Regulation of the 25-Hydroxyvitamin D_3 24-Hydroxylase (CYP24) gene promoter expression by a novel binding site in response to $1,25(OH)_2D_3$

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

By
Josef Steven Kaplan

Department of Molecular Biosciences
University of Adelaide

Adelaide, South Australia

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ABREVIATIONS

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

1,25D	$1,25(OH)_2D_3$ or 1,25-dihydroxyvitamin D_3
$25(OH)D_3$	25-hydroxyvitamin D_3
$24,25(OH)_2D_3$	24,25-dihydroxyvitamin D_3
CYP1α	25-hydroxyvitamin D_3 1α-hydroxylase
CYP24	25-hydroxyvitamin D_3 24-hydroxylase
PTH	Parathyroid Hormone
VDR	vitamin D_3 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
RORα	Retinoid-related orphan receptor
RORE	RORα 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

$1,25(OH)_2D_3$ (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_3 1α-Hydroxylase (CYP27B1) and the 25-Hydroxyvitamin D_3 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_3 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, ERRα, RORα1, Rev-erbα 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 RORα1 reduced both basal and 1,25D induced transcriptional activity of –186 bp CYP24 promoter constructs. Furthermore the use of pharmacological antagonists of ERRα, ERRβ, and ERRγ receptor transcriptional activity, and studies with supershifting antibodies recognising all RORα 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 EBS 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.