors exists as a mechanism to control gene expression. Supershifted antibodies recognising multiple isoforms of ROR $\alpha$  failed to detect binding of these proteins to the CBS in cells not transfected and cells transfected with pSG5-ROR $\alpha$ 1 further demonstrating that ROR $\alpha$ 1 is not involved in CYP24 gene promoter activation. Three supershifted bands were detected upon binding the RORE oligonucleotide using 293T extracts transfected with ROR $\alpha$ 1. The reason for three supershifted complexes is unclear but may represent different conformations of ROR $\alpha$  proteins that result following the binding of the antibody to its epitope located at the C-terminus of ROR $\alpha$ . Of note was the finding that oligonucleotide probes encompassing binding sites for all the orphan receptors analysed in transfection studies failed to compete for the binding of CHEP to the WT-CBS oligonucleotide probe (data not shown). This occurs despite the fact that the mouse N-myc gene RORE containing the ROR $\alpha$  binding sequence TAATGTAGGTCA is similar to but longer in length to the CBS. This oligonucleotide having "TGT" as part of the ROR $\alpha$ 1 5'-flanking sequence when used as an unlabeled competitor in competition EMSA experiments did not prevent the binding of CHEP to WT-CBS oligonucleotide probe (data not shown). In hindsight, this result is expected. Exchanging 'C' in the core of the CBS to 'A' (generating 5'-TGTAGGTCA-3') which now resembles more closely the RORE, compromised the ability of this oligonucleotide to compete for the binding of CHEP to the WT-CBS oligonucleotide probe (Figure 3.7). Therefore, the binding of CHEP to the CBS is critically dependent on 'C' at position 1 of the core. It would be interesting to examine what effect the over-expression of each of the monomeric orphan receptors tested in this study have on CYP24 constructs in which the 'C' in the CBS was changed to 'A'.

It also seemed possible that ERR receptors could be involved because a) they have wide tissue expression patterns, b) there are three subtypes of ERR, ERR $\alpha$ , ERR $\beta$  and ERR  $\gamma$ , c) and they activate bone specific genes in ROS 17/2.8 cells (Vanacker *et al.*, 1998). Interestingly, ERR receptors bind to the 5'-extension sequence 5'-TCA-3'. This 5'-extension is similar in length but not identical to the 5'-extension of the CBS (5'-TGT-3'). Collectively, these facts formed the basis to investigate if ERR receptors could act through the CBS in the CYP24 promoter. In a separate approach, the antagonists 4-OHT and DES which disrupt the interaction of coactivators with the AF-2 domain of ERR receptors, was used to support the data of over-expressing ERR $\alpha$  on wild-type CYP24 constructs. The treatment of ROS 17/2.8 cells and 293T cells with DES and 4-OHT in transient transfection assays ruled out the possibility that ERR $\beta$  and ERR $\gamma$  receptors participated in CYP24 promoter activation in response to 1,25D. These results support the results which demonstrate that ERR $\alpha$  over-expression has no effect on 1,25D induced CYP24 promoter activity. Despite the routine use of 4-OHT and DES in transient assays, the ability of the compounds 4-OHT and DES to antagonize an ERR $\alpha$  responsive reporter gene construct transfected in 293T and ROS 17/2.8 cells should also be investigated in future.

In conclusion, the studies in this chapter have demonstrated that the CBS (5'-TGTCGGCTA-3') can function independent of sequences elsewhere in the CYP24 promoter to enhance 1,25D mediated gene expression from VDRE-1. These results also demonstrate that the CBS sequence is not a VDRE or part of VDRE in the CYP24 promoter. The CBS does not alter basal expression of TK constructs. The level of transcriptional enhancement attributed to the CBS appears greater for the osteoblastic cells (ROS 17/2.8 and UMR-106 [data not shown]) compared with kidney 293T cells. All 5'-flanking nucleotides, in particular the central 'G' in 5'-TGT-3', are important for the binding of CHEP to the CBS. Conversion of the CBS core 5'-CGGTCA-3' to a perfect consensus nuclear receptor half-site AGGTCA reduced, but did not abolish the binding of CHEP. The conversion of the CBS core to a perfect consensus half-site had no effect on the functionality of the CBS in response to 1,25D. The over-expression of different monomeric orphan receptor cDNA clones, the use of antibody experiments and pharmacological modulators of orphan receptor function, had no effect on the 1,25D inducibility of wild-type

CYP24 promoter constructs. Therefore the CBS apparently represents a binding site distinct from those monomeric orphan receptors analysed in this study.

## CHAPTER 4

# **Investigation of the effects of 1,25D and phorbol ester treatment on CYP24 gene promoter expression**

## 4. Investigation of the effects of 1,25D and phorbol ester treatment on CYP24 gene promoter expression

### 4.1 Introduction

It is well established that the phorbol ester, phorbol 12-myristate 13-acetate (PMA), is a potent inducer of various protein kinase C isoenzymes. Protein kinase C (PKC) isoenzyme activation by PMA varies from cell type to cell type but the effects of PMA are principally to modulate the activity of various transcription factors such as Ets-1 and Sp1 (Orzechowski *et al.*, 2001, Chou *et al.*, 2003, Lindemann *et al.*, 2003) but mostly the AP-1 family of transcription factors (Karin and Shaulian *et al.*, 2001, Reddy *et al.*, 2003). The AP-1 family of transcription factors consists of dimers composed of Jun and Fos proteins. The Jun family members are c-Jun, Jun B, Jun D and the Fos family members consist of c-Fos, Fos B, Fra-1 and Fra-2. It has been known for many years that 1,25D and PMA synergistically stimulate expression of CYP24 and that this involves PKC activity (Omdahl and May, 1997). This synergistic action appears to be cell type specific. The PMA and 1,25D induced transcriptional synergy of CYP24 has been shown in kidney cells (Mandla *et al.*, 1990; Chen *et al.*, 1993a) and intestinal cells (Koyama *et al.*, 1994; Armbrecht *et al.*, 1993; Armbrecht *et al.*, 2001) but not in the osteoblast cells (Yang *et al.*, 2001). Studies have also shown that PMA and 1,25D induced transcriptional synergy of CYP24 promoter activity is lacking in ROS 17/2.8 osteoblast cells (Prem Dwivedi, personal communication). The molecular mechanism by which PMA stimulates 1,25D-induced CYP24 promoter activity has remained elusive (Omdahl and May, 1997).

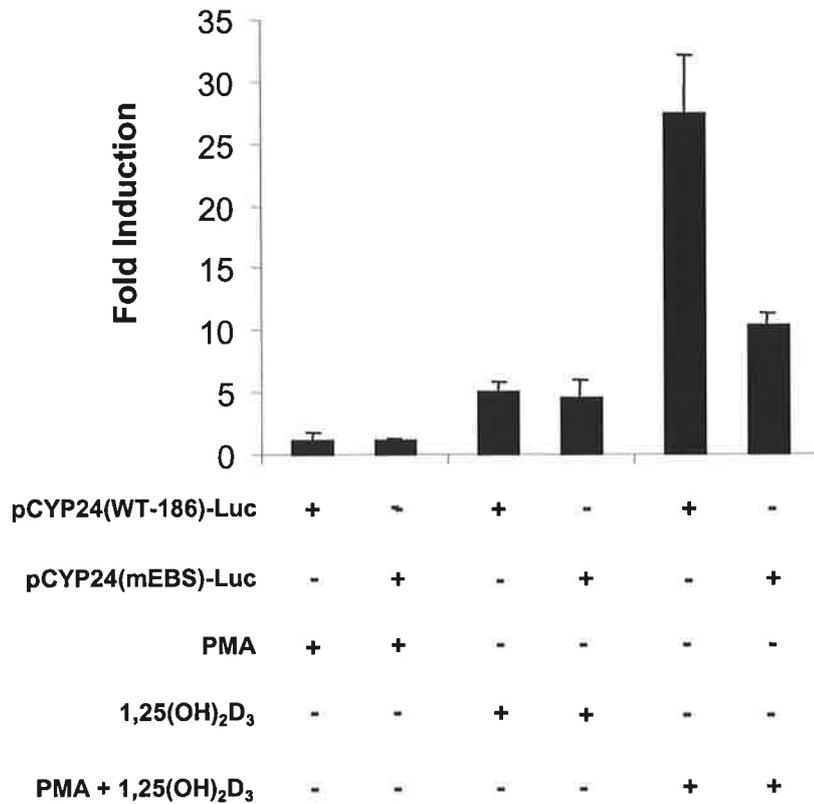
A major aim of the work in this chapter was to determine if the CBS was responsive to PMA. All PMA studies described in this chapter were performed in kidney 293T cells because PMA and 1,25D dependent transcriptional synergy is observed in these cells but not in ROS 17/2.8 cells (as stated above). As will be described shortly, the studies in this chapter demonstrate that CBS is PMA responsive. This raised the possibility that the CBS could be a binding site for the AP-1 protein. Later experiments in this chapter therefore describe the use of EMSA experiments in conjunction with supershifting antibodies directed against all AP-1 proteins, to determine if AP-1 proteins bind to the CBS.

## Results

### 4.2 Investigating the effect of PMA action on the CBS

Transient expression studies performed already by Dr. Dwivedi and Barbara Nutchey in our laboratory had demonstrated that treatment of 293T cells with 1,25D ( $10^{-7}$ M or  $10^{-8}$ M) and PMA (160nM) for 2 hours leads to a synergistic enhancement of 1,25D induced CYP24 promoter activity. The work described in Figures 4.1 and 4.2 was performed in collaboration with Barbara Nutchey. As shown in Figure 4.1, wild-type (pCYP24(WT-186)-Luc) and pCYP24(mEBS)-Luc constructs in which the EBS is inactivated, were transfected into 293T cells. Following transfection of 293T cells, these cells were treated with either PMA (160nM) alone for 2 hours, 1,25D ( $10^{-7}$ M) alone for 24 hours and together (PMA for 2 hours and 1,25D for 24 hrs). A 160nM concentration of PMA was used in all experiments because this concentration is similar to those used in this field of study (Yang *et al.*, 2001; Xing *et al.*, 2002). Secondly, of the various doses of PMA tested in our laboratory, this concentration was shown to significantly induce 1,25D-mediated induction of wild-type promoter constructs. In all dual luciferase experiments, the vector used for the correction of transfection efficiency, pRL/TK-Renilla luciferase, expresses the Renilla luciferase gene under the control of the thymidine kinase promoter. It was found that PMA increased the expression of the pRL/TK-Luc gene by about 2-fold and therefore, in all transfection experiments examining the effect of PMA, the pRL/null-Luc vector (Promega) was employed as the internal control for the dual luciferase assay. The pRL-null vector lacks a eukaryotic promoter and was found not to be modulated by PMA treatment.

As shown in Figure 4.1, wildtype promoter activity was induced to a  $4.96 \pm 0.84$ -fold level upon 1,25D treatment. Mutagenesis of the EBS did not significantly affect 1,25D-mediated induction in this experiment. However, mutagenesis of the EBS was found to give variable results. In some experiments inactivation of the EBS marginally affected 1,25D induction of CYP24 promoter constructs and this can be seen in Figure 4.2. By comparison, mutagenesis of the EBS in COS-1 cells reduces fold 1,25D induction by approximately 50% (Dwivedi *et al.*, 2000; Dwivedi



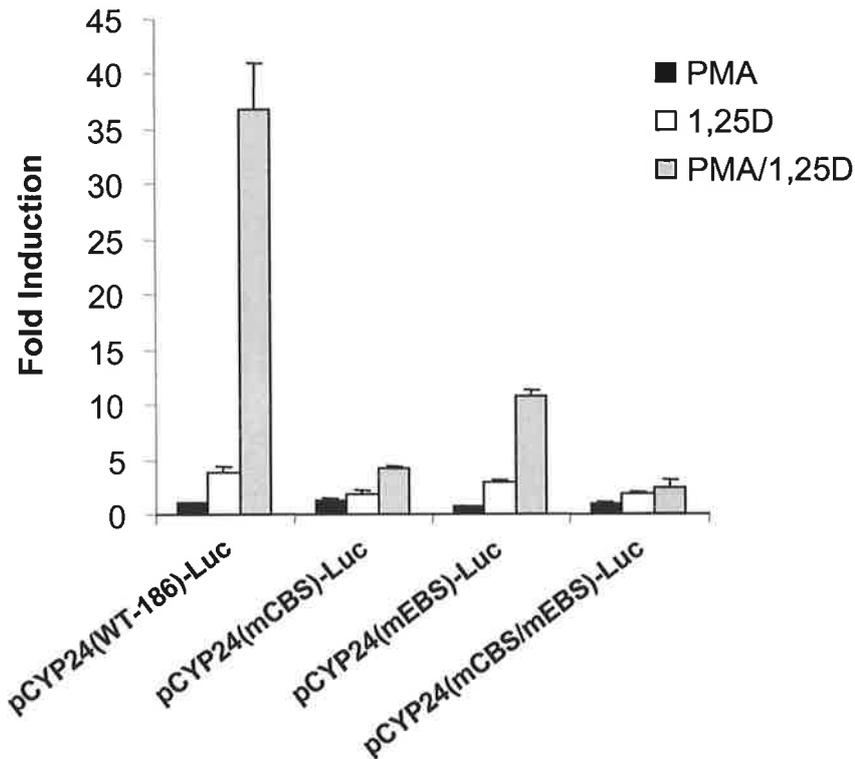
**Figure 4.1: Examining the response of the EBS to PMA and 1,25D treatment in 293T cells.**

Wildtype (pCYP24(WT-186)-Luc) and mutant EBS (pCYP24(mEBS)-Luc) constructs were examined for PMA responsiveness in 293T cells. 200ng of each construct together with 50ng of control pRL/null-*Renilla* (containing no eukaryotic promoter sequences) instead of the pRL/TK-*Renilla* vector, that corrects for transfection efficiency, were transiently transfected into 293T cells. Cells were treated with either PMA (162nM) alone for 2 hours, 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-7</sup>M) alone for 24 hours or PMA (2 hours) and 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-7</sup>M) together. Activity was normalised against the expression of cotransfected *Renilla* luciferase activity. The levels of induction (fold) are calculated from the ratio of luciferase activity from treated cells to that from untreated (ethanol) cells. Data presented in the experiment are the means ± SD of a representative experiment. The experiment was performed three times with the same trend obtained. This work was performed in collaboration with Dr. Prem Dwivedi and Barbara Nutchey.

*et al.*, 2002). The expression of pCYP24(WT-186)-Luc and pCYP(mEBS)-Luc constructs was not affected by PMA treatment alone. PMA and 1,25D cotreatment of 293T cells resulted in wild-type CYP24 promoter activity being increased from  $4.96 \pm 0.84$ -fold to  $27.31 \pm 4.84$ -fold. The induction of wild-type constructs in the presence of PMA and 1,25D ( $27.31$ -fold) is greater than the sum of induction of wild-type constructs treated with PMA alone and 1,25D alone ( $1.11 + 4.96 = 6.07$ -fold). Therefore an approximate 4.5-fold level of synergy arises when pCYP24(WT-186)-Luc constructs are treated with PMA and 1,25D together. Mutagenesis of the EBS clearly reduced the synergistic enhancement of CYP24 induction by PMA and 1,25D treatment. A  $10.43 \pm 0.88$ -fold level of induction was observed upon mutagenesis of the EBS halving the synergy from 4.5-fold in wildtype constructs to 2.3-fold. Therefore the synergistic effect of PMA and 1,25D cotreatment appears to act partially through the EBS.

The same experiment described above and shown in Figure 4.1 was repeated and the CYP24 promoter construct lacking a functional CBS was also included in the experiment (Figure 4.2). Treatment of wild-type and mutant CBS, EBS and the double mutant CBS/EBS constructs with PMA alone did not affect CYP24 promoter activity. 1,25D treatment of the wild-type construct resulted in a  $3.77 \pm 0.67$ -fold level of induction that was reduced to a  $1.81 \pm 0.4$  level upon mutagenesis of the CBS. Mutagenesis of the EBS in pCYP24(mEBS)-Luc had a marginal effect on CYP24 promoter activity with a  $2.96 \pm 0.12$ -fold level of induction observed. The effect upon mutagenesis of the EBS was found to be variable in 293T cells and small compared with inactivation of the EBS in COS-1 cells as already discussed. Mutagenesis of both the CBS and EBS together in pCYP24(mCBS/mEBS)-Luc lowered the induction of pCYP24(mCBS/mEBS)-Luc to  $1.75 \pm 0.27$ -fold, a level similar to the activity of constructs lacking a functional CBS only. These results demonstrated the higher dependency of CYP24 promoter expression on the CBS compared with the EBS.

PMA and 1,25D cotreatment activated the wild-type construct from  $3.77 \pm 0.67$ -fold to  $36.86 \pm 4.22$ -fold. This is an approximate 7.5-fold level of transcriptional synergy, somewhat higher in this experiment compared with Figure 4.1. Significantly, in the presence of PMA and 1,25D, mutagenesis of the CBS lowered



**Figure 4.2: The response of CYP24 constructs to PMA and 1,25D treatment upon mutagenesis of the EBS and CBS alone and together in 293T cells.**

Wildtype (pCYP24(WT-186)-Luc) mutant CBS (pCYP24(mCBS)-Luc) and mutant EBS (pCYP24(mEBS)-Luc) constructs were examined for PMA responsiveness in 293T cells. 200ng of each construct together with 50ng of control pRL/null-*Renilla* (promoter less) instead of pRL/TK-*Renilla* vector that corrects for transfection efficiency were transiently transfected into 293T cells. Cells were treated with either PMA (162nM) alone for 2 hours, 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-7</sup>M) alone for 24 hours or PMA (2 hours) and 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-7</sup>M) together. Activity was normalised against the expression of cotransfected *Renilla* luciferase activity. The levels of induction (fold) are calculated from the ratio of luciferase activity from treated cells to that from untreated (ethanol) cells. Data presented in the experiment are the means ± SD of a representative experiment. The experiment was performed three times with the same trend obtained. This work was done in collaboration with Dr. Prem Dwivedi and Barbara Nutchey.

induction of the wildtype construct from  $35.68 \pm 4.22$ -fold to  $4.19 \pm 0.27$ -fold. The  $4.19 \pm 0.27$ -fold level of induction of pCYP24(mCBS)-Luc is similar to the sum of fold activity of pCYP24(mCBS)-Luc in the presence of PMA alone and 1,25D alone ( $1.21+1.81$ , respectively). These results demonstrate that mutagenesis of the CBS abrogates PMA and 1,25D induced transcriptional synergy of CYP24 promoter constructs. Mutagenesis of the EBS lowered PMA and 1,25D induced wild type induction from  $35.68 \pm 4.22$ -fold to  $10.81 \pm 0.41$ -fold. The level of synergy is therefore reduced from 7.5 fold to 3-fold when the EBS is inactivated. This data are in agreement with the results presented in Figure 4.1 and demonstrate that the EBS partially mediates PMA induced 1,25D dependent transcriptional synergy. Mutagenesis of both the CBS and EBS together gave a  $2.43 \pm 0.62$ -fold level of induction in response to PMA and 1,25D, similar to the activity of the pCYP24(mCBS)-Luc construct ( $1.75 \pm 0.27$ -fold) lacking only a functional CBS. Again, PMA induced 1,25D dependent transcriptional synergy is abolished upon mutagenesis of the CBS and EBS together. This was an expected result since mutagenesis of the CBS alone abrogated PMA induced 1,25D dependent synergy. It can be noted that treatment of the pCYP24(mCBS/mEBS)-Luc construct with PMA and 1,25D together increased fold induction of this construct to levels similar to constructs treated with 1,25D alone. This result supports the finding that the effect of PMA and 1,25D appears to act only through the CBS and the EBS.

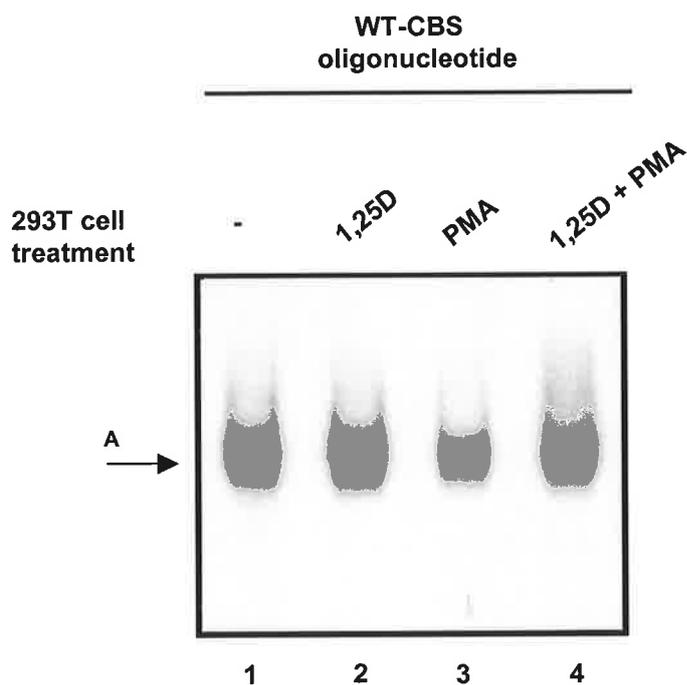
In conclusion these results demonstrate the importance of both the CBS and EBS in PMA and 1,25D mediated transcriptional synergy of CYP24 promoter expression. Importantly, the effect of PMA in the presence of 1,25D is partially mediated through the EBS although in the presence of 1,25D alone the requirement for a functional EBS is marginal in 293T cells. Strikingly, mutagenesis of the CBS abrogates the transcriptional synergism induced by PMA and 1,25D cotreatment. This result also reflects the importance of a functional CBS during 1,25D-mediated induction of CYP24 promoter activity.

#### **4.2.1 EMSA analysis of proteins binding to the CBS using nuclear extracts from cells treated with PMA and 1,25D**

EMSA experiments were undertaken to determine if the synergistic effect observed with PMA and 1,25D cotreatment of 293T cells was the result of altered CHEP binding to the CBS. For this work, nuclear extracts were isolated from 293T cells treated with either PMA or 1,25D alone and PMA and 1,25D together under the same conditions used in transient transfection assays. That is, cells were pretreated with PMA for 2 hours before 1,25D addition at  $10^{-7}$ M for 24 hours. For EMSA experiments, a double-stranded WT-CBS oligonucleotide probe was incubated with 293T nuclear extracts isolated from cells treated with PMA alone, 1,25D alone, and PMA and 1,25D together. As shown in Figure 4.3, the binding of the major complex, complex A (or CHEP), to the WT-CBS oligonucleotide was not altered. Despite the fact that the synergy between PMA and 1,25D is not observed in ROS 17/2.8 cells (as mentioned earlier). An EMSA experiment was carried out with nuclear extracts isolated from ROS17/2.8 cells treated with PMA alone, 1,25D alone, and PMA and 1,25D together. Two complexes were observed (as seen earlier in Figure 3.3) but the binding of these complexes was not altered with these extracts (data not shown). It may be of interest to re-examine the binding of proteins to the WT-CBS in the experiment shown in Figure 4.3 but to include less nuclear extract in the binding reactions. The reason behind this is that binding of proteins to the CBS appears quite intense (see Figure 4.3) and this may therefore mask any small increase in the binding of proteins to the WT-CBS oligonucleotide. However, these results demonstrate that the synergistic effect of PMA and 1,25D induction of CYP24 promoter activity does not appear to act by substantially increasing the amount of CHEP bound to the CBS.

#### **4.2.2 Identification of a sequence highly homologous to the CBS**

Xing *et al.* (2002) characterised a sequence termed RE-3, present in the promoter of the follicle-stimulating hormone (FSH) receptor gene. As shown in Figure 4.4A, the RE-3 sequence (5'-TGACCCACA-3') differs at one position from the CBS sequence on the noncoding strand (5'-TGACCGACA-3'). EMSA experiments utilising supershifting antibodies demonstrated that RE-3 bound c-Jun/c-Fos, SF-1, USF-1/2 and COUP-TF proteins. Interestingly, transient transfection analysis demonstrated that the RE-3 sequence was PMA responsive in both JC-410 and 15P1 gonadal cell lines and mutagenesis of this element abrogated the PMA response (Xing *et al.*, 2002). Since RE-3 bears similarity to the consensus AP-1

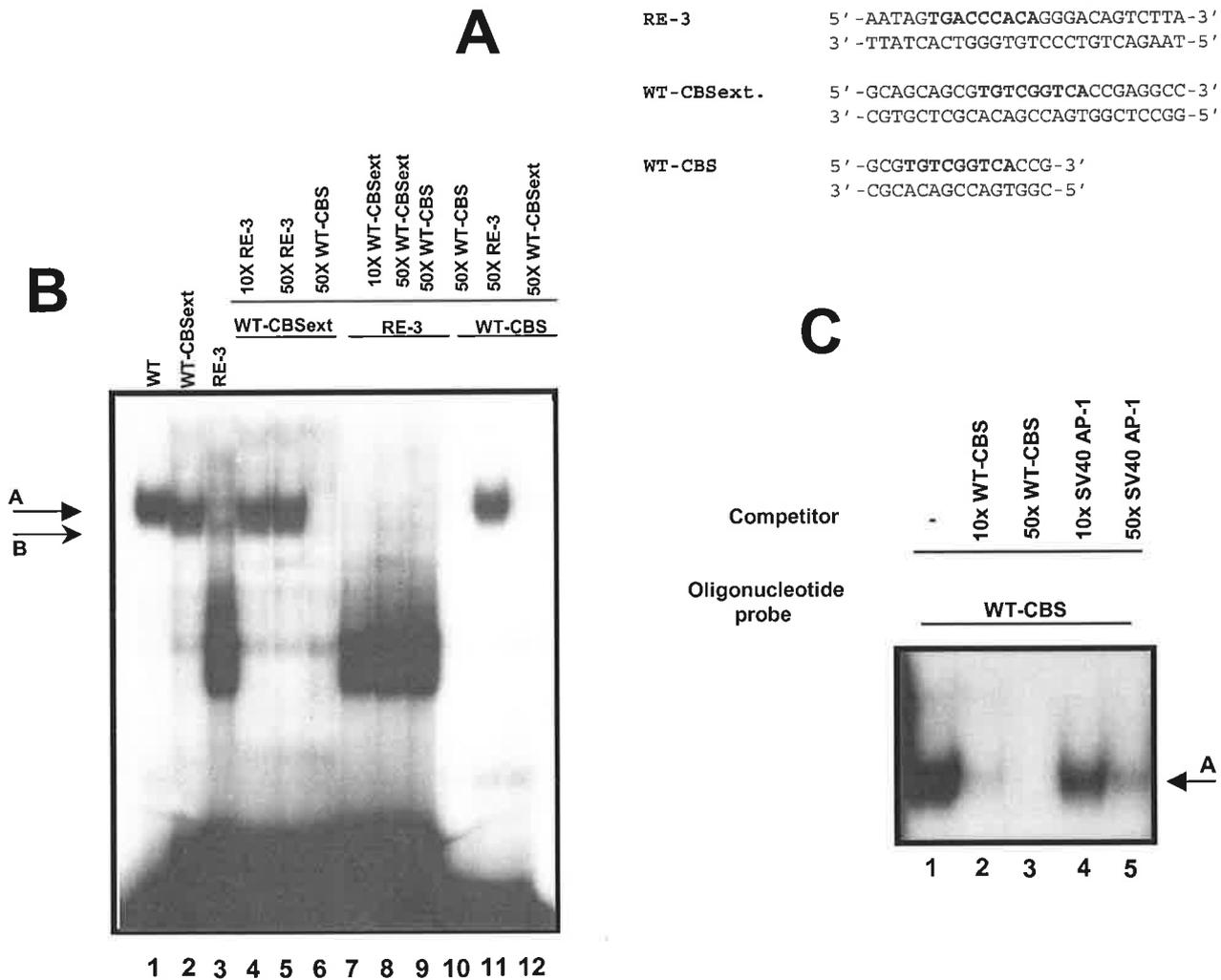


**Figure 4.3: The binding of proteins to the CBS in response to PMA, 1,25D or PMA and 1,25D treatment.**

Nuclear extracts from 293T cells were prepared from cells untreated and cells treated with either 162nM PMA for 2hrs,  $1,25(\text{OH})_2\text{D}_3$  at  $10^{-7}\text{M}$  for 24hrs or PMA + 1,25D for 2hrs and 24hrs, respectively. To examine if the binding of proteins is altered upon exposure to the various treatment groups, nuclear extracts were incubated with the labeled WT-CBS oligonucleotide probe (5'-GCGTGTCGGTCACCG-3') and the retarded complexes formed were compared with WT-CBS oligonucleotide probe incubated with untreated 293T nuclear extracts. Complex A denotes the single band retarded on the WT-CBS oligonucleotide.

binding site (compare RE-3 5'-TGACCCACA-3' with AP-1 consensus 5'-TGAC/GTCA-3'), and binds c-Jun and c-Fos and responds to PMA, Xing *et al.* (2002) concluded that the RE-3 sequence could function as an AP-1 site by binding c-Jun and c-Fos protein. As discussed earlier, it is well established that AP-1 is a downstream target following PKC activation by PMA. AP-1 proteins consist of dimers of either Jun/Fos or Jun/Jun proteins which bind to the consensus sequence 5'-TGAC/GTCA-3'. PMA dependent PKC activation and consequently MAP kinase activation can result in the modulation of the AP-1 complex. For example, the phosphorylation of the AP-1 complex, particularly Jun proteins (Franklin and Kraft, 1995, Carter *et al.*, 2001) and modulation of Jun/Fos mRNA levels (Chen *et al.*, 2001; Won *et al.*, 1998; Clerk *et al.*, 2002) can lead to the alteration of the transcriptional response by AP-1.

Because of the similarity in sequence between the RE-3 and the CBS, and the knowledge that the CBS is responsive to PMA (in the presence of 1,25D), EMSA experiments were undertaken to compare protein binding to the RE-3 and CBS using nuclear extracts prepared from 293T cells not treated with 1,25D or PMA. To accomplish this, a new double-stranded WT-CBS oligonucleotide probe termed WT-CBSext. was synthesized so that it was identical in length to the RE-3 oligonucleotide probe, as shown in Figure 4.4A. As shown in Figure 4.4B, the WT-CBS oligonucleotide retarded a single complex termed complex A, (lane 1). The WT-CBSext. oligonucleotide retarded a complex similar to complex A and another complex with slightly higher mobility termed complex B (lane 2). Complex B was only observed upon lengthening the WT-CBS oligonucleotide. The RE-3 oligonucleotide retarded several high mobility complexes (seen in the bottom half of the gel) and three weak binding complexes (lane 3). The three weak binding complexes are seen more clearly in Figure 4.5, lane 4. Of the three weak binding complexes detected using the RE-3 oligonucleotide, the complex of intermediate mobility appears to have mobility similar to complex A binding to the WT-CBS and WT-CBSext. oligonucleotide. A complex with similar mobility to complex B binding to the WT-CBSext. oligonucleotide was also observed binding to the RE-3 oligonucleotide (compare lane 3 with lane 2). The binding of complex A and B to the WT-CBSext. oligonucleotide was not competed when a 10-fold or a 50-fold molar excess of unlabeled competitor RE-3 oligonucleotide was added to the binding reaction (lane 4 and lane 5). Competition with a 50-fold molar excess of



**Figure 4.4: Comparison of the binding of proteins to the RE-3 element characterised by Xing *et al.* (2002) and the SV40 AP-1 oligonucleotide with the binding of proteins to the WT-CBS oligonucleotide.**

(A), Comparison of the RE-3 element [characterised by Xing *et al.* (2002)] WT-CBS and WT-CBSext. Antibody EMSA experiments by Xing *et al.* (2002) demonstrated that AP-1 (c-Jun/c-Fos), SF-1 and USF-1/2 transcription factors bound the RE-3 oligonucleotide. The sequence highlighted in the RE-3 oligonucleotide in bold appears as the reverse complement to the CBS. The single nucleotide mismatch replaces 'C' with 'G' in the 5'-CGGTCA-3' core of the CBS to give 5'-GGGTCA-3' found in the composite RE-3 element. WT-CBS oligonucleotide probes were extended in length using additional native promoter sequences to generate the WT-CBSext. oligonucleotide. The WT-CBSext. oligonucleotide is identical in length to the RE-3 oligonucleotide used by Xing *et al.* (2002). (B), The binding of proteins to the RE-3 oligonucleotide and the WT-CBSext. oligonucleotide were compared in EMSA experiments. 293T cell nuclear extracts were incubated with either WT-CBS (lane 1), WT-CBSext. (lane 2) or RE-3 (lane 3) oligonucleotide probes to compare the binding pattern profile. Arrow A depicts specific CHEP/CBS complex formed using 293T nuclear extracts. Arrow B denotes a faster migrating complex present only upon binding the WT-CBSext. oligonucleotide. To determine if the same complex bound to the RE-3 and the WT-CBSext. oligonucleotide, competition assays using a 10-fold and 50-fold molar excess of unlabeled oligonucleotide (as specified in figure) was included in the binding reaction. (C), Nuclear extracts were incubated with [<sup>32</sup>P]-labeled WT-CBS oligonucleotide. A 10-fold or 50-fold molar excess of unlabeled self (WT-CBS) (lanes 2 and 3) or SV40 AP-1 (sequence highlighted in bold, 5'-TCGACGCTTGAT**GACTCAGCCGGAAC**-3') (lanes 4 and 5) were added to the binding reactions prior to the addition of labeled probe. Complex A denotes single retarded complex routinely binding the CBS. This is a preliminary experiment performed as a single experiment.

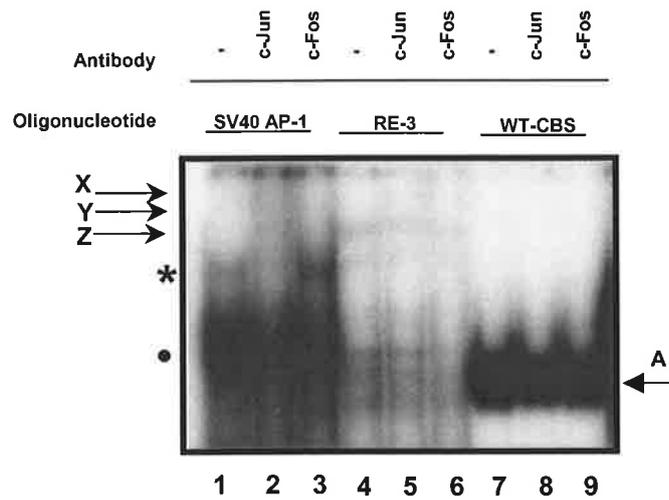
unlabeled WT-CBS competed effectively with the binding of complex A and B to the WT-CBS<sub>ext.</sub> oligonucleotide. Interestingly, competition with unlabeled WT-CBS<sub>ext.</sub> oligonucleotide appeared to compete with the binding of the three complexes of mobility similar to complexes A and B on the RE-3 oligonucleotide (compare lane 7 and lane 8 with lane 3). Competition with unlabeled WT-CBS oligonucleotide appeared to reduce the binding of the same three complexes to the RE-3 oligonucleotide (lane 9). These results suggest two possibilities. Firstly, WT-CBS and WT-CBS<sub>ext.</sub> oligonucleotide may bind similar proteins to the RE-3 oligonucleotide. Secondly, the competition for binding of proteins to the RE-3 oligonucleotide by the WT-CBS oligonucleotide could be due to the protein binding the CBS having low affinity for the RE-3, or the competition is due to non-specific competition in the EMSA. On this note, a 50-fold molar excess of unlabeled RE-3 oligonucleotide did not affect the binding of complex A to the WT-CBS oligonucleotide (lane 11) although, competition with self oligonucleotide abolished the formation of the complex (lane 10 and lane 12). In conclusion, the important points drawn from these results are as follows. Firstly, the WT-CBS oligonucleotide appears to bind complex A quite strongly. The WT-CBS<sub>ext.</sub> oligonucleotide appears to bind complex A quite strongly as well as retarding complex B which has mobility slightly higher than complex A. Secondly, the RE-3 oligonucleotide retards three weak binding complexes, the lower band having mobility similar to complex B and the middle complex having mobility similar to complex A. Lastly, the three weak complexes retarded on the RE-3 oligonucleotide appear to be competed by unlabeled WT-CBS<sub>ext.</sub> oligonucleotide. It is difficult to clearly interpret these results, but of the three complexes retarded on the RE-3 oligonucleotide, two appear to have similar mobility to complex A and therefore could be the same proteins binding to the CBS.

Xing *et al.* (2002) demonstrated that c-Jun and c-Fos were two of several proteins that weakly bound the RE-3 oligonucleotide. On this basis, a control oligonucleotide encompassing an AP-1 binding site from the SV40 promoter (Santa Cruz Biotechnology) was employed in competition EMSAs. As shown in Figure 4.4C, a 10-fold molar excess of unlabeled SV40 AP-1 oligonucleotide reduced slightly the binding of complex A to the WT-CBS oligonucleotide (lane 4). The binding of complex A to the WT-CBS was further reduced in intensity upon addition of a 50-fold molar excess of SV40 AP-1 oligonucleotide. These SV40 and

RE-3 oligonucleotide competition EMSA experiments appear to suggest that complex A binding to the WT-CBS oligonucleotide could consist of AP-1 proteins.

It is not known in the experiments performed in Figure 4.4, whether any of the complexes binding the RE-3 oligonucleotide from nuclear extracts prepared from 293T cells consist of AP-1. Therefore EMSA experiments using supershifting c-Jun and c-Fos antibodies were employed to investigate complex binding to the RE-3 oligonucleotide. A control oligonucleotide encompassing the SV40 AP-1 sequence (Santa Cruz Biotechnology) was also included in the EMSA experiment.

As shown in Figure 4.5, the SV40 AP-1 oligonucleotide retarded several different complexes using 293T nuclear extracts (lane 1). Inclusion of c-Jun antibody produced three faint supershifted complexes (X, Y, and Z in lane 2) which are difficult to see in this gel photo. To better visualise the supershifted complexes, the reader is referred to figure 4.7B (lane 2 and lane 3), where these supershifted complexes observed with c-Jun antibody can be clearly seen. The complex marked with an asterisk is supershifted upon the addition of c-Jun antibody and therefore this band represents a complex containing AP-1 proteins (see lane 1 in Figures 4.5 and 4.7B). Surprisingly, no supershifted bands were detected using c-Fos antibody (Figure 4.5, lane 3) and this is further supported in the results shown in Figure 4.7B. The RE-3 oligonucleotide retarded three faint complexes (lane 4) similar to the three complexes seen in Figure 4.4C. These three complexes binding to the RE-3 oligonucleotide run with higher mobility compared with to c-Jun containing complex binding to the SV40 AP-1 oligonucleotide in lane 1. Interestingly, none of the three complexes retarded on the RE-3 oligonucleotide was supershifted upon the addition of c-Jun supershifting antibody (lane 5). Furthermore, no supershifted complexes were detected with c-Fos supershifting antibody (lane 6). Although difficult to see, of the three faint complexes retarded with the RE-3 oligonucleotide, the binding of the complex of lowest mobility marked with a closed circle at the left hand side of the gel, is almost completely abolished in the presence of supershifting c-Fos antibody (compare lanes 4 and 6). Similarly, and as shown in Figures 4.7A, 4.8 and 4.9, the inclusion of either c-Jun or c-Fos supershifting antibody did not supershift complex A binding to the WT-CBS oligonucleotide using 293T nuclear extracts. It was also noted that



**Figure 4.5: Analysis of c-Jun and c-Fos binding to the SV40 AP-1, RE-3 and the WT-CBS oligonucleotide probe using nuclear extracts from 293T cells.**

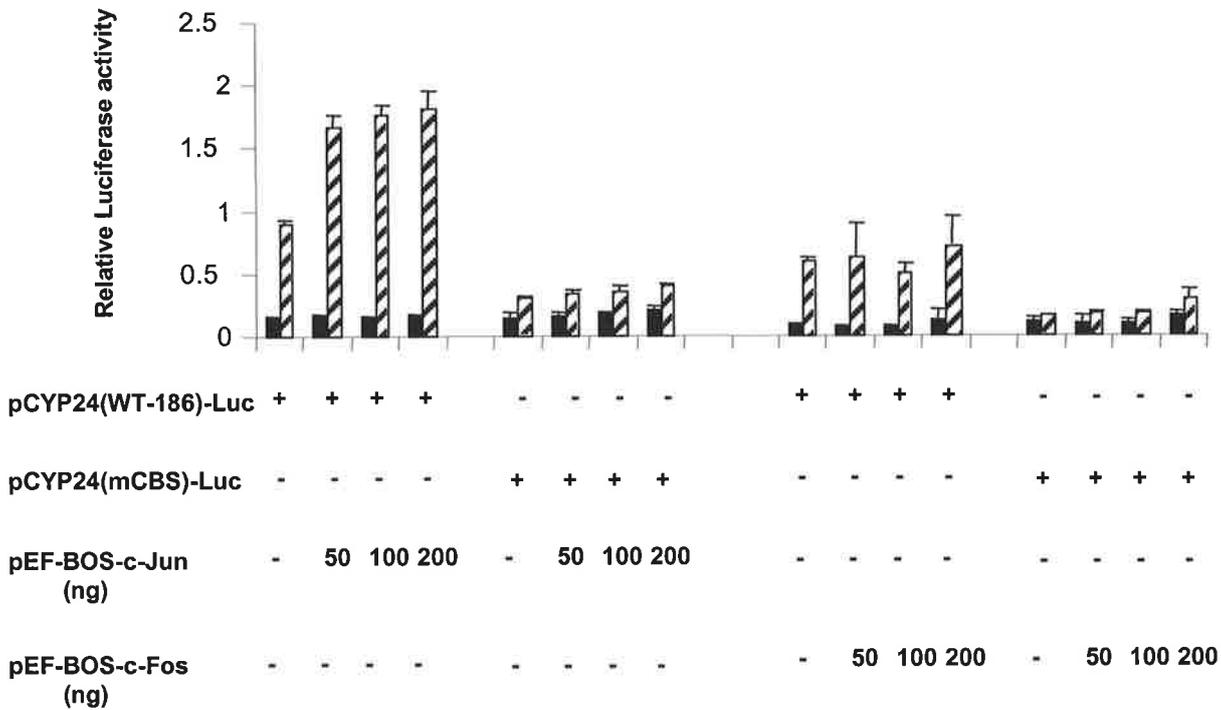
EMSA experiments analysing the binding of 293T nuclear extracts to [<sup>32</sup>P]-labeled control SV40 AP-1 oligonucleotide (lanes 1-3), RE-3 oligonucleotide (lanes 4-6) and the WT-CBS oligonucleotide (lanes 7-9). c-Jun or c-Fos polyclonal supershifting antibody was included in the binding reaction to detect c-Jun or c-Fos binding to each of the oligonucleotide probes. *Asterisk* denotes the AP-1 complex predominantly consisting of c-Jun proteins (see Figure 4.7 also), *Arrows* labeled X, Y, Z indicate supershifted c-Jun complexes which are very difficult to see in this photo, but are better visualised in Figure 4.7, *Arrow A* depicts the CHEP/CBS complex and the *closed circle* denotes the band that is abolished upon incubation of 293T nuclear extracts with supershifting c-Fos antibodies. A very faint inconsistently appearing band binding to the RE-3 oligonucleotide of mobility in between *arrow Y* and *arrow Z* is also seen but not affected by either c-Jun or c-Fos supershifting antibody. This is a preliminary result and has only been performed once.

complex A has a mobility higher than the c-Jun containing complex denoted with an asterisk in lane 1.

In conclusion, these results suggest that c-Jun binding to the SV40 AP-1 oligonucleotide can be detected in 293T nuclear extracts and this is further supported by the data in Figures 4.7B and 4.8. Interestingly, only c-Fos binding to the RE-3 oligonucleotide characterised by Xing *et al.* (2002) was detected in nuclear extracts prepared from 293T cells. The reason for not detecting c-Jun binding to the RE-3 oligonucleotide is unclear. It may be due to very weak binding of c-Jun because c-Jun binding can be detected using the SV40 AP-1 oligonucleotide. Importantly, c-Jun and c-Fos antibodies failed to supershift complex A that is CHEP, retarded on the WT-CBS oligonucleotide. Furthermore the complex binding to the RE-3 oligonucleotide that was abolished upon incubation with c-Fos antibody also has a mobility lower than complex A binding to the CBS. At the same time, the c-Jun containing complex (denoted with asterisk) bound to the SV40 probe has a mobility much lower than complex A implying that this complex is distinct from that binding to the CBS. It seems likely that this complex (denoted with the asterisk) represents AP-1. However, the situation is complicated by the fact that no binding of c-Fos was detected. This suggested that either the newly purchased antibody was inactive or that there was insufficient c-Fos in these nuclear extracts for detection on the SV40 oligonucleotide. Overall, the data suggest that c-Jun and c-Fos do not bind to the CBS in nuclear extracts prepared from 293T cells and that CBS may be a different size from AP-1

#### **4.2.3 Over-expression of c-Jun potentiates 1,25D activity of –186 bp CYP24 promoter constructs**

To test the idea more directly that AP-1 proteins could bind to the CBS, cDNA expression clones for c-Jun (pEF-Bos-c-Jun) and also for c-Fos (pEF-Bos-c-Fos) were over-expressed in 293T cells in the presence of pCYP24(WT-186)-Luc and pCYP24(mCBS)-Luc constructs. As shown in Figure 4.6, over-expression of 50ng of pEFBOS-c-Jun led to an approximate 2-fold increase in the relative level of 1,25D induced expression of pCYP24(WT-186)-Luc compared to vector alone. There was no significant effect on basal expression upon over-expression of either c-Jun or c-Fos proteins. An approximate 2-fold increase in 1,25D induced relative



**Figure 4.6: c-Jun and c-Fos overexpression on wildtype and mutant CBS CYP24 promoter constructs in the presence of 1,25D.**

293T cells were transfected with 200ng of pCYP24(WT-186)-Luc or pCYP24(mCBS)-Luc promoter constructs and the effect of cotransfecting increasing amounts (50ng, 100ng and 200ng) of pEF-BOS-c-Jun or pEF-BOS-c-Fos on basal (black bars) and 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-7</sup>M) induced activity (hatched bars) evaluated. The amount of DNA was balanced in all experiments using empty pEF-BOS vector. Activity was normalised against the expression of cotransfected *Renilla* luciferase activity. Results are expressed as Relative luciferase activity. Data presented are the means ± SD of triplicate samples from a representative experiment. The experiment was performed three times with the same trend obtained.

luciferase activity of pCYP24(WT-186)-Luc expression was also observed upon transfection of 100ng or 200ng of pEFBOS-c-Jun. At all concentrations of c-Jun tested, the enhancement by c-Jun over-expression observed on wild-type constructs was not observed upon mutagenesis of the CBS (Figure 4.6). Jun proteins homodimerise with other Jun family proteins but also form heterodimers with the c-Fos family of proteins. Therefore the over-expression of c-Fos was also evaluated in the same experiment. As shown in Figure 4.6, over-expression of 50-200ng of c-Fos did not significantly affect CYP24 promoter activity in 293T cells. In a separate experiment the effect of coexpressing c-Jun and c-Fos together in the presence of pCYP24(WT-186)-Luc was investigated. A  $5.50 \pm 0.8$ -fold level of 1,25D induction with transfected empty pEF-Bos expression vector was obtained in 293T cells. Coexpression of 100ng of c-Jun and 100ng of c-Fos together resulted in an  $8.89 \pm 0.64$ -fold level of 1,25D dependent CYP24 promoter activity which is similar to the  $9.12 \pm 1.41$ -fold level observed upon transfection of c-Jun only in 293T cells. Therefore the over-expression of c-Fos alone or together with c-Jun does not appear to have any effect on 1,25D dependent CYP24 activity in 293T cells. In a control experiment, the over-expression of c-Jun or c-Fos had no effect on 1,25D dependent activity of the pCYP24(WT-186)-Luc construct in ROS 17/2.8 cells (data not shown).

The data therefore showed that over-expressed c-Jun appears to enhance 1,25D mediated activity of CYP24 promoter constructs. The 1,25D dependent enhancement of CYP24 promoter activity is abolished upon mutagenesis of the CBS. Therefore the CBS may be a complex containing c-Jun protein. This finding was not expected and is difficult to explain based on the EMSA experiments described earlier (and in the next section) which show no binding of Jun/Fos proteins to the CBS.

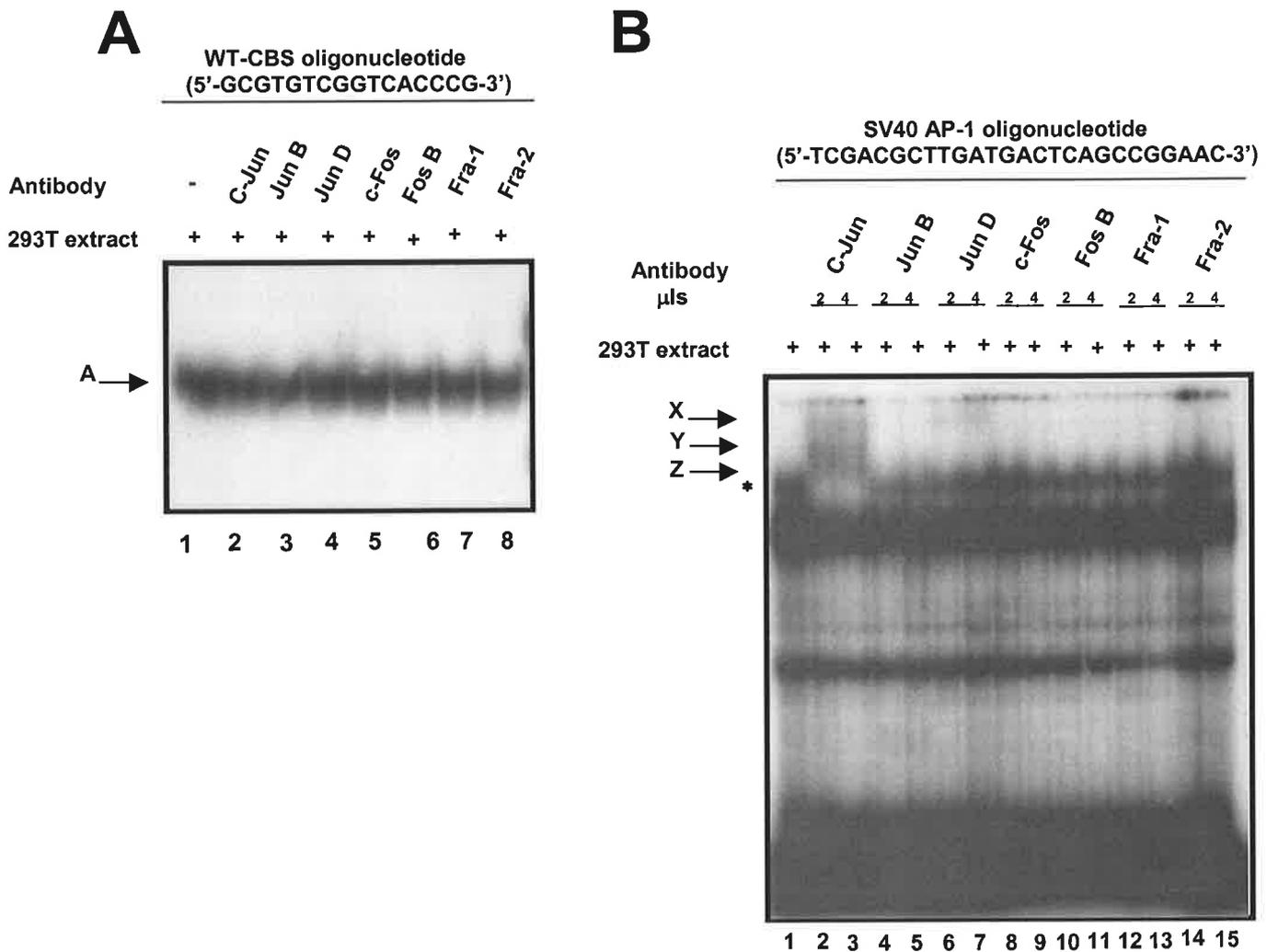
#### **4.2.4 Analysis of the binding of c-Jun and c-Fos proteins to the CBS**

Studies thus far have implicated but not clearly shown that the CBS may in fact contain AP-1 proteins, in particular c-Jun. As already discussed, AP-1 proteins consist of Jun/Jun, Jun/Fos and also Jun/ATF dimers. The data in Figure 4.5 using supershifting antibodies suggested that c-Jun and c-Fos do not bind the CBS. A

sequence highly homologous to the CBS was previously shown to bind c-Jun and c-Fos (Xing *et al.*, 2002) but in the current study, c-Jun binding to the CBS was not detected. Therefore to further examine whether AP-1 binds the CBS, EMSA experiments utilising supershifting antibodies directed against all AP-1 protein members were carried out using the WT-CBS oligonucleotide and the SV40 AP-1 oligonucleotide and nuclear extracts prepared from 293T cells not treated with either 1,25D or PMA.

Antibodies directed against c-Jun (sc-45), Jun B (sc-46) and Jun D (sc-74), c-Fos (sc-52), Fos B (sc-48), Fra-1 (sc-183) and Fra-2 (sc-171) were utilised in these EMSA experiments. Each Jun or Fos antibody was added to binding reactions consisting of WT-CBS oligonucleotide probe and nuclear extracts. For the supershifting EMSA studies, 2 $\mu$ l or 4 $\mu$ l of supershifting antibody was included in the binding reactions. This concentration of Jun and Fos antibody has been used by others in such experiments (Lai and Cheng, 2002; Kannan-Thulasiraman, 2002). As a control for the antibody, the ability of the antibodies to supershift Jun or Fos proteins was analysed using an oligonucleotide probe containing the functional AP-1 sequence highlighted in bold (5'-TCGACGCTTGAT**GACTCAGCCGGAAC**-3') and present in the SV40 promoter (Santa Cruz Biotechnology). As shown in Figure 4.7A, the inclusion of polyclonal antibodies for all members of the Jun or Fos family of proteins to the binding reactions did not supershift complex A bound to the WT-CBS oligonucleotide probe. This was a surprising result due to the fact that ectopic expression of c-Jun potentiated 1,25D inducibility of pCYP(WT-186)-Luc promoter constructs in 293T cells (Figure 4.6).

As shown in Figure 4.7B, the binding of Jun and Fos proteins to the SV40 AP-1 oligonucleotide probe using the same untreated 293T nuclear extracts as in Figure 4.7A was investigated. Antibody (2 $\mu$ l and 4 $\mu$ l) was included in the binding reactions containing the SV40 AP-1 oligonucleotide. Incubation of the SV40 AP-1 oligonucleotide with 293T nuclear extracts resulted in a number of retarded complexes differing in both intensity and mobility (Figure 4.7B, lane 1). Inclusion of anti-c-Jun antibody resulted in the formation of three weak supershifted complexes denoted by arrows labeled X, Y and Z in Figure 4.7B (lane 2 and 3). These three supershifted complexes are similar to the supershifted complexes observed in



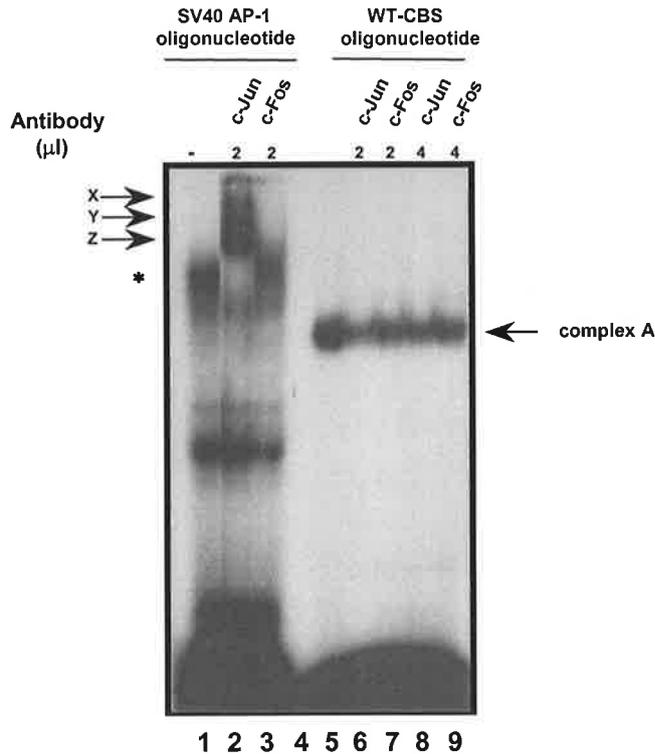
**Figure 4.7: Analysis of c-Jun and c-Fos protein binding to the CBS in 293T cells.**

(A), Nuclear extracts from 293T cells were incubated with [<sup>32</sup>P]-labeled WT-CBS oligonucleotide probe. For the detection of supershifts, 2 μl of polyclonal supershift antibody recognising each AP-1 protein family member (shown in each lane) was incubated with the nuclear extracts prior to the addition of labeled oligonucleotide probe. Complex A denotes the CHEP/CBS complex.

(B), Nuclear extracts from 293T cells were used as in (A) except the binding of proteins from 293T cell nuclear extracts was analysed using the control SV40 AP-1 oligonucleotide (sequence highlighted in bold) (5'-TCGACGCTTGAT**GACTC**AGCCGGAAC-3'). For the detection of supershifts, 2 μl and 4 μl of each polyclonal supershift antibody used in (A) was added to the binding reaction prior to the addition of the labeled SV40 AP-1 oligonucleotide. Three supershifted complexes labeled arrows X, Y and Z were detected using anti-c-Jun and complex X using anti-Jun D antibodies (lanes 2, 3 and lanes 6, 7, respectively). Asterisk depicts the AP-1 protein/DNA complex.

Figure 4.5, lane 2. The concomitant disappearance of the complex denoted by the asterisk in lane 1 demonstrates that this complex contains c-Jun protein. Furthermore, anti-Jun D antibody supershifted a single complex having mobility equal to the supershifted complex X observed with the anti-c-Jun antibody (Figure 4.7B, compare lanes 2 & 3 with lanes 6 & 7). The supershifted complex, complex X, formed upon inclusion of Jun D antibody appeared slightly stronger with 4 $\mu$ l of antibody. Because some supershifted complexes appeared weak, two different volumes of antibody were used in some experiments. All other antibodies tested did not detect any supershifted complexes. Longer exposure of films did not reveal evidence for more supershifted complexes (data not shown). These results demonstrate that in 293T cells, c-Jun and Jun D appear to be the predominant AP-1 proteins binding to the SV40 AP-1 oligonucleotide. Furthermore, these proteins can be immunologically detected using the antibodies.

The failure to detect supershifts with c-Jun antibodies could be the result of weak binding of c-Jun to the CBS. Similarly, the binding of c-Fos may be weak or the levels of c-Fos protein could also be low and hence result in the failure to observe any supershifts with anti-c-Fos antibody. Nuclear extracts prepared from 293T cells over-expressing c-Fos could overcome this problem of not being able to detect the binding of c-Fos proteins in 293T cell nuclear extract. Alternatively, weak binding to both the control SV40 AP-1 oligonucleotide and the WT-CBS oligonucleotide by c-Jun and c-Fos is also a possibility. On this basis, anti-c-Fos and anti-c-Jun antibodies were examined for the ability to supershift proteins binding to the WT-CBS oligonucleotide using extracts prepared from 293T cells over-expressing both c-Fos and c-Jun. Therefore 293T nuclear extracts over-expressing both c-Jun and c-Fos (kindly provided by Dr. Prem Dwivedi) were utilised as a source of proteins for supershift experiments using anti-c-Jun and anti-c-Fos antibodies. The results are shown in Figure 4.8. In this figure the regions between lane 1 and lane 2 were excised from the original EMSA gel. The results are from the same experiment and not from two separate experiments. As shown in Figure 4.8, a c-Jun containing complex binding to the control SV40 AP-1 oligonucleotide probe was supershifted with anti-c-Jun antibody forming three supershifted complexes X, Y, Z (lane 2). This is similar to the three supershifted complexes in 293T cells not transfected with either c-Jun or c-Fos expression vectors shown in Figure 4.7B, lanes 2 and 3. Incubation of the anti-c-Fos antibody



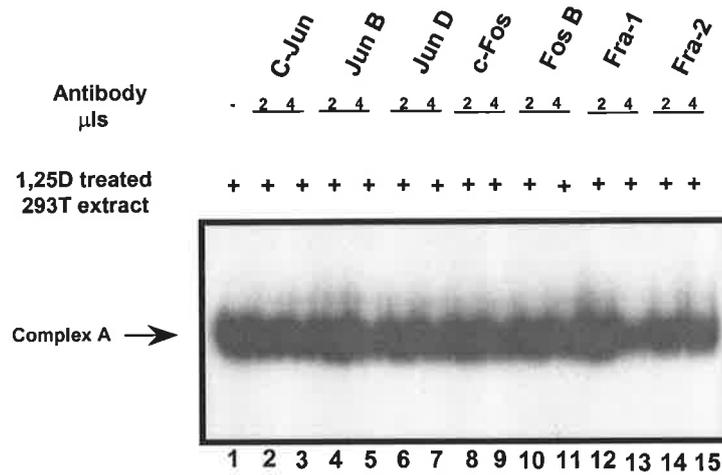
**Figure 4.8: Analysis of c-Jun and c-Fos binding to the CBS oligonucleotide using 293T nuclear extracts transfected with c-Jun and c-Fos.**

(A), 293T nuclear extracts were prepared from cells cotransfected with pEF-BOS-c-Jun and pEF-BOS-c-Fos (gift from Prem Dwivedi, our laboratory). To detect c-Jun and c-Fos binding, supershift experiments were performed using the control SV40 AP-1 oligonucleotide probe with anti-c-Jun (lane 2) or anti-c-fos (lane 3) antibodies included in the binding reaction. Three supershifted complexes denoted X, Y and Z were detected with anti-c-Jun antibodies (lane 2). AP-1 DNA complex is depicted by *asterisk* and the *arrow* labeled complex A depicts the CHEP/CBS complex formed with the WT-CBS oligonucleotide probe. As described in the text, regions between lane 1 and lane 2 in this EMSA figure were excised from the original EMSA gel to generate the figure shown above. It must be emphasized that this EMSA is from the same experiment and not a photograph of two gels from different experiments. All EMSA gels in this experiment were set up identically and therefore all gels would require the excision of irrelevant binding reaction electrophoresed in this gel.

with the SV40 AP-1 oligonucleotide did not supershift the complex (denoted by the asterisk, lane 3). However, the intensity of this band was weakened (see lane 3), strongly suggesting that the complex is AP-1. Inclusion of either anti c-Jun (2 $\mu$ l or 4 $\mu$ l) or anti-c-Fos (2 $\mu$ l or 4 $\mu$ l) antibody did not supershift complex A binding to the WT-CBS oligonucleotide probe (Figure 4.8, lanes 6, 7 and lanes 8, 9, respectively). Longer exposure of films did not detect any supershifted complexes that were not already visible. Again, it should be noted that there is a relative difference in the migration of the CHEP and AP-1 complexes. The AP-1 complex has lower mobility compared with complex A binding to the WT-CBS oligonucleotide which migrates faster through the polyacrylamide gel.

As shown in Figure 4.6, c-Jun over-expression potentiated 1,25D induced activity of wild-type CYP24 promoter constructs. It is possible that upon 1,25D exposure, c-Jun and even c-Fos may become post-translationally modified through the non-genomic effect of 1,25D action. This post-translational modification may alter the conformation of the Jun or Fos proteins making up the AP-1 complex and subsequently alter the affinity of the AP-1 antibodies for their epitopes. Therefore the EMSA supershift experiments were repeated using the WT-CBS oligonucleotide with nuclear extracts from 293T cells treated with 1,25D ( $10^{-7}$ M) for 24hrs. As shown in Figure 4.9, inclusion of 2 $\mu$ l or 4 $\mu$ l of antibody recognising each c-Jun and c-Fos family member to the binding reactions did not supershift complex A. This is in agreement with the results observed using 293T nuclear extracts not treated with 1,25D. The inability to observe any supershifted complexes binding to the WT-CBS oligonucleotide meant that the examination of the proteins binding to the control SV40 AP-1 oligonucleotides using 1,25D treated 293T nuclear extracts was deemed unnecessary and therefore not further analysed.

The results from EMSA experiments using polyclonal antibodies directed against each AP-1 family member suggested that neither c-Jun nor c-Fos proteins bind directly to the WT-CBS oligonucleotide probe. c-Jun and Jun D binding to the control SV40 AP-1 oligonucleotide probe was detected in 293T nuclear extracts. The binding of c-Jun and Jun D to the WT-CBS was not detected. Similarly, the binding of c-Fos to the SV40 Ap-1 was not clearly detected by a supershifted complex. Rather it appeared that c-Fos antibody weakened the intensity of the SV40 AP-1 complex shown in Figure 4.8. The binding of the c-Fos antibody to c-



**Figure 4.9: Analysis of anti-c-Jun and anti-c-Fos antibodies for the binding of proteins to the CBS proteins 293T nuclear extracts treated with 1,25D.**

293T nuclear extracts treated with  $1,25(\text{OH})_2\text{D}_3$  at  $10^{-7}\text{M}$  were incubated with the  $^{32}\text{P}$ -labeled WT-CBS oligonucleotide. For the detection of supershifts,  $2\mu\text{l}$  and  $4\mu\text{l}$  of polyclonal supershifting antibody directed against each AP-1 protein family member (shown in each lane) was incubated with nuclear extracts prior to the addition of the labeled oligonucleotide probe. *Arrow A* depicts the CHEP/CBS complex.

Fos could destabilise the c-Jun/c-Fos complex during electrophoresis. This has been shown by others, where c-Fos antibody (sc-52X) appeared to neutralize rather than supershift a c-Fos containing AP-1 complex using MCF-7 nuclear extracts (Petz *et al.*, 2002). The data from the EMSA experiments are in contrast to the potentiation by c-Jun of 1,25D induced CYP24 promoter activation seen in 293T cells (Figure 4.6).

### 4.3 Discussion

The mechanism by which PMA synergises with 1,25D to stimulate induction of the CYP24 promoter has been of interest for many years. Experiments with PKC inhibitors implicated PKC activity in the synergistic response but no AP-1 site could be identified in the CYP24 promoter (Omdahl and May, 1997). It is now appreciated that PMA functions not only through AP-1 but also through other sites such as Ets protein binding sites (Orzechowski *et al.*, 2001; Lindemann *et al.*, 2003). In this regard, studies in our laboratory (and shown in Figure 4.1) have demonstrated that the EBS present in the CYP24 promoter is partially responsive to the synergistic action of PMA and 1,25D. Inactivation of this site lowers synergy by about 50%. However, the most important result described in this chapter is the finding that the CBS is the predominant target for the stimulation of promoter activity by PMA in 1,25D-treated 293T cells. In the absence of a functional CBS, PMA and 1,25D induced synergy is almost completely abrogated highlighting the critical importance of this site.

The precise mechanism by which PMA induces transcriptional synergy of CYP24 promoter constructs in the presence of 1,25D is under investigation in our laboratory. The finding in this chapter that the response is entirely elicited through the CBS and EBS sites eliminates the possibility that PMA treatment acts through a VDRE by stimulating expression of VDR, as supported by Yang *et al.* (2001). This is supported in separate experiments (not presented in this chapter), which showed that a VDRE-containing TK-Luc construct responded to 1,25D but no synergy was observed with PMA addition. It has been well established that PMA

activates PKC and numerous downstream signaling pathways such as MAP kinases and in particular the JNK pathway (Reinhardt and Horst, 1994; Kumar and Bernstein, 2001; Franklin and Kraft, 1995; Lindemann *et al.*, 2003; Wen-Sheng *et al.*, 2003). PMA activation of MAP kinases could result in the phosphorylation and activation of Jun, Fos or ATF-2 subunits composing the AP-1 complex (Karin and Shaulin, 2001; Xing *et al.*, 2002; Chinenov and Kerppola, 2001; Murakami *et al.*, 1999; Cirillo *et al.*, 1999; Kumar *et al.*, 1997). Similarly, PMA has been shown to signal through the transcription factors Sp1 and Ets-1 (Orzechowski *et al.*, 2001; Chou *et al.*, 2003; Lindemann *et al.*, 2003). On this note, supershift antibody experiments using Ets-1 and Sp1 did not detect these proteins binding to the CBS using 293T nuclear extracts (data not shown). 1,25D treatment of different cell types has been shown to activate signaling pathways including PKC and the MAP kinases ERK1/2 and ERK5 (Johansen *et al.*, 2003; Chen *et al.*, 1999; Dwivedi *et al.*, 2002; Yang *et al.*, 2001). Furthermore, 1,25D induces ERK5 dependent phosphorylation of Ets-1 in a 1,25D dependent manner and this is critical for activation of the CYP24 promoter in COS-1 cells (Dwivedi *et al.*, 2002).

A study of ERK2 activity levels is currently being undertaken in 293T cells in the presence of 1,25D (Barbara Nutchey). ERK2 activity is very low in these cells, with addition of 1,25D having little effect (Barbara Nutchey, personal communication). This is in marked contrast to the situation found in COS-1 cells where 1,25D treatment markedly increased the activities of ERK1/2/5 (Dwivedi *et al.*, 2002). However, in the presence of both 1,25D and PMA there is a substantial increase in ERK2 activity in 293T cells (Barbara Nutchey, personal communication). This raises the possibility that in response to PMA, PKC activity is increased leading to an enhanced activation of ERK2. This in turn may phosphorylate proteins bound on both CBS and EBS. While it has been shown that ERK5 can phosphorylate Ets-1 (as mentioned above) this protein can also be phosphorylated by ERK1 and ERK2 (Waas and Dalby, 2001; Goetze *et al.*, 2001; Seidel and Graves, 2002). The reason for the absence of PMA induced 1,25D dependent transcriptional synergy on CYP24 promoter constructs in osteoblastic UMR-106 and ROS 17/2.8 cells is not clear. ERK activities in the presence of 1,25D and in the presence of both 1,25D and PMA may shed some light on this and these experiments are underway in the laboratory.

In the work described in this chapter, the addition of PMA alone has had no effect on the basal expression of the CYP24 promoter in 293T cells. Neither the CBS nor the EBS contributes to basal expression of the CYP24 promoter. The most likely explanation is the presence of an inhibitory corepressor complex that binds to unliganded VDR/RXR in the absence of 1,25D and sterically interferes with protein binding to the CBS and EBS (Dwivedi *et al.*, 1998; Polly *et al.*, 2000).

At the time of this work, a sequence highly homologous to the CBS was reported in the literature (Xing *et al.*, 2002). The authors showed that this sequence (RE-3) responded to PMA and *in vitro* bound several proteins including c-Jun and c-Fos proteins. It was suggested that AP-1 binds to RE-3 and is the target of PMA action. While reasonable, no evidence other than the EMSA data was presented by these authors to substantiate this proposal. On the basis of their work, it was possible that the CBS may be an AP-1 binding site. To investigate this possibility, three separate approaches were undertaken. Firstly, EMSA experiments were employed using RE-3 and CBS oligonucleotides together with a control SV40 AP-1 oligonucleotide. Secondly, the effect of c-Jun and c-Fos over-expression on pCYP24(WT-186)-Luc constructs in 293T cells was investigated. Thirdly, EMSA experiments utilising supershifting antibodies recognising all the Jun and Fos family of proteins were carried out. The data from these experiments strongly suggest that the CBS does not bind AP-1. These points are as follows. EMSA experiments demonstrated that an AP-1 complex bound to the SV40 AP-1 oligonucleotide as indicated by a c-Jun supershifted complex and a reduction in the complex following addition of c-Fos antibody. Importantly, this AP-1 complex runs with a much lower mobility compared to complex A binding to the WT-CBS oligonucleotide (Figure 4.5). Similarly, of the three protein complexes retarded by the RE-3 oligonucleotide, the complex affected with c-Fos antibody has mobility different to complex A (Figure 4.5). Supershifting antibodies recognising all members of the Jun and Fos family of AP-1 proteins did not affect complex A binding to the WT-CBS oligonucleotide (Figure 4.7 and 4.9). Lastly, the binding of proteins using nuclear extracts prepared from 293T cells transfected with c-Jun and c-Fos did not detect c-Jun or c-Fos binding to this oligonucleotide (Figure 4.8). These results suggest that the CBS is not a binding site for AP-1.

On the other hand, it is possible that c-Jun and c-Fos may bind to the CBS but the retarded AP-1 complex is either too faint or too unstable to be detected by EMSA. Perhaps more sensitive approaches such as the binding of purified c-Jun and c-Fos protein or *in vitro* translated proteins could be employed to further pursue this. However it seems highly probable that complex A represents the authentic CBS binding protein since there is a tight correlation between the loss of promoter activity and complex A binding in EMSA experiments when mutations are introduced into the CBS sequence (see chapter 3).

While the EMSA data strongly indicate that AP-1 does not bind to the CBS, there is some evidence to the contrary. The binding of complex A in EMSA experiments to the CBS was competed with the SV40 AP-1 oligonucleotide. Perhaps this represents a non-specific competition. Also over-expression studies showed that exogenous c-Jun, but not c-Fos, enhanced 1,25D dependent stimulation of CYP24 promoter constructs and that this occurred through the functional CBS. The over-expression data could be the result of c-Jun 'piggy-backing' with the protein bound at the CBS and enhancing its activity; in this case c-Jun must be lost in EMSA experiments since it is not detected in the supershift experiments. ATF-2 can form homodimers with itself and heterodimers with c-Jun, and is PMA responsive and binds to an extended AP-1 binding site (5'-TGACNTCA-3') with Jun (Van Dam and Castellazzi, 2001; Cirillo *et al.*, 1999). It is possible that an ATF-2 homodimer may bind the CBS in nuclear extracts prepared from 293T cells. Antibodies directed against ATF-2 were ordered but not analysed in EMSA experiments due to time constraints. Therefore it will be of interest to test the ATF-2 antibodies in EMSA experiments. Studies presented in this thesis do not support the proposal that CBS is a binding site for AP-1 even though the site is PMA responsive and the sequence has some similarity to the consensus AP-1 site. The comparative study of the CBS and the RE-3 sequences did not clarify the situation, while the c-Jun over expression data appears to further complicate the situation. Plans are now underway to isolate the complex A binding to CBS using affinity chromatography.

**Studies into the functional role of the  
CBS site in 1,25D mediated  
transcriptional control of the CYP24 gene  
promoter**

## 5. Studies into the functional role of the CBS site in 1,25D mediated transcriptional control of the CYP24 gene promoter

### 5.1 Introduction

As discussed in Chapter 1, the rat CYP24 promoter contains two 1,25D response elements (VDREs) that drive the expression of the CYP24 gene promoter in response to 1,25D. We and others have characterised these VDRE's at the molecular level (Kerry *et al.*, 1996; Hahn *et al.*, 1994; Ohyama *et al.*, 1996; Zierold *et al.*, 1995). Our laboratory has demonstrated that VDRE-1 and VDRE-2 harbor unique transcriptional characteristics and our studies of these sites have led to the proposal that transcriptional cooperation exists between the VDR/RXR heterodimeric complex bound at VDRE-1 and surrounding transcription factors. In support of this a functional *ras* responsive Ets-1 binding site (-128/-119) downstream of VDRE-1 was identified (Dwivedi *et al.*, 2000). As described in chapter 3, the identification of CBS, an apparently novel transcription factor binding site (5'-TGTCGGTCA-3' at position -171/-169) upstream of the proximal VDRE, strongly supported the notion that transcriptional activation from VDRE-1 in response to 1,25D does require the cooperation of liganded VDR/RXR complexes with surrounding transcription factors. As described in Chapter 3, mutagenesis of the CBS almost completely abolishes 1,25D responsiveness of -186bp CYP24 promoter constructs demonstrating a key role for the CBS binding protein, CHEP, in the inductive mechanism.

The aim of this chapter was to further investigate the role of the CBS in the transcriptional activation of the CYP24 promoter in response to 1,25D in ROS 17/2.8 cells. In particular, experiments were undertaken to determine if the CBS specifically enhances only the activity of the more closely located VDRE-1. Secondly, work was undertaken to determine if the CBS participates in the transcriptional synergy between VDRE-1 and VDRE-2 and lastly, to determine the response of wild-type and CBS mutant CYP24 promoter constructs to exogenously expressed coactivators.

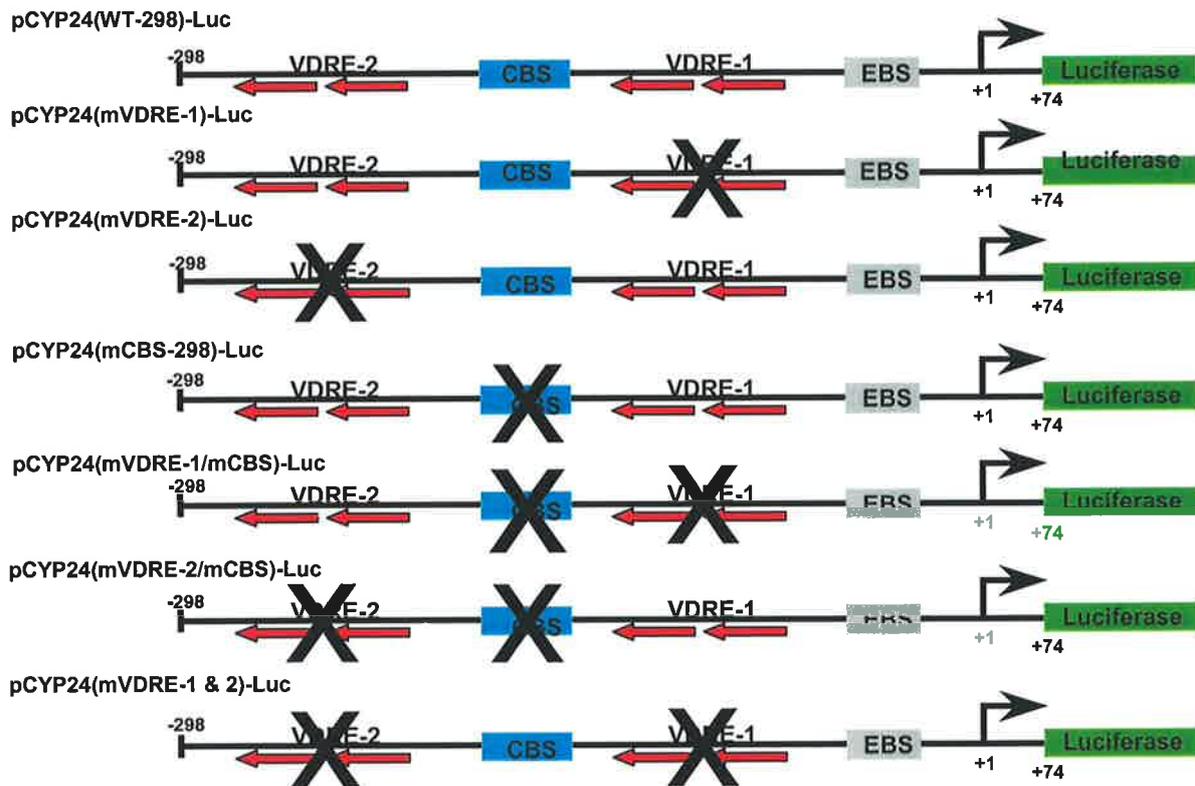
## 5.2 Results

### 5.2.1 Investigating the role of the CBS in the transcriptional synergy between VDRE-1 and VDRE-2 in ROS 17/2.8 cells

The CYP24 gene promoter appears to be the most responsive to 1,25D so far reported with levels of induction of 30 to 80-fold observed in kidney and osteoblast cell lines in our laboratory (unpublished data). This level of induction is attributed to the unique presence of two VDREs that synergise in response to 1,25D exposure as already discussed in Chapter 1. Work conducted during my Honours year established that mutagenesis of the CBS in -298bp CYP24 promoter constructs encompassing both VDRE-1 and VDRE-2 greatly reduced 1,25D dependent promoter activity. For example in ROS 17/2.8 cells, a 40-fold level of 1,25D induction of pCYP24(WT-298)-Luc was lowered to an 8-fold level upon mutagenesis of the CBS. However, these expression studies did not examine whether the CBS played any role in the transcriptional synergy mediated by VDRE-1 and VDRE-2 in response to 1,25D.

Therefore the first aim was to determine whether the CBS cooperated with VDRE-1 or VDRE-2 during 1,25D induction. To examine this, -298bp CYP24 promoter constructs were synthesised with each VDRE inactivated using site-directed mutagenesis, with and without a functional CBS. These constructs are designated pCYP24(WT-298)-Luc, pCYP24(mVDRE-1)-Luc, pCYP24(mVDRE-2)-Luc, pCYP24(mCBS-298)-Luc, pCYP24(mVDRE-1/mCBS)-Luc and pCYP24(mVDRE-2/mCBS)-Luc and are shown in Figure 5.1. Inducibility of these constructs in response to 1,25D at  $10^{-7}$ M was examined in transiently transfected ROS 17/2.8 cells. All experiments with ROS 17/2.8 cells were performed without cotransfecting human VDR. ROS 17/2.8 cells contain a high endogenous level of VDR (Kerry *et al.*, 1996) and cotransfection of the pRSV-hVDR expression vector under these experimental conditions did not further induce the activity of CYP24 promoter constructs (data not shown).

In transfected ROS 17/2.8 cells, mutagenesis of either VDRE-1 or VDRE-2 lowered wild-type induction from  $32.1 \pm 2.23$ -fold to  $2.15 \pm 0.17$ -fold and  $10.27 \pm 1.65$ -fold respectively, confirming the greater contribution of VDRE-1 towards



**Figure 5.1: -298bp CYP24 reporter constructs used to determine the role of the CBS in the synergy mediated between VDRE-1 and VDRE-2.**

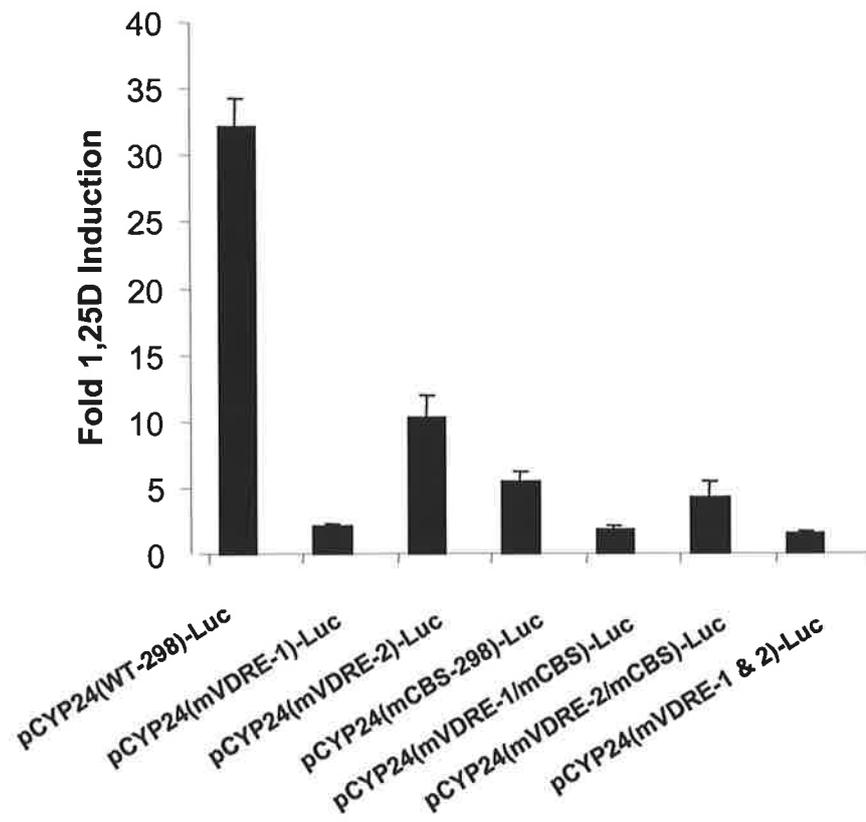
-298bp CYP24 promoter constructs containing both VDRE-1 and VDRE-2 (depicted by arrows) and the CBS were used to investigate the role of the CBS in the synergy mediated between these two VDREs. Site directed mutagenesis was used to inactivate the binding sites for either VDRE-1, VDRE-2 and the CBS. pCYP24(mVDRE-1)-Luc, pCYP24(mVDRE-2)-Luc and pCYP24(mVDRE-1 & 2)-Luc are reporter constructs examining the activity of each VDRE in the presence of an intact CBS. pCYP24(mCBS)-Luc, pCYP24(mVDRE-1/mCBS)-Luc, and pCYP24(mVDRE-2/mCBS)-Luc are reporter constructs examining the functionality of each VDRE in the absence of a functional CBS.

CYP24 promoter induction (Figure 5.2). These two VDREs give rise to a 2.5-fold level of synergy because the sum of their individual inductions is less than their combined inductions observed in wild-type constructs ( $2.15\text{-fold} + 10.27 < 32\text{-fold}$ ) (Figure 5.2). Mutagenesis of the CBS in pCYP24(mCBS-298)-Luc lowered induction from  $32.1 \pm 2.23\text{-fold}$  to  $5.42 \pm 0.81\text{-fold}$  clearly highlighting the importance of this sequence for 1,25D responsiveness of the promoter. In combination with the mutated CBS, mutagenesis of VDRE-1 (pCYP24 (mVDRE-1/mCBS)-Luc) or VDRE-2 (pCYP24(mVDRE-2/mCBS-298)-Luc), lowered induction to  $1.77 \pm 0.29\text{-fold}$  and  $4.16 \pm 1.22\text{-fold}$  respectively. A comparison of the sum of activities for each VDRE in the presence of an inactivated CBS (5.9-fold) compared to the sum of activities of the constructs containing both VDREs but lacking the CBS (5.4-fold) demonstrates a loss of 1,25D-induced transcriptional synergy. Hence the CBS is involved in the synergy between VDRE-1 and VDRE-2 in response to 1,25D. A comparison of the activity between pCYP24(mVDRE-2)-Luc ( $10.27 \pm 1.65\text{-fold}$ ) and pCYP24(mVDRE-2/mCBS)-Luc ( $4.16 \pm 1.22\text{-fold}$ ) constructs demonstrates that the CBS appears to cooperate with VDRE-1 but not the more distal VDRE-2.

Therefore these results demonstrate that in ROS 17/2.8 cells, mutagenesis of the CBS, abrogates the synergy induced by 1,25D between VDRE-1 and VDRE-2. The transactivation function of the CBS appears to act through VDRE-1 and not the more distant VDRE-2. This is the first report to describe the role of a transcription factor in the transcriptional synergy between VDRE-1 and VDRE-2 in the presence of 1,25D.

### 5.2.2 Replacement of VDRE-1 with VDRE-2 in -186bp constructs

With the CBS positioned in close proximity to VDRE-1 and with the effects of the CBS appearing to work through this VDRE, the following questions were addressed using -186 bp CYP24 promoter constructs containing only VDRE-1 and not VDRE-2. Firstly, will replacement of the low affinity VDRE-1 with the higher affinity VDRE-2 sequence in -186bp construct reduce or even abolish the contribution from the nearby CBS? Secondly, will replacement of VDRE-1 with VDRE-2 also affect the contribution of the CBS?



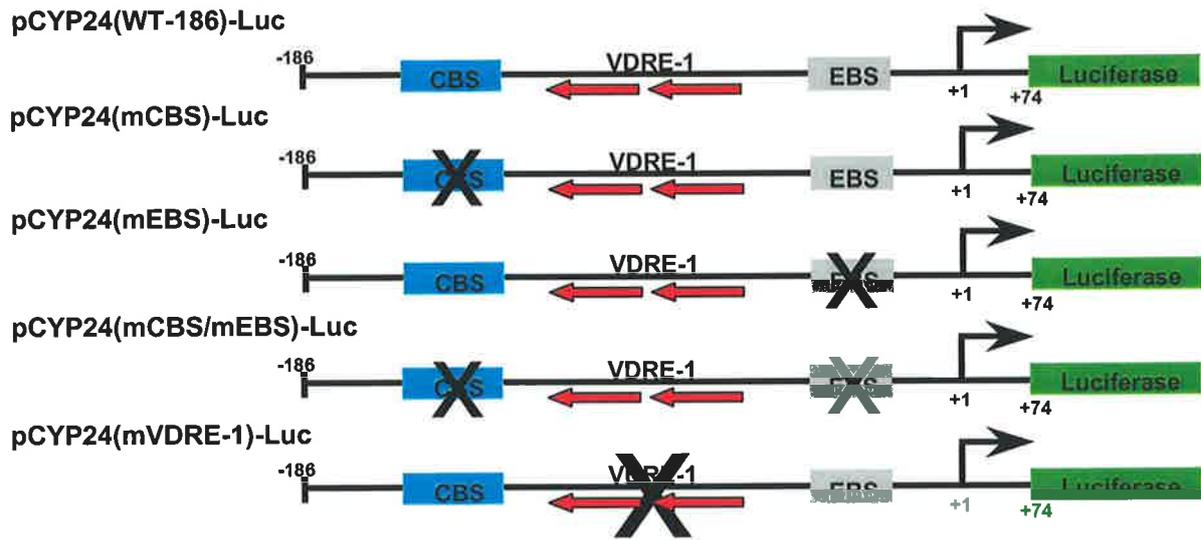
**Figure 5.2: Mutagenesis of the CBS and its role in 1,25D dependent synergy between VDRE-1 and VDRE-2 in ROS 17/2.8 cells.**

Wildtype (pCYP24(WT-298)-Luc) and mutant -298bp CYP24 promoter constructs (200ng) shown in Figure 5.1 were transiently transfected into ROS 17/2.8 cells. The ability of both VDRE-1 and VDRE-2 to synergise in the presence (pCYP24(WT-298)-Luc, pCYP24(mVDRE-1)-Luc and pCYP24(mVDRE-2)-Luc ) and absence (pCYP24(mCBS)-Luc, pCYP24(mCBS/mVDRE-1)-Luc, and pCYP24(mCBS/mVDRE-2)-Luc) of a functional CBS was examined by treating ROS 17/2.8 cells treated with 1,25D at  $10^{-7}$ M. Data presented are the means  $\pm$  SD of triplicate samples from a representative experiment. The experiment was performed three times with the same trend obtained.

Depicted in Figure 5.3A are the constructs used in this experiment. pCYP24(WT-186)-Luc constructs with mutated CBS (pCYP24(mCBS)-Luc), mutated EBS (pCYP24(mEBS)-Luc) and mutated EBS and CBS together (pCYP24(mCBS/mEBS)-Luc) were used as templates in the mutagenesis experiments where the nucleotide sequences of VDRE-1 were mutated into VDRE-2 as shown in Figure 5.3B. These constructs pCYP24(WT)<sup>VDRE-2</sup>-Luc are identical to native constructs except that VDRE-2 now drives the response of this gene-reporter construct in response to 1,25D at 10<sup>-7</sup>M.

In ROS 17/2.8 cells, mutagenesis of the CBS lowered induction from 20.97 ± 1.94-fold for wild-type to 3.45 ± 0.77-fold in pCYP24(mCBS)-Luc constructs (Figure 5.4). This is a reduction of approximately 80% when the CBS is inactivated in -186 bp CYP24 promoter constructs containing VDRE-1. Inactivation of the EBS in pCYP24(mEBS)-Luc gave rise to a 10.11 ± 0.45-fold level of induction. Hence, the CBS plays a far more prominent role in the response of VDRE-1 to 1,25D induction. There was no effect on basal expression upon mutagenesis of the CBS or the EBS (data not shown). Simultaneous inactivation of both binding sites in pCYP24(mCBS/mEBS)-Luc gave a 3.21 ± 0.11-fold level of induction that is similar to the CBS alone (3.45 ± 0.77-fold). Mutagenesis of VDRE-1 abolished the 1,25D inductive response (Figure 5.4). These results demonstrate that VDRE-1 requires the participation of the CBS and EBS for maximal 1,25D inducibility. However the CBS plays a more pronounced role in the 1,25D mediated enhancement from VDRE-1 compared with the EBS.

Mutagenesis of VDRE-1 to VDRE-2 gave a 21.49 ± 2.27-fold level of induction that was similar to that observed with the wild-type construct (20.97 ± 1.94-fold). This result was unexpected because VDRE-2 has an apparent higher affinity for VDR/RXR as determined from gel shift analysis experiments. Inactivation of the CBS in this construct (pCYP24(WT)<sup>VDRE-2</sup>-Luc) decreased significantly the induction from 21.49 ± 2.27-fold to 6.67 ± 1.19-fold, an approximate 70% decrease in promoter activity. This is slightly less than that observed with the wild-type construct (80% decrease). Mutagenesis of the EBS resulted in almost no loss of 1,25D induction with a 17.98 ± 0.51-fold level of induction observed. This is in

**A****B**

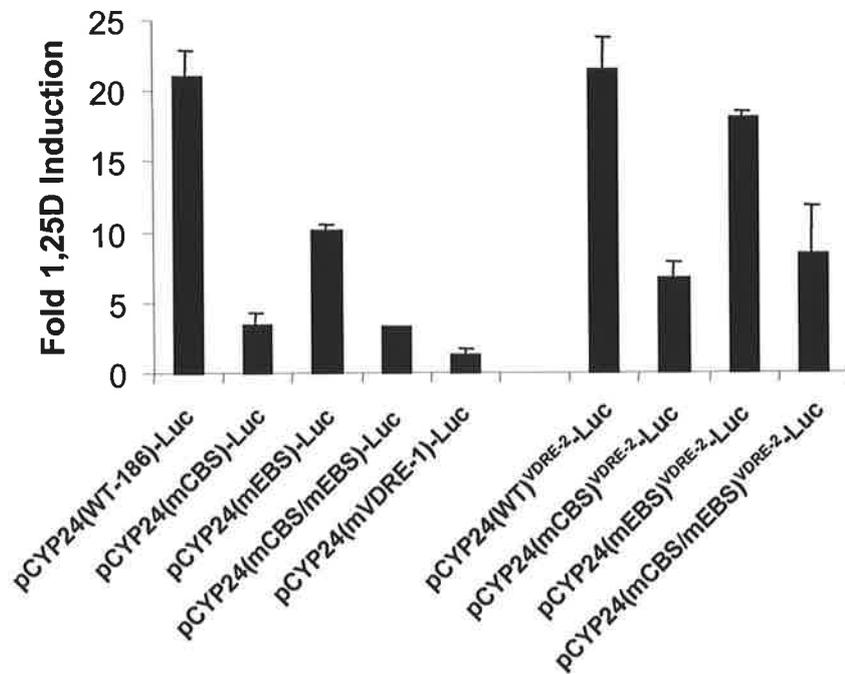
VDRE-1      5-CGC**CCT** CAC **TCACCT**-3'

VDRE-2      5-CGCACC CGC TGAACC-3'

**Figure 5.3: -186bp CYP24 promoter constructs used for replacing VDRE-1 with VDRE-2.**

(A), -186bp promoter constructs used to determine the dependence of VDRE-1 for either the CBS (pCYP24(mCBS)-Luc) and EBS (pCYP24(mEBS)-Luc) alone or together (pCYP24(mCBS/mEBS)-Luc) in transfected cells are depicted. -186 CYP24 constructs where VDRE-1 is replaced with VDRE-2 are termed, pCYP24(WT)<sup>VDRE-2</sup>-Luc. Constructs designated pCYP24(mCBS)<sup>VDRE-2</sup>-Luc, pCYP24(mEBS)<sup>VDRE-2</sup>-Luc and pCYP24(mCBS/mEBS)<sup>VDRE-2</sup>-Luc contain mutations in the CBS, EBS and CBS/EBS respectively in the presence of VDRE-2.

(B), Comparison of the sense strand sequence of VDRE-1 with VDRE-2 from the CYP24 promoter. Two rounds of site-directed mutagenesis was used to replace the nucleotide sequences (highlighted in pink) of VDRE-1 with the corresponding nucleotide sequence of VDRE-2 in -186bp CYP24 promoter constructs. The replacement of VDRE-1 nucleotides with the corresponding nucleotides of VDRE-2 was verified by DNA sequencing.



**Figure 5.4: Analysis of the dependence for the CBS and EBS when VDRE-1 is replaced with VDRE-2 in ROS 17/2.8 cells.**

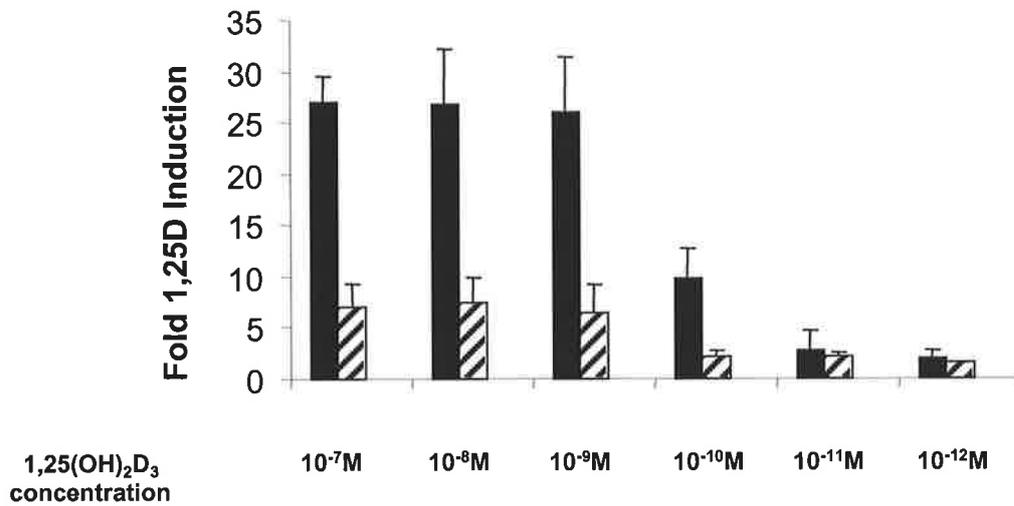
-186bp CYP24 promoter constructs (200ng) depicted in Figure 5.3 (synthesized to examine the role of the CBS and EBS in 1,25D mediated activity from VDRE-1 (pCYP24(WT-186)-Luc) or VDRE-2 (pCYP24(WT)<sup>VDRE-2</sup>-Luc) were transiently transfected into ROS 17/2.8 cells. The functionality of each construct was examined by treating ROS 17/2.8 cells with 1,25D at  $10^{-7}$ M. Data presented are the means  $\pm$  SD of triplicate samples from a representative experiment. This experiment was performed three times with the same trend obtained.

contrast with wild-type constructs where an approximately 50% decrease is observed upon mutagenesis of the EBS. Similarly, mutagenesis of both the CBS and EBS together in pCYP24(mCBS/mEBS)<sup>VDRE-2</sup>-Luc lowered induction to  $8.42 \pm 3.36$ -fold, a level similar to inactivation of the CBS alone in pCYP24(mCBS)<sup>VDRE-2</sup>-Luc. Hence, the requirement for a functional EBS was almost lost when VDRE-1 was replaced with VDRE-2. This suggests that Ets-1 protein bound to the EBS could stabilise the binding of the VDR/RXR heterodimeric complex to VDRE-1 in the wild-type promoter. The slightly reduced requirement for the CBS when VDRE-1 is replaced with VDRE-2 could indicate that there is still some physical interaction between CHEP and VDR/RXR. Furthermore, the lack of effect on basal expression when the CBS or the EBS was inactivated suggests that these two proteins do not contribute towards the basal expression of CYP24 promoter constructs in the absence of 1,25D.

### **5.2.3 The contribution of the CBS to induction of –298bp CYP24 promoter constructs at various concentrations of 1,25D**

All transient transfections in this study have employed a concentration of 1,25D at  $10^{-7}$ M. It was of interest to examine the contribution of the CBS to the induction of pCYP24(WT-298)-Luc constructs in the presence of lower concentrations of 1,25D. As shown in Figure 5.5, ROS 17/2.8 cells were transfected with wild-type (pCYP24(WT-298)-Luc) and mutant CBS (pCYP24(mCBS-298)-Luc) constructs and examined for 1,25D inducibility in response to various concentrations of 1,25D.

At a  $10^{-7}$ M concentration of 1,25D, mutagenesis of the CBS in pCYP24(mCBS(-298)-Luc lowered activity of wild-type constructs from a  $27.0 \pm 2.46$ -fold to  $6.9 \pm 1.9$ -fold as discussed in the introduction to this chapter. pCYP24(WT-298)-Luc and pCYP24(mCBS(-298)-Luc constructs expressed similar levels of induction when treated with 1,25D at  $10^{-8}$ M and  $10^{-9}$ M compared with the level of inductions observed with 1,25D at  $10^{-7}$ M (Figure 5.5). In the presence of 1,25D at  $10^{-10}$ M, pCYP24(WT-298)-Luc activity was lowered to  $9.7 \pm 3.0$ -fold induction and mutagenesis of the CBS lowered this induction to  $2.2 \pm 0.6$ -fold. A further



**Figure 5.5: Analysis of -298bp CYP24 promoter constructs encompassing both VDRE-1 and VDRE-2 on the dependency of the CBS at graded doses of 1,25(OH)<sub>2</sub>D<sub>3</sub>.**

Wildtype pCYP24(WT-298)-Luc (black bars) and mutant CBS pCYP24(mCBS-298)-Luc (200ng) constructs (hatched bars) were transfected into ROS 17/2.8 cells. The expression of wildtype and mutant constructs in response to different concentrations of 1,25(OH)<sub>2</sub>D<sub>3</sub> was analysed by treating ROS 17/2.8 cells with graded doses of 1,25(OH)<sub>2</sub>D<sub>3</sub> ranging from 10<sup>-7</sup>M to 10<sup>-12</sup>M for 24hrs. Data presented are the means ± SD of triplicate samples from a representative experiment. This experiment was performed three times with the same trend obtained.

decrease of wild-type promoter activity to  $2.7 \pm 0.9$ -fold and  $2.0 \pm 0.8$ -fold was observed upon treatment of ROS 17/2.8 cells with 1,25D at  $10^{-11}$ M and  $10^{-12}$ M, respectively. Hence, at these low levels of 1,25D, there appears to be no requirement for the CBS.

#### **5.2.4 Investigation of SRC-1, GRIP-1 and CBP over-expression on wild-type and mutant CBS -186 bp CYP24 promoter constructs**

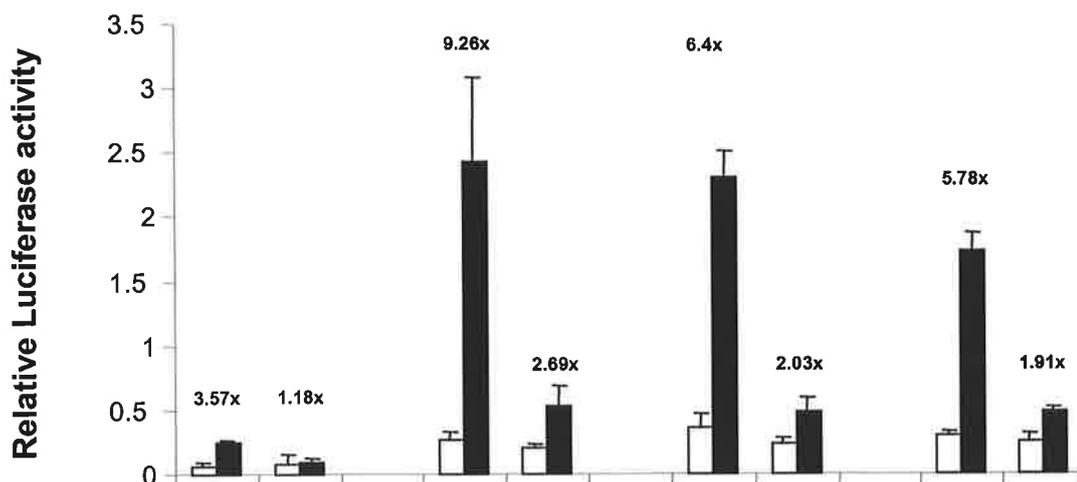
Following the binding of 1,25D to VDR, the transcriptionally repressive corepressor complex is released and a coactivator complex containing a p160 coactivator (MacDonald *et al.*, 2001; Torchia *et al.*, 1998) such as SRC-1 or GRIP-1 (as discussed in Chapter 1) is recruited to the liganded VDR/RXR complex. CBP/p300 is then recruited by SRC-1 or GRIP-1, and with its intrinsic HAT activity (Vo and Goodman, 2001) has been shown on some promoters to acetylate nucleosomal proteins localised allowing the subsequent recruitment and stabilisation of the basal transcriptional machinery and activation of transcription (Chiba *et al.*, 2000). The reduced ability of pCYP24(mCBS)-luc constructs to respond to 1,25D, could indicate that an important role of CHEP bound to the CBS is to interact with the p160 coactivator complex recruited by liganded VDR.

Therefore it was of interest to examine whether over-expression of coactivator proteins such as SRC-1 and GRIP-1 could compensate for the loss of a functional CBS. To investigate this, wild-type (pCYP24(-186)-Luc) and mutant CBS (pCYP24(mCBS)-Luc) constructs were transfected in 293T cells and expression clones for SRC-1, GRIP-1 and CBP over-expressed on these constructs in the presence of 1,25D. In preliminary experiments, it was found that CYP24 promoter constructs transfected into ROS 17/2.8 cells and also into another osteoblast cell line, UMR-106 cells, failed to respond to over-expressed SRC-1, GRIP-1 and CBP at concentrations as high as 500ng (data not shown). This could reflect high endogenous levels of these coactivators in these cell types. However, over-expression of these coactivators in 293T cells enhanced 1,25D induction and so these cells were employed for this study.

As shown in Figure 5.6, pCYP24(WT-186)-Luc was induced  $3.57 \pm 0.37$ -fold by 1,25D and this was lowered to  $1.18 \pm 0.4$ -fold with pCYP24(mCBS)-Luc. Transfection of SRC-1 at 200ng, increased 1,25D induction of the wildtype construct from  $3.57 \pm 0.37$ -fold to  $9.26 \pm 2.54$ -fold. Mutagenesis of the CBS and transfection of SRC-1 resulted in a  $2.69 \pm 0.76$ -fold level of induction similar to the induction of pCYP24(mCBS)-Luc constructs in the absence of transfected coactivator (Figure 5.6). Induction of pCYP24(WT-186)-Luc increased from a  $3.57 \pm 0.37$ -fold level to a  $6.4 \pm 0.54$ -fold upon over-expression of GRIP-1. This fold-level of 1,25D induction was reduced to  $2.03 \pm 0.49$ -fold in the presence of a mutated CBS. The  $2.03 \pm 0.49$ -fold induction observed for pCYP24(mCBS)-Luc in the presence of overexpressed GRIP-1 was again similar to the activity of pCYP24(mCBS)-Luc in the absence of over-expressed coactivator. In the presence of transfected CBP, pCYP24(WT-186)-Luc induction increased from  $3.57 \pm 0.37$ -fold to  $5.78 \pm 0.05$ -fold. The increase of induction observed upon CBP over-expression was again significantly reduced to  $1.91 \pm 0.15$ -fold when the CBS was inactivated in the construct pCYP24(mCBS)-Luc. A small increase of basal activity was observed upon exogenous coactivator expression on wildtype and mutant CBS CYP24 constructs.

In conclusion the exogenous expression of SRC-1, GRIP-1 and CBP has demonstrated two important findings. Firstly, 1,25D mediated induction of -186 bp CYP24 promoter constructs encompassing VDRE-1 are further enhanced upon exogenous expression of SRC-1, GRIP-1 or CBP demonstrating a role for p160 coactivators and CBP during the 1,25D inductive response. Secondly, exogenous expression of the p160 coactivators cannot compensate for the loss of a functional CBS. These results are in keeping with CHEP bound to the CBS physically interacting with both liganded VDR/RXR on VDRE-1 and also with the coactivator complex recruited by VDR/RXR, although direct proof is lacking at this stage and will depend on the identification of CHEP.

### 5.3 Discussion



pCYP24(WT-186)-Luc	+	-	+	-	+	-	+	-
pCYP24(mCBS)-Luc	-	+	-	+	-	+	-	+
pCR3.1-SRC-1 (200ng)	-	-	+	+	-	-	-	-
pSG5-GRIP-1 (200ng)	-	-	-	-	+	+	-	-
pRSV-CBP (200ng)	-	-	-	-	-	-	+	+

**Figure 5.6: Overexpression of SRC-1, GRIP-1 or CBP on wildtype and mutant CBS CYP24 promoter constructs in 293T cells.**

Wildtype (pCYP24(WT-186)-Luc) and mutant CBS (pCYP24(mCBS)-Luc) constructs (200ng) were transiently transfected into 293T cells. pCR3.1-SRC-1, pSG5-GRIP-1 or pRSV-CBP at a concentration of 200ng, was cotransfected into 293T cells and treated with 1,25D at  $10^{-7}$ M (black bars) or vehicle (white bars). This experiment was performed with 293T cells not transfected with pRSV-hVDR. Data presented in the experiment are the means  $\pm$  SD of relative luciferase activity from triplicate samples from a representative experiment. The experiment was performed three times with the same trend obtained. The fold-1,25D induction (ratio of relative luciferase activity of 1,25D/basal) is indicated above each bar

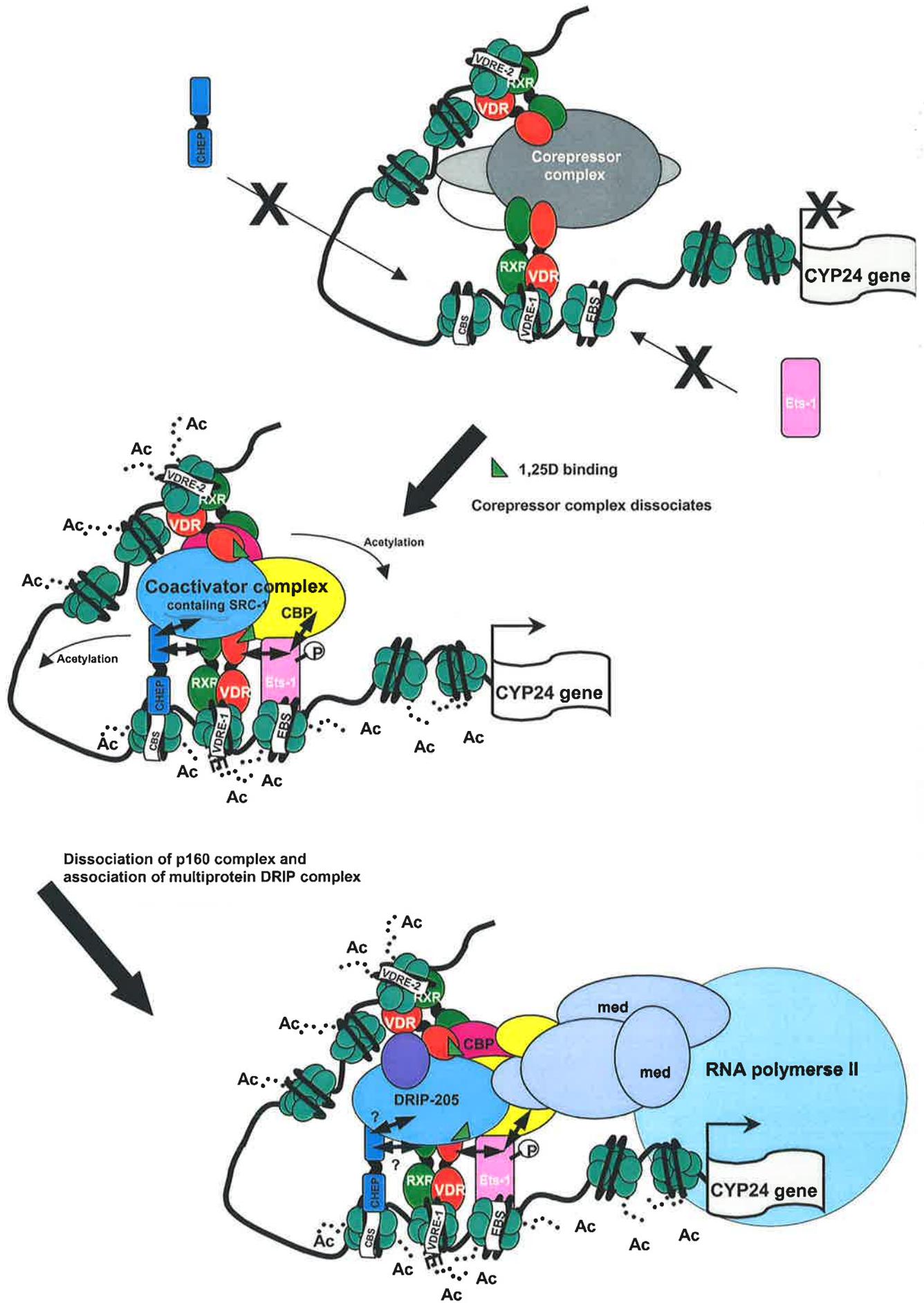
There has been considerable interest in the mechanism by which steroid hormones and the secosteroid 1,25D stimulate gene transcription. In recent years, the identification of coactivator complexes (Collingwood *et al.*, 1999; McKenna *et al.*, 1999; Leo and Chen, 2000; Torchia *et al.*, 1998) and their inhibitory counterparts, corepressors (Zamir *et al.*, 1997; Hu *et al.*, 2001; Nagy *et al.*, 1999; Perissi *et al.*, 1999) have revolutionised our concept of transcriptional activation by nuclear receptors and in particular of VDR. There is evidence that corepressors such as NCoR and RIP13 $\Delta$ 1 (Dwivedi *et al.*, 1998; Polly *et al.*, 2000) can bind to the unliganded VDR/RXR heterodimeric complex on the promoters of 1,25D target genes to lower basal expression. The precise inhibitory mechanism is unclear but is likely to involve histone deacetylase activity associated with these repressive complexes (Jones *et al.*, 2001; Kao *et al.*, 2000; Huang *et al.*, 2000). It is also unclear as to how many of the 1,25D target genes are subject to this control. In any event, the corepressor presumably serves to regulate basal expression at a level acceptable to cellular requirements.

The new insight into 1,25D action that has emerged involves the coactivator complexes. It is well accepted that following the binding of 1,25D to the AF-2 domain of VDR, the corepressor is released and coactivator complexes can dock onto the liganded VDR (Ding *et al.*, 1998; Gill *et al.*, 1998; Liu *et al.*, 2000; Leo *et al.*, 2000; Jimenez-Lara and Aranda, 1999; Baudino *et al.*, 1998; MacDonald *et al.*, 2001). Many coactivators have been identified. Members of the p160 family such as SRC-1 and GRIP-1 interact with the liganded VDR and then recruit another distinct coactivator CBP/p300 (Jimenez-Lara and Aranda, 1999; Ogryzko *et al.*, 1996; Bannister and Kouzarides, 1996; Giles *et al.*, 1998) that has strong histone acetyltransferase activity. Evidence suggests that this activity through acetylation of histones results in chromatin modification in the vicinity of the promoter which allows greater access to transcription factors and importantly to the RNA polymerase II machinery (Kornberg, 1999; Buratowski, 2000; Parvin and Young, 1998). In one attractive scenario, it can be envisaged that a nucleosome is bound at the transcription start site, so that following acetylation and SWI/SNF action (Kitagawa *et al.*, 2003; Kingston and Narlikar, 1999), this nucleosome slides downstream permitting entry of RNA polymerase II. This mechanism is analogous to that elucidated for the interferon beta promoter (Lomvarda and Thanos, 2001). Another distinct coactivator, NCoA-62 also binds at the same time as the p160

coactivators and is required for maximal activity (Barry *et al.*, 2003; MacDonald *et al.*, 2001).

An interesting two step model for coactivator assembly on 1,25D-sensitive promoters has been proposed in which the p160/CBP/NCoA-62 complex is lost following chromatin modification, and this is replaced by a large multisubunit complex called the vitamin D<sub>3</sub> receptor interacting complex (DRIP) (Rachez *et al.*, 1998; Rachez *et al.*, 1999; Rachez *et al.*, 2000; Fondell *et al.*, 1996). It is proposed that DRIP, which has no histone acetyltransferase activity, recruits and interacts with RNA polymerase II. However, whether the two-step mechanism exists *in vivo* on 1,25D target promoters remains to be determined. It is possible that DRIP may act in association with the other coactivators, rather than by a two-step mechanism. Clearly the precise *in vivo* functions of the relevant coactivators need to be determined and the precise temporal order of assembly clarified.

It is within this current understanding of 1,25D action, that the work described in this chapter must be put into context. The CYP24 promoter is as far as we are aware unique in that it contains two VDREs in tandem which give rise to the highest level of induction by 1,25D so far reported. Earlier evidence showed that in the absence of ligand, basal expression of the CYP24 promoter is repressed through unliganded VDR/RXR bound to a VDRE (Dwivedi *et al.*, 1998). Repression is not relieved when either VDRE-1 or VDRE-2 are mutated individually (Prem Dwivedi, personal communication) implying that a repressive complex may assemble on each VDRE. Neither the CBS nor EBS motifs contribute to basal expression of the CYP24 promoter in transfected ROS 17/2.8 and 293T cells. This suggests that the presence of the corepressor complex sterically prevents the binding of proteins to these sites in the absence of 1,25D (Figure 5.7). In the presence of 1,25D, the repressor is presumably lost and coactivator assembly follows, although this has not been definitely established *in vivo* on this promoter. The current work confirms the requirement for either of the p160 family members SRC-1 or GRIP-1 for maximal stimulation of 1,25D-dependent induction of the CYP24 promoter. Over-expression of DRIP-205 (the protein anchoring DRIP to liganded VDR) was not examined in this work but would be of interest. Barry *et al.* (2003) have shown that NCoA-62 stimulates CYP24 promoter activity in the presence of 1,25D. In these studies it was established the



**Figure 5.7: A model depicting the two-step activation of the CYP24 gene promoter in response to 1,25(OH)<sub>2</sub>D<sub>3</sub>.**

In the absence of 1,25D, VDR/RXR are repressed from basal expression through interaction with a corepressor complex. It is unknown if CHEP and Ets-1 are precluded from binding or are already bound but repressed from participating towards basal expression. 1,25(OH)<sub>2</sub>D<sub>3</sub> binding (colored triangle) to the nuclear VDR bound with RXR causes a conformational change in VDR and subsequently releases the corepressor complex allowing the association of liganded VDR/RXR heterodimers with a p160 coactivator complex. The coactivator complex containing histone acetyltransferase activity, consists of SRC-1 or GRIP-1 leads to the acetylation of the histone proteins (depicted by Ac) and possibly other non-histone proteins through recruitment of CBP. In an unknown mechanism, the p160 coactivator complex dissociates and now the large multiprotein DRIP complex binds. The multiprotein complex is anchored to the promoter through the interaction of DRIP-205 subunit with liganded VDR. With its associated mediator (med) proteins, the DRIP complex subsequently recruits and stabilises the RNA polymerase machinery. Following the stabilisation and the subsequent protein-protein interactions that occur, transcriptional synergism between VDRE-1 and VDRE-2 ensues. These events cycle so that the DRIP complex dissociates and the p160 complex is thought to re-associate. This way the promoter is kept in an 'open' configuration to allow multiple rounds of transcriptional re-initiation. Arrows labeled with ? indicate that the CBS may act to stabilise the bound coactivator complex at the adjacent VDRE-1 and/or stabilise VDR/RXR binding to VDRE-1. The orientation of VDR/RXR binding to VDRE-1 and VDRE-2 is not known. Therefore the orientation depicted here is for illustrative purposes only. VDRE, vitamin D<sub>3</sub> response element; EBS, Ets-1 binding site; CBS, CHEP binding site.

over-expression of these coactivators could not overcome the inhibitory effect on 1,25D induction of mutating the CBS. This clearly highlights the importance of the CBS in the induction mechanism and the likely importance of CHEP interaction (Figure 5.7) with these coactivators as discussed further below.

Earlier studies revealed 1,25D-dependent transcriptional synergy between the two VDREs, as determined by separate inactivation of these sites in the CYP24 promoter and transfection of COS-1 and JTC-12 cells (Kerry *et al.*, 1996). In the current study, an approximate 2-3-fold level of transcriptional synergy was observed between the VDREs in transfected ROS17/2.8 cells. Interestingly, inactivation of the CBS abrogated this synergistic response. The mechanism underlying this transcriptional synergy is not known. In one possible scenario, it could be postulated that liganded VDR/RXR, on VDRE-1, together with the neighboring Ets-1 and CHEP interact with the coactivator complex, altering its conformation so that binding of liganded VDR/RXR on VDRE-2 to the complex is enhanced. This could lead to a more efficient modification of chromatin allowing greater access to the RNA polymerase II machinery. It is also possible that the p160/CBP coactivator complex interacts with the transcription machinery and synergy results from an increased interaction with this complex, with the DRIP perhaps contributing at the same time. It is also possible that a separate p160/CBP coactivator complex assembles on each VDRE (Figure 5.7) and synergy reflects an interaction between these complexes. Chromatin immunoprecipitation (ChIP) using antibody against VDR to immunoprecipitate the cross linked proteins bound to the CYP24 promoter followed by detection of p160 coactivator proteins may possibly shed light on this matter. On the other hand, if the two-step mechanism mentioned earlier occurs, then synergy could result from a cooperative interplay between the VDREs which leads to a greater binding of the DRIP/mediator complex rather than the p160/CBP complexes. The reason why mutagenesis of the CBS results in the loss of synergy, is not easily explained. Perhaps the binding protein CHEP interacts with the coactivator and alters its local conformation so that the binding of liganded VDR/RXR on VDRE-1 is increased (Figure 5.7). Additionally, CHEP could interact with VDR/RXR heterodimer increasing the affinity of the heterodimer for VDRE-1. In keeping with these suggestions is the finding in this chapter, that CBS only functions through VDRE-1

and not the more distant VDRE-2, and as described further below there is a continued dependence of the CBS when VDRE-1 is replaced with VDRE-2.

Surprisingly, replacement of VDRE-1 with VDRE-2 in –186 bp CYP24 promoter constructs resulted in a similar level of 1,25D induced expression. This is despite the fact that VDRE-2 apparently binds VDR and RXR with higher affinity (Kerry *et al.*, 1996). In –186 bp constructs in which VDRE-1 was replaced with VDRE-2, 1,25D mediated activity from VDRE-2 was still dependent on a functional CBS although to a slightly lesser extent than the requirement on the native promoter. This may indicate that the major role of CHEP on the CBS is not to stabilize the binding of VDR/RXR on VDRE-1 in the native promoter but rather to interact with the coactivator complex. This is depicted in Figure 5.7. This role may also reflect why the CBS contributes to a greater extent than the EBS in the 1,25D inductive response from VDRE-1 in –186bp constructs. In contrast to the CBS, the requirement for Ets-1 binding to the EBS was almost abolished upon replacement of VDRE-1 with VDRE-2. Ets-1 has been shown to interact directly with VDR/RXR (Dwivedi *et al.*, 2002; Tolon *et al.*, 2000) and it seems probable that this interaction becomes less important on VDRE-2 if it strongly binds VDR/RXR. In addition, Ets-1 has been shown to also bind to the coactivator CBP (Pastorcic and Das, 2000; Yang *et al.*, 1998; Jayaraman *et al.*, 1999). Therefore it is possible that CBP recruitment to VDRE-2 is stabilised to a greater extent compared with recruitment to VDRE-1. This could reduce the dependency of VDRE-2 for the nearby stabilising effect contributed by Ets-1. The binding of VDR and RXR to VDREs occurs mostly such that RXR occupies the 5' half-site and VDR the 3' half-site (Quelo *et al.*, 1994; Schrader *et al.*, 1995). Interestingly, the orientation of VDR/RXR binding to VDRE-1 and VDRE-2 is not known. There is some similarity between the VDRE from the chicken carbonic anhydrase-II gene and VDRE-1 from the CYP24 promoter. It was shown that VDR binds to the 5' half-site and RXR to the 3' half-site of the carbonic anhydrase-II VDRE. On this basis it is predicted that VDR binds to the 5' half-site and RXR to the 3' half-site of VDRE-1. This could be determined using gapped Brd-U substituted VDRE-1 oligonucleotide and UV cross-linking VDR and RXR protein to the oligonucleotide, the same strategy employed by Quelo *et al.* (1994). In the model presented in Figures 5.7, the orientation of VDR/RXR shown is shown such that VDR occupies the 5' half-site and RXR binds to the 3' half site. It is not known if both VDRE-1 and VDRE-2

bind VDR/RXR in similar or opposite orientations. A reduction for a functional EBS was almost abrogated when VDRE-1 was replaced with VDRE-2. This could imply the orientation of VDR/RXR binding is different such that RXR binds to the 5' half-site in VDRE-2 and interacts weakly with Ets-1.

Both VDRE-1 and VDRE-2 synergise in response to high levels of 1,25D. At lower concentrations of 1,25D, VDRE-1 is preferentially used. Higher levels of 1,25D result in the activation of VDRE-2, that subsequently results in transcriptional synergy arising between VDRE-1 and VDRE-2 (Kerry *et al.*, 1996). The dependence of the -298 bp CYP24 promoter for a functional CBS at relatively high ( $10^{-7}$ M- $10^{-10}$ M) concentrations but not at low ( $10^{-11}$ M and  $10^{-12}$ M) was a surprising result. All experiments described in this study have demonstrated that VDRE-1 functionality is strongly dependent upon CHEP binding to the CBS. Furthermore, as stated, VDRE-1 is preferentially used when 1,25D levels are low. This is difficult to interpret. It may appear that at low levels of 1,25D, the activity driven by VDRE-1 may instead be dependent upon the adjacent EBS. Studies by Dwivedi *et al.* (2000) demonstrated that at low concentrations of 1,25D, mutagenesis of the EBS in -298 bp constructs abolished the activity driven from VDRE-1 as tested in COS-1 cells. Therefore at low 1,25D hormone levels, there may be a switch in dependency by VDRE-1 from the CBS at higher hormone levels to the EBS when hormone levels are reduced. The contribution of the CBS during high 1,25D levels of circulating 1,25D would ensue that VDRE-1 and VDRE-2 transcriptionally synergise to increase the level of CYP24 catabolising activity. In *vivo*, the action of 1,25D to synergistically enhance the transcriptional regulation of the CYP24 promoter and hence more CYP24 protein synthesised would ensure adequate removal of potentially toxic levels of hormone.

In conclusion, the studies in this chapter have confirmed the role of coactivators in the 1,25D induction mechanism of the CYP24 promoter. There is synergy between the VDREs at higher concentrations of 1,25D and this synergy is dependent on the CBS. A model to explain synergy and the role of CBS emphasizes a likely important interaction between CHEP and the coactivator complex. Evidence is also presented which suggests that Ets-1 interacts with VDR/RXR on VDRE-1 and this interaction may become significant at low levels of 1,25D when the CBS is

apparently non-functional; however, the mechanism for this discrimination at low hormone levels is not clear.

## CHAPTER 6

# FINAL DISCUSSION

## 6. FINAL DISCUSSION

1,25D plays important roles predominantly in calcium homeostasis and bone mineralisation and recent studies have implicated a role for 1,25D in cell proliferation and differentiation, immune modulation and secondary bile acid metabolism. CYP27B1 and CYP24 represent the two crucial enzymes necessary for the synthesis and degradation of active 1,25D respectively. Several factors including 1,25D, parathyroid hormone and calcitonin act to regulate the expression of these two enzymes resulting in the regulation of ambient serum and cellular levels of 1,25D. Other factors such as dexamethasone and the isoflavonoid, genistein, can directly regulate CYP24 promoter expression and therefore it is pivotal that the transcriptional control mechanism underlying CYP24 gene expression be elucidated. The study in this thesis has focused on the molecular mechanisms by which 1,25D regulates CYP24 promoter expression and for the first time identified an apparently novel transcription factor binding site involved during the inductive process.

As pointed out previously, the CYP24 gene promoter is unique because it contains two vitamin D<sub>3</sub> response elements (VDREs) within the first 300bp of the promoter. Previous studies (Kerry *et al.*, 1996) have demonstrated that each VDRE binds VDR/RXR with VDRE-2 binding VDR/RXR with greater affinity than VDRE-1. 1,25D mediated activity is however significantly stronger from VDRE-1 than VDRE-2. This led to the proposal that the cooperation with surrounding transcription factors contributes to the greater activity observed by VDRE-1 in response to 1,25D. A functional Ets-1 binding site (EBS) located at position -128/-119, downstream of VDRE-1 was shown to be important for maximal activity of VDRE-1 (Dwivedi *et al.*, 2000). The EBS does not contribute to basal expression and in response to the non-genomic activation of MAP kinases is phosphorylated by 1,25D activated ERK5 (Dwivedi *et al.*, 2002). Similarly, RXR was also phosphorylated by 1,25D activated ERK2 therefore linking the rapid non-genomic actions of 1,25D to transcriptional activation of the CYP24 gene. Upstream from VDRE-1 an apparently novel binding site termed the CYP24 Hydroxylase enhancing protein (CHEP) binding site (CBS), was identified during my Honours year. The sequence of this site located at position -171/-163 was 5'-TGTCGGTCA-3' and was shown to be critical for maximal activation of native CYP24 promoter

constructs encompassing VDRE-1 in response to 1,25D with the contribution of the site being more significant in osteoblastic ROS 17/2.8 cells than in kidney 293T cells. Therefore the current study focused on the further characterisation of this sequence in both native and heterologous promoter constructs transfected into 293T and ROS 17/2.8 cells.

Cloning the CBS into heterologous thymidine kinase (TK) promoter constructs established that the sequence 5'-TGTCGGTCA-3' could enhance the activity of a VDRE-1 driven TK-luciferase reporter to a greater extent in ROS 17/2.8 cells than in 293T cells. Interestingly, these studies established three important facts. Firstly, the CBS alone in TK-luciferase constructs is unresponsive to 1,25D treatment. Secondly, similar to the EBS, the CBS did not affect basal expression of native or heterologous promoter constructs. Lastly, the CBS does not represent a cryptic VDRE in the native CYP24 promoter because the CBS itself is not responsive to 1,25D treatment and antibodies against VDR or RXR fail to detect these proteins binding to the CBS in EMSA experiments (data not shown). Collectively, experiments utilising heterologous promoter constructs have defined the sequence 5'-TGTCGGTCA-3' as being the only sequence necessary for the functioning of CHEP. Database searches of transcription factor binding sites did not identify the CBS as a known transcription factor binding site. A closer inspection of the CBS revealed that the CBS appeared to have similar characteristics to a monomeric orphan receptor binding site. As discussed, monomeric orphan receptor binding sites are composed of a core half-site of the sequence 5'-AGGTCA-3' preceded by a 5'-extension of nucleotides. In comparison, the CBS appears to contain a core half-site of the sequence 5'-CGGTCA-3' and the 5'-extension 5'-TGT-3'. Mutagenesis of each single nucleotide in the 5'-extension 5'-TGT-3' revealed that all nucleotides are important for the binding of CHEP in EMSA experiments using nuclear extracts prepared from 293T cells. However the functionality of these mutations was not examined in native CYP24 promoter constructs and future experiments will need to address this. Similarly, mutagenesis of the CBS core into a perfect consensus half-site did not affect 1,25D inducibility of -186 bp CYP24 promoter constructs. These results strongly implied that the CBS contains a core half-site of the consensus 5'-AGGTCA-3' type which is flanked by the 5'-extension of nucleotides 5'-TGT-3'.

UV cross-linking experiments demonstrated that CHEP appears to have a molecular weight of approximately 60 kDa which falls in the molecular weight range for many transcription factors including monomeric orphan receptors (Giguere, 1999). The overexpression of the monomeric orphan receptors SF-1, ROR $\alpha$ , Rev-erb $\alpha$ , NGFI-B and ERR $\alpha$  in 293T and ROS 17/2.8 cells transiently transfected with -186 bp CYP24 promoter constructs did not enhance 1,25D mediated activity. Similarly, EMSA experiments using unlabeled competitor oligonucleotide probes encompassing known DNA binding sites for each monomeric orphan receptor analysed did not affect the binding of CHEP to the CBS. Exogenous expression of ROR $\alpha$  repressed both basal and 1,25D mediated activity of -186 bp CYP24 promoter constructs in the presence and absence of a functional CBS. The relevance and the molecular basis for this repression is unclear but ROR $\alpha$  over-expression has been shown to repress 1,25D mediated transcription of the osteocalcin gene (Meyer *et al.*, 2000). ROR $\alpha$  and ERR $\alpha$  were possible candidates for binding to the CBS due to their A/T rich 5'-extension and their expression in osteoblasts and kidney cells. EMSA experiments utilising a supershifting antibody recognising all ROR $\alpha$  isoforms failed to supershift any complexes binding to the CBS. Furthermore, pharmacological antagonists for ERR $\alpha$ , ERR $\beta$  and ERR $\gamma$  transcriptional function, namely 4-Hydroxytamoxifen (4-OHT) and Diethylstilbestrol (DES) did not affect CYP24 promoter expression in the presence or absence of added 1,25D in transiently transfected 293T and ROS 17/2.8 cells. Collectively, these data demonstrate that the CBS does not appear to be a monomeric orphan receptor binding site and that the CBS may bind an apparently novel transcription factor.

It has been known for many years that the phorbol ester, phorbol 12-myristate 13-acetate (PMA) can activate PKC and various MAP kinase pathways and together with 1,25D synergistically activate CYP24 gene expression in kidney and intestinal cells but not in osteoblast cells (this study, Omdahl and May, 1997; Chen *et al.*, 1993a; Armbrecht *et al.*, 1993; Yang *et al.*, 2001). The reason for the absence of PMA induced 1,25D dependent transcriptional synergy of CYP24 promoter constructs is not clear. A deficiency in a PMA dependent signalling pathway has been noted in other cell types (Carter *et al.*, 2001). MAP kinase activation in osteoblasts in the presence of 1,25D and in the presence of both 1,25D and PMA

may shed light on this matter and this is currently under investigation. The synergistic effect of PMA and 1,25D on the transcriptional activation of the CYP24 gene has been recently shown to be mediated the CBS (Prem Dwivedi, submitted) and in this study also. The synergistic activation of -186 bp CYP24 constructs was observed only in kidney cells and not osteoblast cells supporting previous findings. Interestingly, mutagenesis of the CBS completely abolished PMA and 1,25D transcriptional synergy highlighting the critical importance of this site not only for the 1,25D inductive process but also for transcriptional synergy in response to PMA and 1,25D. These are the first reports that describe the identity of the factors mediating the response to PMA on the CYP24 promoter. Secondly, the identification that the CBS is responsive to PMA provided the first clue towards the possible identity of the proteins binding to the CBS. It is well established that PMA primarily mediates its transcriptional effects through the modulation of AP-1 protein levels and activity (Karin and Shaulin, 2001; Murakami *et al.*, 1999; Kumar *et al.*, 1997; Cirillo *et al.*, 1999). Concomitant to this study, a sequence highly homologous to the CBS was identified and shown to bind many different proteins including c-Jun and c-Fos (Xing *et al.*, 2002). On the basis of their work coupled with the PMA responsiveness of the CBS in this study, c-Jun and c-Fos were over-expressed on -186 bp CYP24 constructs transiently transfected into 293T cells. Exogenous expression of c-Jun potentiated 1,25D mediated activity of the CYP24 promoter that was dependent on a functional CBS. Exogenous expression of c-Fos had no effect. Supershifted antibodies directed against all Jun and Fos family members did not produce any supershifted complexes suggesting that the CBS does not support the direct binding of c-Jun. EMSA experiments utilising supershifted AP-1 antibodies coupled to competition with a cold unlabeled oligonucleotide encompassing the sequence characterised by Xing *et al.* (2002) demonstrated that the CBS binds a distinct protein.

The overwhelming data generated in this study suggest that the CBS is not a binding site for c-Jun or c-Fos. It is envisaged that the potentiation of 1,25D mediated CYP24 promoter expression by c-Jun may be the result of c-Jun interacting with the protein bound at the CBS. Whether c-Jun actually binds to the CBS *in vivo* can only be determined either using chromatin immunoprecipitation (ChIP) assays or *in vitro* translating c-Jun protein and analysing its binding to the CBS oligonucleotide in EMSA experiments. Interestingly, ATF-2 can form

homodimers with itself and heterodimers with c-Jun, is PMA responsive and can bind to an extended AP-1 binding site (van Dam and Castellazzi, 2001; Cirillo *et al.*, 1999). The possibility that ATF-2 may bind to the CBS is currently under investigation. The studies in this thesis do not appear support the proposal that the CBS is an AP-1 binding site despite the fact that the CBS is PMA responsive and the sequence bears some similarity to the consensus AP-1 site. The comparative study using the sequence characterised by Xing *et al.* (2002) did not clarify the situation whilst the c-Jun over-expression data appear to complicate the situation further. Plans are currently underway in the laboratory to isolate the protein binding to the CBS. The approach for the isolation of the protein binding to the CBS will involve a DNA pull-down strategy using CBS oligonucleotide probes attached to streptavidin coated magnetic beads. Following isolation, the protein will be fractionated on SDS-PAGE followed by limited proteolytic cleavage and cleavage products analysed by matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) MS.

Recently it has been shown that dexamethasone together with 1,25D increases both CYP24 mRNA and enzyme activity in both kidney and osteoblast cells, a process the authors concluded may possibly involve activation of the AP-1 transcription factor (Akeno *et al.*, 2000; Kurahashi *et al.*, 2003). It will be of great interest to determine if the CBS is also responsive to dexamethasone treatment. Barbara Nutchey from our laboratory is currently investigating this possibility.

As discussed, a unique feature of the CYP24 promoter is the presence of two VDREs termed VDRE-1 and VDRE-2 that synergise in response to 1,25D. The synergistic activation of CYP24 expression would ensure the rapid removal of potentially toxic levels of 1,25D from occurring. In osteoblast cells, the local production of 1,25D by CYP27B1 is important for osteoblast proliferation, differentiation and bone mineralisation processes. The correct maintenance of 1,25D levels through the activation of CYP24 expression is likely to be important in these processes and is highlighted by the abnormal bone mineralisation defects observed in CYP24 knockout mice (St-Arnaud *et al.*, 2000). Strikingly, mutagenesis of the CBS abolished the 1,25D dependent synergy between VDRE-1 and VDRE-2. This is the first report to describe the involvement of a factor distinct from VDR and RXR to be involved in the synergy between these two

VDREs. The mechanism underlying the transcriptional synergy is not known. In one possible scenario, it may be possible that the liganded VDR/RXR bound at VDRE-1 together with the neighboring Ets-1 and CHEP interact with the coactivator complex altering its conformation so that in some way the binding of liganded VDR/RXR to VDRE-2 is enhanced. Alternatively, it is possible that a p160 coactivator complex (Torchia *et al.*, 1998; MacDonald *et al.*, 2001; McKenna *et al.*, 1999) interacts with the RNA polymerase II machinery and synergy results from an increased interaction with this complex. It is possible that two separate p160 complexes may assemble on each VDRE and transcriptional synergy arises when these two complexes interact. The reason why mutagenesis of the CBS results in the loss of synergy is not easily explained. The binding of CHEP to the CBS could interact with the coactivator complex to alter its conformation so that the binding of liganded VDR/RXR to VDRE-1 is increased. On this note, exogenous expression of the p160 coactivators SRC-1, GRIP-1 or the potent HAT coactivator CBP could not compensate for the loss of 1,25D mediated activity in constructs lacking a functional CBS. Furthermore, CHEP may simultaneously interact with the liganded VDR/RXR heterodimer increasing the affinity of VDR/RXR for VDRE-1. Interestingly, the CBS only appeared to function through VDRE-1 and not the more distant VDRE-1 in native CYP24 promoter constructs. This is presumably a reflection of the ~80 bp separating the CBS and VDRE-2. In -186 bp CYP24 promoter constructs in which VDRE-1 is replaced with the high affinity VDRE-2, 1,25D mediated activity from VDRE-2 was still dependent on a functional CBS although to a slightly lesser extent than the requirement for VDRE-1. This indicates that the major role of CHEP binding to the CBS is not to stabilise the binding of VDR/RXR but rather interact with the coactivator complex and may explain why the CBS contributes significantly greater in the activity mediated by VDRE-1 than Ets-1. In contrast, 1,25D mediated activity from VDRE-2 appeared to be independent of a functional CBS. This is an interesting finding since Ets-1 can interact with VDR (Dwivedi *et al.*, 2002; Tolon *et al.*, 2000) and also the coactivator CBP (Pastorcic and Das, 2000; Jayaraman *et al.*, 1999). Therefore it is clear that CHEP plays a critical role in the 1,25D inductive process from VDRE-1 and that the action of CHEP is dominant over the CBS. The expression of CYP24 promoter constructs encompassing VDRE-1 and VDRE-2 are dependent on a functional CBS at various doses of 1,25D. However at very low levels, the dependence of the CYP24 promoter for the CBS is lost. In contrast, at low doses of 1,25D, the CBS

has been shown to be important for CYP24 promoter activity in transiently transfected COS-1 cells (Dwivedi *et al.*, 2000). Therefore at low levels of hormone there appears to be a switch in dependency by VDRE-1 from the CBS to the EBS. The molecular basis for this is unknown but the contribution of the CBS during high levels of 1,25D ensures that transcriptional synergy ensues between VDRE-1 and VDRE-2 leading to the efficient detoxification of potentially toxic levels of 1,25D hormone.

\* In conclusion to the studies presented in this thesis have characterised the CBS located upstream of VDRE-1 as being critical for 1,25D responsiveness of the CYP24 promoter in osteoblast cells. Therefore a model can be proposed for the role of CHEP during the 1,25D inductive process. In the absence of ligand, VDR/RXR is associated with a corepressor complex (Dwivedi *et al.*, 1998; Polly *et al.*, 2000). This may sterically prevent the binding of CHEP and Ets-1 to their cognate sequence and explain the lack of contribution of CHEP and Ets-1 towards basal expression of CYP24 promoter constructs. 1,25D binding results in a conformational change causing the dissociation of the corepressor complex and association with a p160 coactivator complex containing histone acetyltransferase (HAT) activity. The resultant HAT activity would serve to open up the repressive chromatin structure allowing greater access to transcription factors and importantly the RNA polymerase machinery. It is here that the CBS would appear to play a critical role possibly through interacting and stabilising the coactivator complex bound to the VDR/RXR heterodimeric complex at VDRE-1. Following recruitment and stabilisation of the coactivator complex transcriptional synergism between VDRE-1 and VDRE-2 would ensue. In an unknown step following chromatin modification, the p160 coactivator complex dissociates and the larger multisubunit complex termed the DRIP complex associates with liganded VDR recruits and interacts with the RNA polymerase II machinery. Whether this two-step mechanism of coactivator signalling occurs on all 1,25D target genes *in vivo*, and in particular on the CYP24 promoter, remains to be elucidated together with the functional role of CHEP in this process.

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**Amendments to the thesis entitled "Regulation of the 25-hydroxyvitamin D3 24-hydroxylase (CYP24) gene promoter expression by a novel binding site in response to 1,25D"**

***Corrections:***

Page 41, line 23; delete 'band-aid'

Page 48, line 18; 'passive lysis buffer' is a trademark of Promega

Page 55, line 22; should read, 'Mutagenesis of 5'-TGT-3' and competition with a 10-fold or 50-fold molar excess prevented the formation of complex B leaving complex A (Figure 3.3B, lanes 4 and 5)'

Page 102, paragraph 3, line 2; ( $20.97 \pm 1.94$ -fold) should read ( $20.97 \pm 1.94$ -fold)

Page 118, line 19; VDRE-1 should read VDRE-2

***Additional comments to Figure 3.8.***

A marked difference in the binding of CHEP in nuclear extracts isolated from 293T was observed for the different mutations in the 5'-flanking sequence. To rule out the possibility that the decrease or inhibition of CHEP binding to the labeled oligonucleotide probes is not due to differentially labeled oligonucleotide probes, titration experiments using various concentrations of cold unlabelled oligonucleotide probe must also be examined in future experiments.

***Additional comments to Figure 4.3, 4.4 and 4.5.***

It must also be noted that the protein complex binding to the WT-CBS oligonucleotide probe may consist of a number of different proteins. The treatment of 293T cells with PMA or PMA and 1,25D together may alter the composition of the protein/DNA complex without altering its intensity as seen in Figure 4.3.

In Figures 4.4 and 4.5, nuclear extracts prepared from 293T cells treated with  $10^{-7}$ M 1,25D may not contain significant amounts of AP-1 protein to detect using supershifting AP-1 antibody. Therefore future experiments should be repeated using nuclear extracts isolated from 293T cells treated with PMA and PMA and 1,25D together.

***Clarification of Figure 5.6.***

It was stated that the data graphed in a preliminary experiment shown in Figure 5.6, mutagenesis of the CBS and overexpression of SRC-1 has no effect on 1,25D induction of the pCYP(mCBS)-Luc construct. It should read that overexpression of SRC-1 results in a slight increase and to a lesser extent with GRIP-1, in Luciferase gene reporter activity upon inactivation of the CBS.

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November 12, 2003