Identification of a vitamin D responsive element in the promoter of the rat cytochrome P450\textsubscript{24} gene

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ABSTRACT

Mitochondrial cytochrome P450\textsubscript{24} expression in the vitamin D-degradation pathway is induced by 1,25-dihydroxyvitamin D\textsubscript{3} [1,25-(OH)\textsubscript{2}D\textsubscript{3}]. The molecular basis of this enzyme regulation was investigated by isolating the rat P450\textsubscript{24} gene and examining the 5'-flanking region for possible cis-acting regulatory elements involved in the induction process. Constructs containing different lengths of 5'-flanking region of the gene were linked to a luciferase reporter gene and transiently co-transfected with a human vitamin D receptor (hVDR) expression vector (pRSV-hVDR) into COS-1 cells. These experiments showed that the flanking region from -298 to -122 directed a 24-fold increase in luciferase activity in response to 1,25-(OH)\textsubscript{2}D\textsubscript{3} provided that the cells were co-transfected with pRSV-hVDR. Within this region, the sequence from position -171 to -123 conferred 1,25-(OH)\textsubscript{2}D\textsubscript{3} responsiveness to both the native P450\textsubscript{24} promoter and the heterologous thymidine kinase promoter. Mutagenesis revealed that the sequence from position -150 to -136 is required for induction by 1,25-(OH)\textsubscript{2}D\textsubscript{3} and that this sequence shares similarity to other vitamin D responsive elements (VDREs) reported for other genes. Gel shift mobility assays showed this region specifically bound a nuclear protein complex from position -171 to -123 that had been co-transfected with pRSV-hVDR. This retarded band was specifically competed with the well characterized VDRE from the mouse osteopontin gene. A VDRE at position -150 to -136 in the promoter of the rat P450\textsubscript{24} gene is identified in this study and found to be important in mediating the enhanced expression of the gene by 1,25-(OH)\textsubscript{2}D\textsubscript{3}.

INTRODUCTION

Cytochromes P450 direct the bioactivation and metabolic degradation of the vitamin D secosteroid through sequential hydroxylations (1–4). The vitamin D pathway begins with a 25-hydroxylation step that is localized predominantly in the liver. Subsequent bioactivation occurs principally in the kidney where the 1-hydroxylation of 25-hydroxyvitamin D [25-(OH)\textsubscript{2}D\textsubscript{3}] results in production of the hormonally active metabolite 1,25-dihydroxyvitamin D\textsubscript{3} [1,25-(OH)\textsubscript{2}D\textsubscript{3}] (4). This hormone functions in the control of calcium homeostasis and also regulates cellular growth and differentiation in a number of normal and malignant hematopoietic cells (1,5–7). In addition, 1,25-(OH)\textsubscript{2}D\textsubscript{3} acts as an immunomodulatory regulatory hormone (8). Lowered bioactivity has been reported for other hydroxylated metabolites of 25-(OH)\textsubscript{2}D\textsubscript{3}; however, 1,25-(OH)\textsubscript{2}D\textsubscript{3} occupies the central role as mediator of the biological functions of vitamin D (9). Most known cellular actions of 1,25-(OH)\textsubscript{2}D\textsubscript{3} are transcription dependent and occur through the specific high-affinity binding of the hormone to the vitamin D receptor (VDR). The VDR most likely functions as a heterodimer complex with retinoid X receptor (RXR); a sub-member of the nuclear receptor family that binds 9-cis retinoic acid as its ligand (10–15). RXR may facilitate binding of the VDR to the vitamin D response element (VDRE) in the promoter of genes that respond to 1,25-(OH)\textsubscript{2}D\textsubscript{3} and the resulting heterodimer then activates gene transcription.

The steady-state level of 1,25-(OH)\textsubscript{2}D\textsubscript{3} is determined by the balance between biosynthesis and degradation. The rate controlling step in the biosynthesis occurs through the action of 25-hydroxyvitamin D\textsubscript{3} 1-hydroxylase (cytochrome P450\textsubscript{27}) whereas degradation is initiated through the C-24 oxidation pathway that directs subsequent 1,25-(OH)\textsubscript{2}D\textsubscript{3} side-chain oxidation and cleavage (16,17). Entry into the C-24 oxidation pathway is directed by 25-hydroxyvitamin D\textsubscript{3} 24-hydroxylase (P450\textsubscript{24}), a mitochondrial enzyme that is known to be up-regulated by 1,25-(OH)\textsubscript{2}D\textsubscript{3} (17,18). The action of 1,25-(OH)\textsubscript{2}D\textsubscript{3} to feedback activate its own degradation is initiated at the transcriptional level (19) and results in increased expression of P450\textsubscript{24} mRNA in target tissues (20). In order to understand this action of 1,25-(OH)\textsubscript{2}D\textsubscript{3} at the molecular level, we have isolated a genomic clone for rat P450\textsubscript{24} and characterized 5'-flanking region sequence together with the first exon and intron. A regulatory element was identified that responds to 1,25-(OH)\textsubscript{2}D\textsubscript{3} and participates in the up-regulation of P450\textsubscript{24} gene expression. This identified VDRE is located about 150 bp upstream of the transcription start site and has an imperfect-repeat motif that is characteristic of vitamin D-dependent genes.

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MATERIAL AND METHODS

Materials

The luciferase plasmid pGL2-Basic, luciferase assay kit and cell culture lysis reagent were obtained from Promega. Stratagene was the source of pBluescript KS+ and the λ EMBL3-S6/T7 rat genomic library was obtained from Clontech. The pBLCAT2 vector was a gift from Gunther Schutz (Heidelberg, FRG) (21) and a VDR cDNA clone (pAXVD) was kindly supplied by Nigel Morrison, St Vincent’s Hospital (Sydney, Australia). 1.25-Ο(ΟH)2D3 was generously supplied by Hoffmann LaRoche (Nutley, NJ). A Sequenase Version 2.0 sequencing kit was obtained from United States Biochemical Corporation. Synthetic oligonucleotides were obtained from Bresatec (Adelaide, Australia), Protein Chemistry Laboratory at the University of New Mexico (Albuquerque, NM) and IDT Technologies (Coralville, IA).

Isolation, characterization and sequencing of genomic clones

Oligonucleotide primers were designed for PCR amplification of the 5'-end of the rat P45024α gene. The oligonucleotides 5'-GGGGATCCGAGCTTTATCTGACGCTC-3' and 5'-GGGGATCCGAGCTTTATCTGACGCTC-3' were derived from the rat P45024α cDNA sequence and were located at nucleotide positions 19-42 and 524-547 (22). They contained BamHI sites (underlined bases) and were designed to give a 529 bp fragment. The PCR fragment was cut with BamHI, cloned into pBluescript KS+ and sequence verified. The radiolabeled 529 bp fragment (Mega-Prime Kit, Amersham) was used to probe a rat genomic λ EMBL3-S6/T7 library (Clontech) using standard hybridization techniques (23). Five positive plaque plagues were purified and restriction mapped by Southern analysis. One clone contained ~ 4 kb of 5’-flanking region and a 4.5 kb EcoRI fragment that hybridized to the 529 PCR product. This EcoRI product was sequenced, extensively restriction mapped and used in subsequent promoter studies. The nucleotide sequence data of rat P45024α will appear in the EMBL Data Library (accession number Z28351).

Plasmid constructs

pGL24H-298, pGL24H-644 and pGL24H-1401. A series of constructs were made in which various lengths of the P45024α gene 5'-flanking region were cloned upstream of the luciferase reporter gene in pGL2-Basic. The nested set of luciferase constructs begins at +74 and contained 298, 644 and 1401 bp fragments of 5'-flanking region. The fragments were produced by cutting with Sall (+74) and either PvdII (-298), HincII (-644) or Sall (-1401) and blunt-end cloned into the SmaI site of pGL2-Basic.

pGL24Hprom and pGLTKprom. A construct containing the P45024α minimal promoter was made by PCR amplifying the promoter region using oligonucleotides 5'-TAGCTCGAGCC-ACACCGCCGGGCG-3' and 5'-GGGAAGTGTGCCTGC-TACCTCC-CCCT-3' and pGL24H-298 as DNA template. The first oligonucleotide contains sequence from -116 to -100 of the P45024α promoter (see Fig. 4). The second oligonucleotide hybridizes to the polylinker of pGL2-Basic (Promega) so that the 6 nucleotides incorporate the Sall/Sall (+74) cloning interface between pGL-Basic and the P45024α gene promoter. The 222 bp PCR product was restricted with XhoI and HindIII (underlined bases) and directionally cloned into pGL2-Basic resulting in a construct containing 122 bp of rat P45024α gene 5'-flanking region designated pGL24Hprom. The thymidine kinase promoter was subcloned from pBLCAT2 (BamHI/BglII) into the BglII site of pGL2-Basic (pGLTKprom).

pGL24HWT, pGL24HM1, pGL24HM2, pGL24HM3 and pGLTKWT. A series of double-stranded oligonucleotides were made to the putative vitamin D responsive region from -171 to -123 and to mutated half-sites within this region. Sequences for these oligonucleotides are given in the following list in which WT represents the wild-type oligonucleotide from -171 to -123 and M1, M2 and M3 have mutations (mutated bases underlined) in putative VDRE half-sites within WT (underlined on complementary strand). The double-stranded oligonucleotides were cloned into XhoI sites of pGL24Hprom or pGLTKprom as a single copy in the native orientation.

WT: 5'-TCGACTGTCGCTACGAGGCCCCGGCGGCTGCACTACCTCCTGTCATCGTTACATCC-3'; 3'-GACAGCCAGTGGTCATCGCCGCGGGGGCGAGTTGGAGCTGAGGCTGAGAACTGGC-5'

M1: 5'-TCGACTGTCGCTACGAGGCCCCGGCGGCTGCACTACCTCCTGTCATCGTTACATCC-3'; 3'-GACAGCCAGTGGTCATCGCCGCGGGGGCGAGTTGGAGCTGAGGCTGAGAACTGGC-5'

M2: 5'-TCGACTCCTACGAGGCCCCGGCGGCTGCACTACCTCCTGTCATCGTTACATCC-3'; 3'-GACAGCCAGTGGTCATCGCCGCGGGGGCGAGTTGGAGCTGAGGCTGAGAACTGGC-5'

M3: 5'-TCGACTCATGTCGCTACGAGGCCCCGGCGGCTGCACTACCTCCTGTCATCGTTACATCC-3'; 3'-GACAGCCAGTGGTCATCGCCGCGGGGGCGAGTTGGAGCTGAGGCTGAGAACTGGC-5'

The rec A' host E.coli DH5α was used for plasmid cloning in order to prevent multimeric-insert cloning. Plasmid DNA was prepared by alkaline lysis and CsCl/ethidium bromide equilibrium density gradients (23). All construct DNA was quantified by spectrophotometry followed by 1% agarose-gel analysis to ensure consistency between preparations with regard to quantity and supercoiling.

Oligomer DNA constructs

Two double-stranded oligonucleotides (ΔWT and OSP) were prepared for use in gel retardation assays. ΔWT is a shortened WT fragment from -153 to -133 and OSP is the VDRE from the mouse osteopontin gene promoter (24) (half sites are underlined on their respective strands). Each double-stranded oligonucleotide contained terminal Sall and XhoI sites for use in determining insert orientation and were cloned into the XhoI site of pGL24Hprom or pGLTKprom as a single copy in the native orientation.

ΔWT: 5'-TCGACTGTCGCTACGAGGCCCCGGCGGCTGCACTACCTCCTGTCATCGTTACATCC-3'; 3'-GCGGGATCCGAGCTTTATCTGACGCTC-5'

OSP: 5'-TCGACTGTCGCTACGAGGCCCCGGCGGCTGCACTACCTCCTGTCATCGTTACATCC-3'; 3'-GCGGGATCCGAGCTTTATCTGACGCTC-5'

Culture and transfection of COS-1 cells

COS-1 cells were maintained in Dulbecco-Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS). In preparation for electroporation, cells were grown to 80-90% confluency, collected by trypsinization, washed once with phosphate buffered saline (PBS) and resuspended (1 x 10⁶ cells/ml) in ice cold 20 mM HEPES (pH 7.05) containing 137 mM NaCl, 5 mM KCl, 0.7 mM NaHPO₄, 6 mM dextrose (23) and 500 μg/ml of sheared salmon sperm DNA. COS-1 cells (5 x 10⁶) and 3 pmol of construct DNA were electroporated at 300 V, 960 μF using a Bio-Rad Gene Pulser with Capacitance Extender. Following electroporation, the samples were placed...
on ice for 10 min and aliquoted (2.5 × 10^6 cells) into two 60 mm diameter petri dishes containing 5 ml of DMEM-10% FCS. COS-1 cells were allowed to recover at 37°C for 20 h and then switched to RPMI medium supplemented with 12% charcoal stripped FCS for 2 h. Paired samples were treated with 1,25-(OH)2D3 (10-6 M) or carrier ethanol and further incubated for 24 h before harvesting.

**Luciferase assay**

COS-1 cells were washed once with PBS and treated with 200 μl of Cell Culture Lysis Reagent (Promega). The cells were scraped off immediately, transferred to an Eppendorf tube and placed on ice for 15 min. To aid lysis, the cells were frozen on dry ice for 5 min, thawed on ice and vortexed vigorously. After centrifugation for 5 sec, the supernatant was removed and assayed for protein concentration. Luciferase activity was determined in 50 μg of lysate protein using a Luciferase Assay Kit (Promega).

**Gel mobility shift assay**

COS-1 cell lysates were prepared essentially as described by Schreiber et al. (26). COS-1 cells (3 cassettes each with 1 × 10^7 cells) were either transfected by electroporation as described above with 3 μg of the expression plasmid pRSV-hVDR or simply plated untransfected into 100 mm diameter petri dishes containing 12.5 ml DMEM-10% FCS. After overnight incubation, these cells were treated with 1,25-(OH)2D3 (10-6 M) and further incubated for 24 h. The cells were washed twice with PBS, harvested by scraping in 1 ml of PBS, pelleted in an Eppendorf tube, washed with 1 ml of PBS and resuspended in 800 μl of ice cold Lysis buffer (10 mM Tris-HCl pH7.9, 10 mM KCI, 1 mM dithiothreitol, 1.5 mM MgCl2 and 0.5% Nonidet NP40). After 15 min on ice to allow for the cells to swell and lyse, the nuclei were pelleted for 1 min at 4°C and then washed with 800 μl of ice cold Buffer A (10 mM Tris-HCl pH7.9, 10 mM KCl, 1 mM dithiothreitol and 1.5 mM MgCl2). Nuclei were resuspended in 400 μl of ice cold Buffer B (50 mM Tris-HCl pH7.5, 500 mM KCI, 2 mM dithiothreitol, 5 mM MgCl2, 0.1 mM EDTA, 10% sucrose and 20% glycerol) and agitated for 1 h at 4°C to extract the nuclear proteins. Following centrifugation (12,000 × g) for 15 min at 4°C, the supernatant was dialyzed twice with at least 50 volumes of TM-1 buffer (25 mM Tris-HCl pH7.6, 100 mM KCl, 0.5 mM dithiothreitol, 5 mM MgCl2, 0.5 mM EDTA and 10% glycerol) for 1 h at 4°C. The dialysate was centrifuged (12,000 × g) for 5 min at 4°C to pellet nuclei and nuclear remnants and the supernatant assayed for protein. Nuclear extracts were aliquoted and stored at -80°C. Protease inhibitors were added to the above buffers just prior to use as follows: leupeptin (1 μg/ml), aprotinin (1 μg/ml), pepstatin (1 μg/ml) and phenylmethylsulfonyl fluoride (100 mM). Binding reactions were carried out as described by Liao et al. (27) at room temperature with approximately 1 ng (i.e. 70,000–100,000 copies) of radiolabeled probe, 1 μg of poly(dI-dC) and 5 μg of nuclear protein in Binding buffer (25 mM Tris-HCl pH7.9, 15 mM HEPES, 5.5 mM KCl, 0.5 mM dithiothreitol, 3 mM MgCl2, 4.5 mM EDTA, 6% glycerol and 0.08% Tween 20). DNA/protein complexes were separated on a 5% non-denaturing polyacrylamide gel run at 80 V/cm in 50 mM Tris/380 mM glycine buffer, pH 8.2 at 4°C. Shifted oligonucleotide probes were detected by autoradiography for 1–3 days.

**RESULTS**

**Isolation of genomic clones for rat P45024**

A PCR clone encoding 5'–sequence upstream from the transcription start site of the rat P45024 gene (Materials and Methods) was used to screen 1.2 × 10^6 plaques from a λ EMBL-3 SP6/T7 library and identify five positive clones. The PCR probe was then used to isolate a 4.5 kb EcoRI fragment from one of the clones that contained a 15 kb genomic DNA insert. This fragment was restriction mapped (Fig. 1) and sequence analyzed. Sequence was determined from the HindII site at -644 to the PstI site at +769 in the first exon as shown in Figure 2. The sequenced fragment encompassed 5'-flanking region, the first exon (translation initiation AUG codon located at +357) and part of intron 1. The transcription start site was determined by RNase protection analysis using total RNA from rat kidney and a transcript generated from the MscI fragment.

**Gel mobility shift assay**

COS-1 cell lysates were prepared essentially as described by Schreiber et al. (26). COS-1 cells (3 cassettes each with 1 × 10^7 cells) were either transfected by electroporation as described above with 3 μg of the expression plasmid pRSV-hVDR or simply plated untransfected into 100 mm diameter petri dishes containing 12.5 ml DMEM-10% FCS. After overnight incubation, these cells were treated with 1,25-(OH)2D3 (10-6 M) and further incubated for 24 h. The cells were washed twice with PBS, harvested by scraping in 1 ml of PBS, pelleted in an Eppendorf tube, washed with 1 ml of PBS and resuspended in 800 μl of ice cold Lysis buffer (10 mM Tris-HCl pH7.9, 10 mM KCl, 1 mM dithiothreitol, 1.5 mM MgCl2 and 0.5% Nonidet NP40). After 15 min on ice to allow for the cells to swell and lyse, the nuclei were pelleted for 1 min at 4°C and then washed with 800 μl of ice cold Buffer A (10 mM Tris-HCl pH7.9, 10 mM KCl, 1 mM dithiothreitol and 1.5 mM MgCl2). Nuclei were resuspended in 400 μl of ice cold Buffer B (50 mM Tris-HCl pH7.5, 500 mM KCl, 2 mM dithiothreitol, 5 mM MgCl2, 0.1 mM EDTA, 10% sucrose and 20% glycerol) and agitated for 1 h at 4°C to extract the nuclear proteins. Following centrifugation (12,000 × g) for 15 min at 4°C, the supernatant was dialyzed twice with at least 50 volumes of TM-1 buffer (25 mM Tris-HCl pH7.6, 100 mM KCl, 0.5 mM dithiothreitol, 5 mM MgCl2, 0.5 mM EDTA and 10% glycerol) for 1 h at 4°C. The dialysate was centrifuged (12,000 × g) for 5 min at 4°C to pellet nuclei and nuclear remnants and the supernatant assayed for protein. Nuclear extracts were aliquoted and stored at -80°C. Protease inhibitors were added to the above buffers just prior to use as follows: leupeptin (1 μg/ml), aprotinin (1 μg/ml), pepstatin (1 μg/ml) and phenylmethylsulfonyl fluoride (100 mM). Binding reactions were carried out as described by Liao et al. (27) at room temperature with approximately 1 ng (i.e. 70,000–100,000 copies) of radiolabeled probe, 1 μg of poly(dI-dC) and 5 μg of nuclear protein in Binding buffer (25 mM Tris-HCl pH7.9, 15 mM HEPES, 5.5 mM KCl, 0.5 mM dithiothreitol, 3 mM MgCl2, 4.5 mM EDTA, 6% glycerol and 0.08% Tween 20). DNA/protein complexes were separated on a 5% non-denaturing polyacrylamide gel run at 80 V/cm in 50 mM Tris/380 mM glycine buffer, pH 8.2 at 4°C. Shifted oligonucleotide probes were detected by autoradiography for 1–3 days.

**Figure 1.** Restriction map of the 4.5 kb EcoRI fragment from the rat P45024 genomic clone. The gene transcription start site (+1) and the ATG initiation start codon (asterisk) are shown together with the first exon and intron. The sequences corresponding to the PCR product used as a probe to screen for this gene and the portion of the gene used as a template for RNase protection analysis are indicated.

**Figure 2.** Sequence of the rat P45024 gene including the 5'-flanking region and first intron. The transcription start site is located at +1. Putative CCAAT, GC and AP-1 binding sites are indicated together with a TATA box sequence (boxed). The translation initiation AUG codon (underlined) is located at +357. The first exon is shown in bold-type. The asterisk indicates the T that replaces the CC in the sequence of Ohyama et al. (28).
at −53 to +144 (data not shown). A sequence resembling a TATA box was located 28 bp upstream of the start site. The sequence in Figure 2 extends that reported recently by Ohyama et al. (28) by 131 bp upstream and within the first intron, and agrees with these authors except that at position −77 where there is a T instead of CC. Several possible cis-acting motifs were present in the 5′-flanking region and in the first intron. These include putative GC boxes at −379, −345, −105, +672 and +758; CCAAT boxes at −595, −419 and −54; AP-1 binding sites at −139 and −126 (Fig. 2).

Transgenic transfection of promoter deletion constructs in COS-1 cells

The promoter activity of the 5′-flanking region of the P45024 gene was investigated by deletion analysis. Increasing lengths of 5′-flanking region of the gene were inserted upstream of the firefly luciferase reporter gene (Fig. 3). These chimeric constructs contained −122, −298, −644 and −1401 bp of 5′-flanking region and all terminated at a common StuI site at +74 in the 5′-untranslated region of the P45024 gene (Fig. 3). Constructs were co-transfected into COS-1 cells together with a VDR expression vector (pRSV-hVDR). The cells were incubated in charcoal-treated media, treated with 1,25(OH)2D3 (10−6 M) or ethanol carrier and assayed 24 h later for luciferase activity (Fig. 3). The transient expression analysis demonstrated that the first 122 bp of 5′-flanking sequence did not respond to 1,25(OH)2D3, but a 24-fold induction was observed when the sequence was extended to −298 bp. With the other two plasmids there was a further increase in the response to 1,25(OH)2D3 and a 47-fold level of induction was seen with the longest construct. In separate experiments, expression of the constructs containing −298 and −1401 bp of 5′-flanking sequence were examined in COS-1 cells that had not been transfected with pRSV-hVDR (Fig. 3). There was a substantial decrease in the response to 1,25(OH)2D3 with both constructs and the induction observed was only 2.4- and 2.9-fold compared with 24- and 47-fold, respectively, when assayed in VDR transfected cells (Fig. 3). These experiments, therefore, established that the region from −298 to −122 of the P45024 gene was required for induction by 1,25(OH)2D3. Examination of this region indicated possible VDREs between position −171 to −123, which was investigated further.

Transient analysis of a putative vitamin D responsive region

A double-stranded synthetic oligonucleotide containing the −171/−123 sequence was inserted in the pGLTKprom construct (i.e. pGLTKWT) and analyzed for responsiveness to 1,25(OH)2D3. In three separate experiments, the pGLTKWT

![Figure 3. Transient expression of P45024 constructs in COS-1 cells. (A) Diagrammatic representation of the 5′-flanking region of the 24-hydroxylase (P45024) gene. (B) Induction of luciferase expression by 1,25(OH)2D3 in COS-1 cells following transfection of 5′-flanking deletion constructs of the P45024 gene linked to luciferase reporter gene (Luc). (C) Expression of the construct with −298 and −1401 bp of flanking region in the presence of 1,25(OH)2D3 but absence of transfected pRSV-hVDR. The levels of induction are shown as the ratio of luciferase activity from 1,25(OH)2D3 treated cells versus untreated cells. Data in B are averages of three experiments; data in C are averages of two experiments.](https://academic.oup.com/nar/article-abstract/22/12/2410/2400164/Identification-of-a-vitamin-D-responsive-element/23714)
plasmid showed a 4.1-fold induction to 1,25-(OH)_{2}D_{3} treatment when co-transfected with pRSV-hVDR into COS-1 cells (data not shown). The response of the −171/−123 sequence was investigated in the context of the native promoter. The pGL24HWT construct consistently gave a 7.5-fold stimulation of luciferase activity following 1,25-(OH)_{2}D_{3} treatment (Fig. 4). The pGL24HWT construct re-created the first 171 bp of the native promoter except for the replacement of 3 bp at position −119 to −117 due to the introduced XhoI cloning site. This alteration did not create or destroy any known transcription factor binding site. Since the native −298 construct gave a 24-fold induction (Fig. 3), it becomes apparent that an additional upstream-control-region exists between −298 and −171 that contributes to the promoter induction by 1,25-(OH)_{2}D_{3}.

Within the region from position −171 to −123, three sequences with similarity to known VDREs were located on the complementary strand beginning at positions −140 (GAGTG-AggcCGCCCG), −136 (AGGTGAgtgAGGGCG) and −127 (GAGTCAgagCGGTGA) (Fig. 4). The order of best-to-poorest match to previously identified VDREs is −136, −127 and −140. To investigate their possible roles, three mutant double-stranded synthetic oligonucleotides (M1, M2 and M3) were synthesized from position −171 to −123 with the first half-site of each VDRE mutated separately (Fig. 4). These oligonucleotides together with the wild-type oligonucleotide (WT) were fused to pGL24Hprom (Materials and Methods) and used to measure 1,25-(OH)_{2}D_{3} induction of the transfected luciferase-mutant-constructs. Transient expression of pGL24HM1 showed that mutagenesis of the putative VDRE at −127 lowered the 1,25-(OH)_{2}D_{3} induction of luciferase activity by 36%. However, mutagenesis of the putative VDRE at −136 (pGL24HM2) resulted in the almost complete loss of induction by 1,25-(OH)_{2}D_{3}. The construct pGL24HM3 contained mutations (5’-AGGTGAgggAGGCG-3’ to 5’-AGGTG-AaggTGGCG-3’), which altered the putative second half-site for the VDRE beginning at position −136. Transfection of this construct resulted in a marked inhibition of 1,25-(OH)_{2}D_{3} induction similar to that seen with pGL24HM2. It is evident from these mutation results that the sequence from −150 to −136 contains a potential VDRE, which confers 1,25-(OH)_{2}D_{3} responsiveness to the P450_{24} gene. The mutations introduced into pGL24HM2, which virtually abolished 1,25-(OH)_{2}D_{3} induction, did not alter sequence in the distal −140 half-site. Therefore, it can be concluded that the putative VDRE beginning at −140 is not responsive to 1,25-(OH)_{2}D_{3}.

**In vitro DNA binding studies**

Double stranded synthetic radio-labelled oligonucleotides with sequence from position −171 to −123 (WT) and from −150 to −136 (ΔWT) were used as probes in mobility gel shift assays to determine if the potential VDRE can bind nuclear proteins. These oligonucleotides were incubated with nuclear extracts prepared either from 1,25-(OH)_{2}D_{3} treated COS-1 cells transfected with pRSV-hVDR or from COS-1 cells that were not transfected with this VDR expression vector. Several retarded bands were seen with the longer WT oligonucleotide, but a unique band (i.e. band 3) was consistently observed only with the nuclear extract from COS-1 cells transfected with pRSV-hVDR (Fig. 5). A major retarded-band with the same mobility was also seen with the shorter ΔWT oligonucleotide but only when nuclear extracts were employed from VDR transfected cells (Fig. 5). The intensity of this band was not increased by the inclusion of 1,25-(OH)_{2}D_{3} at 10^{-8} M in the binding assay (result not shown). The major band detected with the ΔWT oligonucleotide (band 3) was of similar electrophoretic mobility to the unique VDR-dependent band seen with the longer oligonucleotide and also to the single band generated by the well characterized VDRE present in mouse osteopontin gene (24). Competition experiments were undertaken using a 10-, 30- and 100-fold excess of unlabelled mouse osteopontin VDRE oligonucleotide. The addition of this oligonucleotide at 10-fold excess effectively and specifically competed with the major band observed for the ΔWT.

Mouse calbindin-D28k  
-199/-184 GGGGGA tgt GAGGAG

Rat calbindin-D9k  
-488/-474 GGTTGCG cgg AAAGCC

Rat osteocalcin  
-456/-438 GGTTGAG atg AGGACA

Mouse osteopontin  
-758/-740 GGTTCA cga GGGTC

Rat 24-hydroxylase  
-150/-136 AGGTTG gtg AGGGCG

**Figure 5.** Gel shift analysis using oligomers to the P450_{24} promoter. Oligomer for position −171 to −123 (WT), oligomer to the putative VDRE at position −150 to −136 (ΔWT) and oligomer for the VDRE from the osteopontin gene (OS) were labeled by end-filling with [γ-^32P]dATP and incubated with nuclear extracts of 1,25-(OH)_{2}D_{3} treated COS-1 cells either mock transfected (−) or transfected with 2 μg of pRSV-hVDR (+) at 24 h earlier. Protein/DNA complexes seen with oligomer (WT) are shown as 3–4. Complex 3 is unique to pRSV-hVDR transfected COS-1 cells. The binding of protein complex 3 to oligomers WT and ΔWT was competed with 10-, 30- and 100-fold excess of unlabelled OSP oligomer.

**Figure 6.** A comparison of the identified VDRE in the rat 24-hydroxylase (P450_{24}) gene with other reported VDREs. The two half-sites of each VDRE are shown in upper case. Sequences are from mouse calbindin gene −199 to −184 (31), rat calbindin gene −488 to −474 (30), rat osteocalcin gene −456 to −438 (29), mouse osteopontin gene −758 to −740 (24) and rat P450_{24} gene (present work) −150 to −136, but on the complementary strand.
oligonucleotide from position −150 to −136 and with the unique VDR-dependent band seen with the WT oligonucleotide from position −171 to −123 (Fig. 5). Conversely, an excess of unlabelled ΔWT oligonucleotide effectively competed with radiolabelled mouse osteopontin VDRE for protein binding whereas a nonspecific-DNA oligonucleotide did not mimic osteopontin in the competition experiments (result not shown). These experiments demonstrate: 1) the presence of promoter sequences in the P450_{24} gene that can bind a specific nuclear-protein-complex in the nuclear extract of cells expressing VDR and 2) the identification of a proximal VDRE in the region from position −150 to −136.

**DISCUSSION**

The molecular regulation of the rat P450_{24} gene has been investigated by transient transfection analysis. We have determined that the 5′-flanking region from −298 to −122 can respond dramatically to 1,25-(OH)_{2}D_{3} resulting in a 24-fold induction in the presence of the hormone. A further induction of up to 47-fold was seen with 1401 bp of 5′-flanking region indicating that there are transcription factor binding sites between position −298 to −1401, which can enhance expression. Further analysis showed that the proximal regulatory region from position −171 to −123 can confer 1,25-(OH)_{2}D_{3} responsiveness when attached to a either a heterologous thymidine kinase promoter or to native P450_{24} gene promoter sequence. A nearly 2-fold higher level of induction was seen with the native-promoter construct compared with the thymidine kinase promoter construct. The increased level of induction with the native promotor may be related to the much lower basal expression of luciferase activity observed with this promoter compared with the thymidine kinase promoter (data not shown). Possible VDREs in the 5′-flanking sequence of the P450_{24} gene (−171 to −123) were investigated by transient expression analysis in COS-1 cells. From these studies, a potential VDRE was located in the complementary strand at position −150 to −136. The mutated putative VDRE half-sites at −141 to −136 or −150 to −145 resulted in the almost complete loss of 1,25-(OH)_{2}D_{3} induction. A more proximal putative VDRE half-site at position −132 to −127 is contained within an AP-1 like element (5′-GGGTA-3′). Mutagenesis of this sequence partially inhibited (36%) the 1,25-(OH)_{2}D_{3} induction response. The possibility that AP-1 binds to this site and participates in the mechanism of induction is currently being investigated.

Gel shift mobility assays were employed to further investigate the potential VDRE at position −150 to −136. When an oligonucleotide encompassing this region was incubated with crude nuclear extracts from COS-1 cells (transfected with pRSV-hVDR), a major retarded-band was identified. This band had the same mobility as the band seen with the well-defined VDRE in the mouse osteopontin gene promoter (24). In addition, the major retarded band seen with oligonucleotide (−150 to −136) was specifically eliminated in competition experiments using the mouse osteopontin VDRE. Since the band seen with the oligonucleotide from −150 to −136 was only observed using nuclear extracts from pRSV-hVDR transfected COS-1 cells, we conclude that VDR is required for interaction in a gel shift assay and that the −150 to −136 sequence is a VDRE.

Several VDREs have been identified in the promoters of genes whose expression is upregulated by 1,25-(OH)_{2}D_{3}. Transfection studies with reporter-gene constructs and in vitro gel shift analysis have been used to identify specific half-site sequences and spacing, as delineated in Figure 6. The VDREs can be considered as two purine-rich half-sites that are direct imperfect repeats and are separated by 3 bp of non-specific sequence. In contrast to the classical direct-repeat half-site paradigm for upregulatory VDREs, a motif with only a single VDRE half-site (GGTTCA) is observed in the downregulatory VDRE of the human parathyroid hormone promoter (32). However, the VDRE identified in this study conforms to the classical VDRE structure, which is consistent with an upregulatory action for the VDRE in the rat P450_{24} gene promoter. Based on the available half-site sequence and spacing data, we propose that the consensus sequence for a VDRE is GGGTGANnn(G/A)(G/A)GNCN in which the second half-site can be considered as a degenerate copy of the first 5′-GGGTTGA-3′ half-site.

The spacing between half-sites of hormone responsive elements in gene promoters is critical for expression of hormone specificity. For example, a spacing of either 3, 4 or 5 bp determines a selective response to vitamin D, thyroid hormone or retinoic acid receptors, respectively (33). There is evidence that the retinoid/thyroid hormone/vitamin D subfamily of nuclear receptors bind to their response elements as heterodimers with the same nuclear factor retinoid X receptor (RXR). Carlberg et al. (10) have shown that the mouse osteopontin VDRE binds VDR and RXR in gel shifts and that expression of this VDRE is enhanced synergistically by these receptors in the presence of 1,25-(OH)_{2}D_{3} and 9-cis retinoic acid. These workers also provided evidence that a VDRE in the human osteocalcin gene consists of two half-sites separated by 6 bp and binds a homodimer of VDR rather than a heterodimer of VDR-RXR (10). In the present work, the identified VDRE consists of two half-sites separated by 3 bp and, therefore, it seems reasonable to assume that the VDRE:nuclear-complex contains VDR and the co-activator RXR. Such an occurrence is consistent with VDR being absent in COS-1 cells while sufficient transactivated RXR exist for 1,25-(OH)_{2}D_{3} induction of transfected constructs.

Direct confirmation of a functional VDR-RXR heterodimer in the P450_{24} VDRE will require purified proteins and their attendant antibodies. Nevertheless, it is evident from this study that the rat P450_{24} gene contains a functional VDRE in its proximal promoter region. From the transient expression analysis, it can be inferred that other upstream control elements will contribute to promoter induction by 1,25(OH)_{2}D_{3} and the identity of such elements is currently being pursued.

The action of the secosteroid hormone 1,25-(OH)_{2}D_{3} to feedback regulate its own ambient concentration is an unusual control loop for physiological cytochromes P450. A major factor in this feedback loop is the transcriptional action of 1,25-(OH)_{2}D_{3} to enhance its own metabolic inactivation. This occurs through the 1,25-(OH)_{2}D_{3} induction of the P450_{24} gene and the associated increase in the hormone's side-chain oxidation. It is now clear that this action of 1,25-(OH)_{2}D_{3} is mediated in part through the upregulatory VDRE in the early promoter region of the P450_{24} gene. The documentation of additional upregulatory elements and the presence of yet-to-be-defined downregulatory elements all contribute to a multi-factorial system whereby the P450_{24} gene is controlled in response to cellular regulatory signals. Defining the interactions between the different control components is fundamental to understanding the gene-level regulation of P450_{24} expression.
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