A Downstream Intergenic Cluster of Regulatory Enhancers Contributes to the Induction of CYP24A1 Expression by 1α,25-Dihydroxyvitamin D3

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CYP24A1 expression is up-regulated by 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) via a vitamin D receptor (VDR)/retinoid X receptor (RXR) heterodimer that binds to two vitamin D response elements (VDREs) located near the proximal promoter. Interestingly, although 1,25(OH)2D3 induced VDR/RXR response elements (VDREs) located near the proximal promoter, the VDR/RXR heterodimer also localized to a cluster of at least four potential enhancers located in intergenic regions 50–69 kb downstream of the human CYP24A1 gene and 35–45 kb downstream of the mouse Cyp24a1 gene as revealed by ChIP-chip and ChIP-seq analyses. To address whether this downstream region and potential VDREs located within mediated CYP24A1 induction, we constructed recombinant wild-type and mutant bacterial artificial chromosome clones that spanned mouse and human loci and contained luciferase reporters inserted into their 3′-untranslated regions. The activity of these clones in stably transfected cells revealed that both the proximal and the putative downstream elements contributed to CYP24A1 up-regulation by 1,25(OH)2D3. Further analysis using transfected enhancer fragments led to the identification of contributing regulatory elements in several of these downstream regions. Additional studies of coregulator recruitment using ChIP-chip analysis revealed both similarities and differences between the region located proximal to and those located downstream of the promoter. Recruitment of these coregulators was likely responsible for the increase in RNA polymerase II and histone H4 acetylation, which was also observed in response to 1,25(OH)2D3 at the enhancer sites across the locus. We conclude that a more complex mechanism is responsible for the striking CYP24A1 up-regulation induced by the vitamin D hormone in target cells.

Vitamin D3 is metabolically activated through two important hydroxylations, first in the liver to 25-hydroxyvitamin D3 and then in the kidney to the active hormone 1,25-dihydroxyvitamin D3 (1,25(OH)2D3)2 (1). These conversions are mediated by several cytochrome P450-containing enzymes that include hepatic CYP2R1 and renal mitochondrial CYP27B1 (2). The latter is considered the most important enzyme associated with vitamin D3 metabolism, because it catalyzes the final step in the conversion of the parent vitamin to its bioactive form. Perhaps of equal importance is CYP24A1, a mitochondrial enzyme in target cells that functions to degrade 1,25(OH)2D3 (2). CYP24A1 facilitates a series of catabolic steps that begins with 24-hydroxylation of 1,25(OH)2D3 and ends in side-chain cleavage of a penultimate metabolic intermediate to produce calcitriol. Thus, both the endocrine synthesis of 1,25(OH)2D3 in the kidney and the degradation of this hormone at peripheral sites of action serve to maintain a homeostatic level of biologically active 1,25(OH)2D3.

The maintenance of active levels of hormone is a dynamic process responsive to a multitude of systemic signals that monitor the mineral status of the organism. 1,25(OH)2D3 production in the kidney is regulated positively by many factors, including parathyroid hormone (3) and regulated negatively by others (4), including the phosphate hormone FGF23 (5, 6). CYP27B1 is also down-regulated by 1,25(OH)2D3 itself, which exerts strong negative feedback control on CYP27B1 transcription to curb 1,25(OH)2D3 synthesis (3, 7–9). Interestingly, this negative feedback mechanism does not appear to be operable in the many extrarenal tissues that are also known to express CYP27B1, albeit at more modest levels (10). 1,25(OH)2D3 also regulates its own degradation as well, because the hormone potently induces CYP24A1 expression in all cells in which it acts (2). Because the magnitude of this induction is generally cell-specific, the regulation of CYP24A1 expression likely represents a mechanism whereby cellular response can be precisely tailored to individual cellular requirements in the face of changing circulating 1,25(OH)2D3 levels. Not surprisingly, reductions in CYP27B1 expression, enhanced synthesis of CYP24A1, or both can lead to a decrease in biologically active 1,25(OH)2D3 levels that results in altered mineral homeostasis and increased disease potential (11). In summary, the collective activity of both renal CYP27B1 and target cell CYP24A1 results in the maintenance of highly dynamic steady-state levels of biologically active 1,25(OH)2D3 in all tissues in which the hormonal ligand is active.

The cloning of Cyp24a1 in 1993 revealed Cyp24a1 as a bone fide member of a large family composed of all the cytochrome
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P450 genes (12). Although the cloning of the structural gene provided an entrée into studies of structure/function, the cloning of the chromosomal gene enabled studies focused upon understanding the underlying mechanisms important to the regulation of CYP24A1 expression by 1,25(OH)₂D₃ and other modulators. 1,25(OH)₂D₃ is known in general terms to regulate gene transcription via the vitamin D receptor (VDR), a nuclear protein that forms a heterodimer with retinoid X receptor (RXR) at specific DNA sequences termed vitamin D response elements (VDREs) and then participates directly in the recruitment of complex molecular machinery that is essential for changing the level of expression of target genes (13, 14). Key to the regulated induction of CYP24A1 by 1,25(OH)₂D₃ are two VDREs located within an enhancer positioned very near the transcriptional start site (TSS) (15, 16). The interaction of the VDR with these regulatory elements and the activities of multiple coregulatory complexes at these sites as well has been the focus of extensive analysis since their initial discoveries. Indeed, we have shown using chromatin immunoprecipitation (ChIP) assays that the VDR and its heterodimer partner bind directly to these elements and recruit coregulatory complexes both in cells in culture and in mouse tissues in vivo (17, 18). Thus, it is well established that CYP24A1 is regulated in most species through an activation event that is mediated in trans by a VDR/RXR heterodimer and its associated cofactors and facilitated in cis through the two VDREs located near the CYP24A1 proximal promoter.

The results of ChIP analysis coupled to either tiled microarray hybridization (ChIP-chip) or deep sequencing techniques (ChIP-seq) now supports the general concept that the expression of numerous genes is regulated by multiple enhancers located not only proximal to the TSS but at sites that are distal as well (19). Indeed, these sites can be located at various positions across an extended gene locus and in many cases are positioned many kilobases from the TSS. While distant in a linear sense, it is worth noting that these enhancers are frequently located in three-dimensional terms immediately adjacent to the promoters that they are known to regulate (20–22). In the current study, we used ChIP-chip and ChIP-seq approaches as well as BAC clone analysis to examine whether the regulation of CYP24A1 by 1,25(OH)₂D₃ was mediated exclusively through the two regulatory elements described above or perhaps involved additional elements. We show herein that CYP24A1 regulation in both mouse and human genomes is indeed more complex and that an extensive downstream intergenic cluster comprised of at least four separate regions is also involved. Several of these regions contain one or more VDREs that function to control the expression of CYP24A1. Our results highlight the additional complexity that is likely typical of many highly regulated genes.

EXPERIMENTAL PROCEDURES

Reagents—1,25(OH)₂D₃ was obtained from Tetrionics, Inc. (Madison, WI). Antibodies to VDR (C-20), RXR (ΔN-197), SRC-1 (M-341), NCoR (C-20), and TRAP220/MED1/DRIP205 (M-255) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). SMRT (PA1–843) antibody was purchased from Affinity Bioreagents/ThermoFisher (Rockford, IL). Anti-tetra-acetyl H4 antibody (06–866) was acquired from Upstate (Charlottesville, VA). Anti-CTCF antibody was obtained from Millipore Corp. (Billerica, MA). Anti-RNA polymerase II (pol II) antibody (8WG16) was obtained from Covance (Emeryville, CA). All quantitative real-time PCR (qPCR) reagents (Power SYBR green) were obtained from ABI (Foster City, CA). All qPCR analyses were conducted on the RealPlex 2.0 from Eppendorf AG (Hamburg, Germany). Primers (unlabeled and FAM- and VIC-labeled) were obtained from IDT (Coralville, IA), and all sequences are freely available upon request.

Cell Culture—Human colonic LS180 and mouse osteoblastic MC3T3-E1 cells were obtained from ATCC (Manassas, VA). LS180 cells were cultured in minimum Eagle’s medium supplemented with 10% non-heat-inactivated fetal bovine serum (FBS) obtained from HyClone (Logan, UT), 1% non-essential amino acids, 1% sodium pyruvate, and 1% penicillin-streptomycin from Invitrogen as previously reported. MC3T3-E1 cells were cultured in α-minimal essential medium supplemented with 10% heat-inactivated FBS obtained from HyClone.

ChIP Assay—ChIP was performed as described previously (17, 23). Briefly, MC3T3-E1 or LS180 cells were treated for the appropriate period of time with either vehicle or the indicated experimental inducer. The treated cells were washed several times with phosphate-buffered saline and then cross-linked for 15 min with 1.5% formaldehyde. After the cross-linking reaction, isolated cell extracts were sonicated to prepare chromatin fragments (average DNA size of 500–600 bp DNA as assessed by agarose gel electrophoresis) using a Fisher Model 100 Sonic Dismembrator at a power setting of 1. Pre-cleared samples were subjected to immunoprecipitation using either a control IgG antibody or the indicated experimental antibody. The precipitated DNA was then washed, cross-links were reversed, and the DNA fragments were purified using Qiagen QIAquick PCR Purification Kits (Valencia, CA). The isolated DNA (or DNA acquired prior to precipitation (input)) was subjected to qPCR using primers designed to amplify fragments of the murine or human CYP24A1 gene promoter and/or for ChIP-DNA microarray (ChIP-chip) or ChIP-deep sequencing (ChIP-seq) analyses.

ChIP-chip Analysis—Output DNA from ChIP was amplified using a ligation-mediated PCR method (24) and then labeled with Cy3 or Cy5. 6 μg of each labeled sample was combined with 2× Hybe Buffer and Hybe component A (Roche NimbleGen), denatured at 95 °C for 5 min, and then hybridized to custom Roche NimbleGen DNA microarrays overnight at 42 °C using a MAUI hybridization system (BioMicro, Salt Lake City, UT). All oligonucleotide sequences for the array were generated using Roche NimbleGen’s design standard, Tₘ-balanced 50–75 oligomers with an approximate spacing of 100–150 bp. Low complexity and repeat regions were excluded from tiling. Following hybridization, arrays were washed and scanned at 5 μm using the GenePix 4000B scanner (Axon/Molecular Devices, Sunnyvale, CA). Data were extracted using NimbleScan (version 2.4, Roche NimbleGen) and normalized using loess or bi-weight mean normalization in R. The log₂ ratios of test versus experimental data were calculated for each point, and peaks were called using CMARRT algorithms (25). Data shown are representative of at least three or more ChIP-chip
analyses performed for each experimental setting and peaks were validated by ChIP qPCR (data not shown). The results of this analysis at the genome-wide level will be reported separately.

ChIP-seq Analysis—Output ChIP DNA was analyzed commercially via deep sequencing using a SOLiD Instrument (ABI, Foster City, CA) at Beckman Coulter Genomics (formerly Agencourt Biosciences, Boston, MA). Samples were gel-purified, and all DNA segments below 2 kb were selected for further analyses. 400 ng of each ChIP sample, VDR (vehicle), and VDR (1,25(OH)₂D₃) was sonicated to 150-bp median fragment size and verified by agarose gel electrophoresis. Libraries were created using the AB SOLiD System 2 Lower Input Fragment Library Preparation protocol (4391587 Rev.C, May, 2008). Samples were verified for integrity, and 50 ng was subjected to sequencing. The resulting 35-bp sequences were mapped to the human hg18 reference genome (UCSC Genome Browser, available on-line). ABI sequence format was extended bioinformatically from 35 bp to the 150-bp median fragment digestion and converted to density files for each chromosomal position. Direct duplicate sequences were removed as artifacts of sequencing as were all adapter sequences. All remaining sequences were merged and counted every 30 bp throughout the genome and listed as density files (tag density) that are observed in Fig. 1C. Peaks of binding were determined using MACS v1.3.5 (26). The results of this analysis at the genome-wide level will be reported separately.

Plasmid Construction and Cloning—The pCH110-β-galactosidase reporter plasmid and the pcDNA-hVDR vector were previously described (23). pTK-hCYP24A1 +69, +66, +60, +50, and +41, promoter (Pro), and pTK-mCyp24a1 +42, +39, +37, and +35 were constructed by cloning the appropriate human or mouse CYP24A1 DNA fragments obtained through DNA amplification of BAC or genomic DNA into the pTK-luc vector using BamHI (or HindIII) and SalI restriction sites. Mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as per the manufacturer’s protocol and recommendations. Mutated sequences in the pTK-hCYP24A1 +66 and +50 reporter vectors map to the following genomic locations (assembly hg18): +66 VDRE1 (chr20: 52157600-14), +66 VDRE2 (chr20: 52157954-68), +50 VDRE1 (chr20: 52173578-92), and +50 VDRE2 (chr20: 52173765-79). Mutagenesis primers were designed to change the first three base pairs of one or both VDRE half-sites to the nucleotide sequence TCC. The pIRES-luciferase reporter constructs was generated using 2 µl of bacterial culture as a template for PCR amplification together with primers that flanked the recombination site. BAC DNA was prepared from the bacterial culture of a positive clone, linearized by NotI digestion, and used for generating stable cell lines (see below). The hCYP24A1 ΔVDRs construct was prepared as a modification of the parental hCYP24A1 wt using the galactokinase (galk) positive/negative selection as described by Warming et al. (30). The targeting construct was generated from the pgalk plasmid by PCR amplification of the galk open reading frame using primers that contain 50 bp of homology to the regions flanking the transcriptionally active promoter region of CYP24A1. The amplified DNA was digested with DpnI for 1 h, gel-purified, and introduced into SW106 cells harboring the parental hCYP24A1 wt construct. The recombinants were selected first on M63 minimal media plates containing galactose, leucine, biotin, and chloramphenicol. Several emerging colonies were further selected on MacConkey agar plates to remove Gal− contaminants. Gal+ colonies were selected and cultured for use in the second stage of the recombineering effort. For hCYP24A1 ΔVDRs, the mutated promoter region from pTK-hCYP24A1 ΔVDRs was employed as a replacement fragment and used for transformation. Removal of the galk cassette resulted in the seamless mutation of individual VDREs. The recombinants were selected on M63 minimal media plates containing 2-deoxygalactose, leucine, biotin, and chloramphenicol. Colonies were analyzed by restriction enzyme digestion and pulse-field gel electrophoresis. A colony with a digestion pattern matching the parent construct was confirmed by PCR analysis and sequencing. DNA for the hCYP24A1 ΔVDRs construct was prepared, linearized through NotI digestion, and then used to generate stable cell lines (see below).

Stable Cell Line Preparation—Stable cell lines were prepared as previously described (29) and by using a Nucleofector electroporator (Lonza, Walkersville, MD). For chemical transfections, MC3T3-E1 cells were seeded into 6-well plates at a concentration of 1.5 × 10⁵ cells/well in α-minimal essential medium containing 10% FBS. Cells were transfected 24 h later with 1–4 µg of BAC-luciferase reporter vector in serum and antibiotic-free medium using Lipofectamine. After transfection, the cells were cultured in medium supplemented with 10% FBS for 24 h. For Nucleofector transfection, cells were grown to 80% confluency in 10-cm dishes and trypsinized, and 1–2 × 10⁶ cells were aliquoted per transfection. 1–4 µg of BAC-luciferase reporter vector was transfected using solution R in program T-20 and plated in 6-cm plates overnight. For both methods,
cells were then collected by trypsinization, re-plated into two 10-cm dishes, and subjected to positive selection 24 h later using G418 (200 μg/ml). Colonies emerging from the three plates after 10–14 days were harvested, pooled, and examined.

**Transient Transfection Luciferase Assay**—MC3T3-E1 cells were seeded into 24-well plates in α-minimal essential medium containing 10% FBS at a concentration of 5.0 × 10^4 cells/well and transfected 24 h later with Lipofectamine PLUS (Invitrogen, Carlsbad, CA) in serum and antibiotic-free medium. Individual wells were cotransfected with 250 ng of a luciferase reporter vector, 50 ng of pCH110-βgal, and 50 ng of pcDNA-hVDR. After transfection, the cells were cultured in medium supplemented with 20% FBS with or without 1,25(OH)₂D₃. Cells were harvested 24 h after treatment, and the lysates were assayed for luciferase and β-galactosidase activities as previously described (31). Luciferase activity was normalized to β-galactosidase activity in all cases.

**Chromosome Conformation Capture Assay**—The chromosome conformation capture (3C) assay was performed as previously reported with few modifications (22, 32). Briefly, LS180 cells were grown to confluency and treated with 100 μM 1,25(OH)₂D₃ for 0, 0.5, 1 or 3 h prior to 3C analysis. Cells were cross-linked with 1% formaldehyde for 10 min and quenched with 0.125 M glycine. Nuclei were collected and Dounce-homogenized with 20 strokes on ice. The nuclei suspension was divided into two tubes per time point with 5 × 10⁵ cells per sample, washed, and resuspended in NEBuffer 3 (1×, New England Biolabs, Ipswich, MA). SDS was added to 0.1% final concentration, nuclei were incubated at 65 °C for 10 min, and Triton X-100 was added to 1% final concentration. Digestion was performed with 400 units of BglII (New England Biolabs) overnight at 37 °C. Enzyme was inactivated by addition of 2% final concentration SDS and further incubated at 65 °C for 10 min, and Tri-reagent was added. The samples were incubated overnight at 65 °C. The DNA was purified through two rounds of phenol:chloroform:isoamyl alcohol (25:24:1) extractions and a final ethanol precipitation. Final DNA was redissolved in TE buffer (10 mM Tris-Cl, pH 8.1, 1 mM EDTA) and treated with 1 μg of RNase A (Promega, Madison, WI). As PCR product size control, the BAC clone RP11-705A3 was digested with BglII overnight, then ligated and purified. This contains all possible PCR products from a BglII digestion across the CYP24A1 locus. PCR was performed using Go-Taq Flexi Green Master Mix (Promega) for 32 cycles. Primers were designed on the forward genomic strand in proximity to the BglII cut site. Analysis was performed from each BglII segment located between the distal elements and proximal promoter (data not shown); a subset of these primers is displayed in Fig. 4.

**RESULTS**

**ChIP-chip and ChIP-seq Analyses Define the CYP24A1 Locus and Reveal a Downstream Intergenic Enhancer Cluster Potentially Important to CYP24A1 Gene Regulation**—1,25(OH)₂D₃ is known to regulate the expression of the CYP24A1 gene through two regulatory elements, which are located immediately upstream of the transcriptional start site (15, 16). To explore the properties of these regulatory elements and to determine whether additional regions might also be involved in CYP24A1 expression, we conducted a ChIP-chip analysis across the CYP24A1 gene locus covering both the regions surrounding the gene and the transcription unit itself, a span of ~100 kb. In this analysis, we treated human colonic LS180 cells for 3 h with either vehicle or 1,25(OH)₂D₃, subjected the lysates to ChIP-chip analysis, and measured the levels of VDR and RXR across the gene locus under both conditions at a resolution of 100 bp. Fig. 1 (A and B) provide the data tracks from these analyses that represent the log₂ ratios of fluorescence obtained from vehicle-treated and input samples precipitated with antibody to VDR (VDR, Veh/Input) (basal VDR) (Fig. 1A) or RXR (RXR, Veh/Input) (basal RXR) (Fig. 1B) and from 1,25(OH)₂D₃-treated and input samples precipitated with antibody to VDR (VDR, 1,25/Input) (activated VDR) (Fig. 1B) or RXR (RXR, 1,25/Input) (activated RXR) (Fig. 1B). As can be seen, although neither VDR nor RXR binding was evident at the CYP24A1 proximal promoter sites in the absence of 1,25(OH)₂D₃, both were strongly up-regulated in the presence of the ligand. This binding activity corresponded to the location of the two previously identified VDREs, although the DNA microarray analysis was unable to resolve the two distinct sites because of their proximity to each other (100 bp). Unexpectedly, as seen in Fig. 1 (A and B), robust VDR and RXR binding was also observed at a cluster of intergenic sites located +41, +50, +60, +66, and +69 kb downstream of the gene. Interestingly, while VDR and RXR binding was largely dependent on the presence of 1,25(OH)₂D₃ at most of these sites, a significant level of VDR and RXR was observed at the +50 and perhaps the +41 kb sites in the absence of 1,25(OH)₂D₃ and simply augmented in its presence. Direct ChIP analysis using specific sets of primers and qPCR confirmed the presence of both VDR and RXR at each of these sites in multiple independent experiments (data not shown).

We also explored the CYP24A1 locus for VDR binding activity using ChIP-seq analysis, an approach capable of higher resolution. Similar ChIP samples derived from either vehicle or 1,25(OH)₂D₃-treated LS180 cells were prepared and subjected to massively parallel DNA sequencing methods as described under “Experimental Procedures.” As is evident in Fig. 1C, although mappable tags for the VDR were largely absent at the VDRE-containing CYP24A1 proximal promoter following vehicle treatment, these tags were strongly induced in this region in the presence of 1,25(OH)₂D₃. Importantly, ChIP-seq analysis also confirmed that VDR was present at each of the intergenic regions located downstream of the CYP24A1 locus as well. Indeed, the binding activity of the VDR at the +50 and +66-kb regions was highly robust and similar to that observed using the ChIP-chip analysis. These data together with the ChIP-chip analyses of both RXR and the VDR suggest the possibility that, in addition to the well established proximal enhancer, a downstream cluster of regulatory sites might also participate in 1,25(OH)₂D₃-induced expression of CYP24A1.

**BAC Clone Analysis Confirms the Regulatory Contribution of the Downstream Enhancer Cluster to Human CYP24A1 Expression**—The location of the potential enhancer cluster downstream of the CYP24A1 transcription unit suggests but does not prove that it is involved in CYP24A1 regulation by
CYP24A1 Distal Regulation by 1,25(OH)\textsubscript{2}D\textsubscript{3}

1,25(OH)\textsubscript{2}D\textsubscript{3}. To address this issue, we obtained a BAC clone containing the human CYP24A1 gene locus, inserted a neomycin-selectable cassette containing an IRES-luciferase reporter into the gene’s 3’-UTR, and evaluated the activity following stable transfection into host cells as described under “Experimental Procedures.” We also examined the activity of a series of mutant BAC clones as well. Collections of at least 30 cell clones from at least three independent transfections per construct were evaluated in triplicate to avoid integration site bias. As can be seen in Fig. 2, The recombined wild-type CYP24A1 BAC clone exhibited both a modest basal activity and, as seen in Fig. 2, a strong inducible response to 1,25(OH)\textsubscript{2}D\textsubscript{3} following treatment for 24 h. Importantly, however, the induction of mutations into the two VDREs located at the proximal promoter reduced but did not eliminate sensitivity to 1,25(OH)\textsubscript{2}D\textsubscript{3}, suggesting that a segment of the gene capable of mediating response to 1,25(OH)\textsubscript{2}D\textsubscript{3} was still present. We then introduced a gross deletion at the CYP24A1 locus, which effectively removed the bulk of the downstream intergenic sites, prepared stable cells, and evaluated the activity of this BAC clone as well. As can be seen, this mutation exhibited a similarly attenuated response to 1,25(OH)\textsubscript{2}D\textsubscript{3}, demonstrating that the downstream region was directly involved in the regulation of CYP24A1. Stimulation by 1,25(OH)\textsubscript{2}D\textsubscript{3} was completely eliminated, however, when both genetic changes were introduced simultaneously into the BAC clone. We conclude from these studies that the intergenic cluster located downstream of the human CYP24A1 gene likely plays a direct and contributory role in 1,25(OH)\textsubscript{2}D\textsubscript{3}-mediated induction of CYP24A1.

The Downstream Regulatory Cluster Is Located near the CYP24A1 Promoter—The 3C assay analysis suggests that many transcription units and the regions that control their expression are often conserved across genomes. Thus, we explored the possibility that the downstream regulatory cluster observed in the human CYP24A1 locus might also be present in and control 1,25(OH)\textsubscript{2}D\textsubscript{3}-mediated induction of the mouse Cyp24a1 gene. We treated mouse MC3T3-E1 osteoblastic cells with either vehicle or 1,25(OH)\textsubscript{2}D\textsubscript{3} and then subjected the cross-linked lysates to ChIP-chip analysis using the antibody to VDR. As can be seen in Fig. 3 (A and B), although VDR binding activity was not apparent across the Cyp24a1 locus in the absence of 1,25(OH)\textsubscript{2}D\textsubscript{3}, its binding activity was strongly induced by the hormone not only at the proximal promoter, as expected, but also at four independent regions located downstream of the Cyp24a1 tran-
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**A.** hCYP24A1 pIRES constructs

| WT | Pro ∆VDREs | CYP24A1 | ∆+50kb | ∆+66kb | ∆+71-37kb | ∆VDREs ∆+71-37kb |
|----|-------------|---------|--------|--------|------------|-------------------|
| BAC51 | IRES | PFD4 | +66 | +50 | +66 | IRES |
| BAC51 | IRES | PFD4 | +66 | +50 | +66 | IRES |
| BAC51 | IRES | PFD4 | +66 | +50 | +66 | IRES |

**B.**

![Diagram](image)

**FIGURE 2.** BAC clone reporter analysis links transcriptional activity of distal segments to CYP24A1 gene. A, schematic diagrams of BAC clone reporter vectors created using recombinant technologies around the CYP24A1 gene locus. Each BAC clone encompasses 190 kb of genomic sequence, including the entire CYP24A1 gene as well as 43 kb upstream (past PFDN4 TSS) and 128 kb downstream (into BAC51). A series of constructs were created to accompany the wild-type (wt) construct that contains a luciferase reporter gene along with a mammalian selectable marker (IRES cassette) inserted into the 3′-UTR of the CYP24A1 gene. Known promoter VDREs were mutated (Pro ∆VDREs), the +50-kb region alone was deleted (∆+50kb), the +66-kb region alone was deleted (∆+66kb), and all downstream regions with VDR activity were deleted (∆+71–37kb) as well as a final double deletion of the promoter and downstream elements (∆VDREs ∆+71–37kb). B, these constructs (A) were stably integrated into MC3T3-E1 cells and assayed for their luciferase activities following treatment with ethanol vehicle (V) or increasing concentrations of 1,25(OH)_{2}D_{3} (10^{-10} to 10^{-8} m). Each point represents the relative light unit (RLU) average normalized to total protein ± S.E. for a triplicate set of assays. *, p < 0.05 as compared with vehicle treatment. The data are representative of at least three independent collections of stable cells that were evaluated for each construct.

with either vehicle or 1,25(OH)_{2}D_{3} and subjected the resulting cell lysates to 3C analysis. The approach is illustrated in Fig. 4A. The results in Fig. 4B reveal that only primers capable of identifying DNA ligase-dependent segments of DNA containing both the TSS and either the segment adjacent to the TSS or to the +50, +60, +66, and +69 segments yielded detectable amplification products of the correct size. Control segments immediately downstream of either the CYP24A1 gene or of the +69 kb segment were not detected. Surprisingly, proximity of the downstream cluster to the CYP24A1 promoter was independent of the presence of 1,25(OH)_{2}D_{3}. We conclude from this experiment that, although located far from the CYP24A1 promoter in linear terms, the downstream intergenic cluster of enhancers is positioned very near the CYP24A1 promoter in living cells, as illustrated in Fig. 4C, and is thus capable of functioning as a traditional enhancer in mediating regulation by 1,25(OH)_{2}D_{3}. Furthermore, analysis with BtgI allowed a finer digestion and resolution at the downstream elements. Through successive assays, each region was found to interact with each other in a ligand independent fashion (data not shown).

Mapping Regulatory VDREs at the Downstream Enhancer Cluster in the CYP24A1 Gene Locus—The presence of the VDR and its RXR partner at multiple sites downstream of the mouse and human CYP24A1 gene suggests the likely presence of specific regulatory elements or VDREs. To determine functionally whether such elements existed, we cloned DNA fragments of 400–1500 bp composed of each of the putative regions from both the mouse and human genes into a viral promoter-containing plasmid as previously described (38, 39) and assessed their capacity to mediate response to 1,25(OH)_{2}D_{3} following transient transfection into MC3T3-E1 cells. The results in Fig. 5 reveal that two of the putative regulatory regions derived from the human gene (+50 and +66) (Fig. 5A), and all four of the regions identified in the mouse gene (+35, +37, +39, and +41) (Fig. 5B) mediated a dose-dependent response to 1,25(OH)_{2}D_{3}. Control segments derived from other regions of both the mouse and human genes, which were negative for VDR/RXR binding, were uniformly unresponsive (data not shown). The absence of transcriptional response to hormone in the +60-and +69-kb regions from the human gene is surprising but not unexpected. This result suggests the possibility that either the VDREs located within are incapable of functioning independently or that their activities are inhibited by adjacent negative regulators. We mapped the functional VDREs located in the +50- and +66-kb regions of the human gene by using in silico analysis to identify putative candidates and transient transfection studies linked to VDRE mutagenesis to identify active regulatory elements. As can be seen in Fig. 5C, mutation of the two putative VDREs located in the +50-kb region each fully compromised enhancer response to 1,25(OH)_{2}D_{3}, whereas two of the putative VDREs located in the +66-kb region significantly reduced but did not eliminate the 1,25(OH)_{2}D_{3} response. These studies identify response elements for the VDR/RXR heterodimer directly in at least two of the downstream regulatory regions in the human gene and support the likelihood that similar elements already identified through in silico analyses are functional in the downstream region of the mouse gene as well. Additional studies will be necessary to define these activities in the four regulatory regions in the mouse gene.

Assessing Cofactor Recruitment across the CYP24A1—Having established the presence of a novel downstream region essential to 1,25(OH)_{2}D_{3} -mediated induction of the mouse and human CYP24A1 genes, we next examined the ability of the VDR/RXR heterodimer to recruit coregulators to these control regions. Cells were treated with either vehicle or 1,25(OH)_{2}D_{3} for 3 h and then subjected to ChIP-chip analysis as described earlier using antibodies to a series of coregulators known to interact with the VDR, including MED1, SRC-1, NCoR, and SMRT (40). As can be seen in Fig. 6, 1,25(OH)_{2}D_{3} induced MED1, SRC-1, and SMRT binding at the CYP24A1 locus, both at the promoter for this gene and at the individual enhancers located downstream at +50, +60, and +66 kb as well. Each of
these binding activities was confirmed using direct ChIP coupled to qPCR analysis (data not shown). Interestingly, residual levels of both MED1 and SRC-1 were also present at the \(50-kb\) region in the absence of \(1,25(OH)_2D_3\), a feature reminiscent of the ligand-independent binding observed for both VDR and RXR at this site. SMRT was also bound at the \(CYP24A1\) promoter in the absence of ligand as well. Surprisingly, NCoR binding was independent of \(1,25(OH)_2D_3\) treatment and generally limited to the \(+50-kb\) region. Although both SMRT and NCoR were originally identified as nuclear receptor corepressors (41–44), more recent studies suggest that these transcription factors are capable of mediating activation as well (45). Individual small interference RNA knockdown studies were performed for each cofactor shown in Fig. 6; however only VDR, SRC-1, and MED1 knockdowns were able to reduce the level of \(CYP24A1\) transcript significantly as we previously reported (data not shown) (18, 46). Interestingly, NCoR and SMRT knockdown assays were unable to reduce the levels of \(CYP24A1\) (data not shown). We conclude from these experiments that \(1,25(OH)_2D_3\), likely via the VDR/RXR heterodimer, stimulates the recruit-
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**A.**

![Diagram of genomic locus](image)

**B.**

![Diagram of time course](image)

**C.**

![Diagram of interaction between loci](image)

**FIGURE 4.** 3C assay places the distal binding sites proximal to the CYP24A1 TSS. A, schematic of the CYP24A1 genomic locus. Regions of VDR binding (TSS, +50, +60, +66, and +69) as well as a control region are indicated. BglII restriction sites within the locus are depicted by vertical red bars. DNA fragments generated by BglII digestion and examined are indicated by horizontal red bars. The location of the primers used are indicated by caretts. A region utilized as a control (Con) is also depicted. B, LS180 cells were treated for 0, 0.5, 1, or 3 h with 10^{-7} M 1,25(OH)_{2}D_{3} and subjected to 3C analysis using BglII as indicated under “Experimental Procedures.” PCR was performed on cross-linked and restricted DNA using the specific TSS primer shown and each individual primer indicated to the left of the PCR data. The positive control primer for ligation dependence is designated Adj.TSS. A BAC control (“B”) was included to show the proper PCR product size. W, water negative control for PCR. The BAC was digested and ligated to form all possible combinations. Negative controls include Con and primers at +104, -14, and -35 kb (not depicted in schematic in A (off-scale)). C, schematic interaction model of +50 through +69 kb distal elements in proximity to the CYP24A1 TSS.

Mention of a series of coregulators that play a role in the ligand’s transcriptional modulating action at the CYP24A1 gene. The recruitment of these factors to the downstream enhancers provides additional support for important regulatory activity at these sites and a role for these enhancers in the regulation of CYP24A1 by 1,25(OH)_{2}D_{3}. Although some cofactor binding differences were apparent across the CYP24A1 I locus, it is clear that neither the promoter nor the downstream enhancers exhibit properties at this level of analysis that are strikingly different from one another.

1,25(OH)_{2}D_{3} Induces the Level of Histone H4 Acetylation (H4ac) across and Stimulates the Recruitment of RNA pol II at the CYP24A1 Locus—Because 1,25(OH)_{2}D_{3} strongly induces CYP24A1 expression in LS180 cells, we assessed in a final set of experiments whether 1,25(OH)_{2}D_{3}-induced VDR/RXR DNA binding and cofactor recruitment were associated with changes at the CYP24A1 locus that could reflect increased transcription of the gene itself. LS180 cells were treated with either vehicle or 1,25(OH)_{2}D_{3} for 3 h and then subjected to ChIP-chip analysis using antibodies to either tetra-acetylated histone H4 or RNA pol II. As can be seen in Fig. 7 (A and B), while basal levels of both H4ac and RNA pol II were undetectable in the absence of 1,25(OH)_{2}D_{3}, treatment with the hormone strongly induced an up-regulation of H4ac across the CYP24A1 locus and a substantial increase in RNA pol II density at the CYP24A1 promoter and across the CYP24A1 transcription unit itself. Interestingly, increased RNA pol II levels were also apparent at several of the downstream intergenic enhancers as well.

Most profound, however, was the dramatic 1,25(OH)_{2}D_{3}-induced increase in H4ac which, although spanning the entire region from +41 to +69, was centered generally at each of the individual downstream enhancers. Interestingly, as seen in Fig. 7C, virtually all of this inducible activity was constrained between two regions located upstream of the CYP24A1 gene locus at 52.140 Mb and downstream at 52.250 Mb that via a ChIP-chip analysis bound significant levels of CCCTC-binding protein (CTCF). While the CTCF protein manifests several distinct activities (47, 48), these CTCF binding regions appear to function as insulators to limit regulatory activity to the CYP24A1 locus; neither the neighboring upstream nor downstream genes were regulated by 1,25(OH)_{2}D_{3} (data not shown). We hypothesize that VDR/RXR binding and cofactor recruitment by this receptor heterodimer at the CYP24A1 promoter/transcription unit and at the enhancer cluster located downstream of the gene results in potential transcription-regulating activities.

**DISCUSSION**

Two closely spaced regulatory elements located proximal to the CYP24A1 gene promoter are known to mediate the ability of 1,25(OH)_{2}D_{3} to induce the expression of CYP24A1 mRNA (15, 16). In the studies described in this report, we show that the regulation of this 1,25(OH)_{2}D_{3}-sensitive gene is much more complex and involves a cluster of distal enhancers located significantly downstream of the final CYP24A1 exon. This region was identified using both ChIP-chip and ChIP-seq analyses of binding sites for VDR and its partner RXR and confirmed using stably transfected recombinant BAC clones that comprised the entire CYP24A1 gene locus. Several of the isolated fragments containing these enhancers mediated independent response to 1,25(OH)_{2}D_{3} in transient transfection assays and thus enabled the functional identification of specific VDREs. Additional studies demonstrated that both this downstream region as well as the proximal promoter itself served as 1,25(OH)_{2}D_{3}-initiated recruitment centers for several coregulators as well as for RNA pol II and were extensively modified through H4ac. These studies reinforce the emerging concept that the expression of many, if not most, highly regulated target genes is controlled by multiple enhancers whose locations are often remote from their promoter (19, 49).

The discovery through unbiased ChIP-chip and ChIP-seq techniques that regulatory enhancers are frequently located many kilobases from their target genes and often upstream,
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Although only two of the four enhancer regions investigated in the human gene were independently active in transient transfections in mediating 1,25(OH)₂D₃ response, all four of the enhancers examined in the mouse gene mediated active hormone induction. These data provide additional support for the enhancer nature of the intergenic regions we have identified in the two species. Based upon the activity observed in the two human enhancers, we mapped the underlying regulatory elements located within. This approach, together with 3C analysis, wherein we demonstrated that this downstream region was likely located in three-dimensional terms near the proximal promoter, provide strong evidence that we have identified a novel region within the CYP24A1 gene locus that contributes to the gene’s induction by 1,25(OH)₂D₃. The lack of effect of the hormone on the cluster’s proximity to the promoter suggests that this three-dimensional relationship may represent a structural feature of the CYP24A1 gene locus.

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context of an isolated gene segment, perhaps because they contain negative regulatory components that inhibit inducibility (50, 51). Restricting the size of an otherwise inactive fragment can, nevertheless, lead to the recovery of an inducible response. Despite this, we conclude that at least two of the enhancers identified in the human gene contribute directly to the induction of CYP24A1 mediated by 1,25(OH)2D3. Additional studies will be required to map the VDREs located in the mouse enhancers.

The authenticity of the downstream enhancers is further bolstered by the ability of 1,25(OH)2D3 to induce the recruitment of a series of coregulators to these regions. Although this recruitment was similar at both the promoter and across the downstream regions, there were some differences noted, primarily involving the corepressor NCoR. Thus, although MED1, SRC-1, and SMRT recruitment upon 1,25(OH)2D3 induction was similar across most of the regulatory regions and at the promoter, NCoR was present only at the +50-kb enhancer and was unaffected by the presence of 1,25(OH)2D3. Interestingly, while the knockdown of both SRC-1 and MED1 as

**FIGURE 6.** Coregulatory molecules are present and/or recruited to both the promoter and the downstream regions of CYP24A1. LS180 cells were treated with ethanol vehicle (Veh) or 10^{-7} M 1,25(OH)2D3 (1,25) for 3 h and subjected to ChIP assay using antibodies to SRC-1, MED1, NCoR, and SMRT. Samples were further analyzed by microarray (ChIP-chip), and the data are displayed as the log2 ratios of the fluorescence obtained following cohybridization of DNA from samples immunoprecipitated under both vehicle versus input (Veh/Input) and 1,25(OH)2D3 versus input (1,25/Input) conditions. Relevant regions are shown. The data are depicted and analyzed as in Fig. 2. These data are representative of at least three similar ChIP-chip analyses and verified through direct qPCR-based ChIP analyses.

**FIGURE 7.** The CYP24A1 gene locus displays a broad histone H4ac and RNA pol II recruitment patterns. LS180 cells were treated with ethanol vehicle or 10^{-7} M 1,25(OH)2D3 for 3 h and then subjected to ChIP-chip analysis using antibodies to tetra-acetylated histone H4 and RNA pol II. Samples were further analyzed by microarrays (ChIP-chip), and the data are displayed as the log2 ratios of fluorescence obtained following cohybridization of samples under both vehicle versus input (Veh/Input) and 1,25(OH)2D3 versus input (1,25/Input) conditions. Gene landmarks and data evaluation are displayed as in Fig. 2. These data are representative of at least three similar ChIP-chip analyses and verified through direct qPCR-based ChIP analyses.
well as the VDR compromised the induction of CYP24A1 mRNA by 1,25(OH)₂D₃ (46), no effect was observed following a similar suppression of the expression of either NCoR or SMRT (data not shown). The roles of these two coregulators remains unclear at present, although it is possible that the protein complexes they nucleate may be involved in maintaining the gene in a silenced state in the absence of 1,25(OH)₂D₃. Residual activity of all of these coregulators at the +50-kb site correlated with the significant VDR/RXR binding that was observed in the absence of 1,25(OH)₂D₃. Regardless of these questions, the ability of 1,25(OH)₂D₃, presumably via the VDR and its partner RXR, to induce the recruitment of coregulators strengthens the argument that these downstream enhancers play a significant role in the up-regulation of CYP24A1 by 1,25(OH)₂D₃.

ChIP-chip analysis revealed that, in addition to VDR/RXR binding and the recruitment of coregulators, 1,25(OH)₂D₃ also induced a striking increase in H4ac, both at the CYP24A1 promoter and across the entire cluster of downstream enhancers. Although the strongest activities in the downstream region were centered on the individual enhancers, increased H4ac was evident across the entire cluster. It is possible that the acetyltransferases activity of SRC-1 underlies the increase in this epi-genetic modification (52), although additional ChIP-chip assays have also shown that both the acetyltransferases p300 and CBP were recruited to these regions as well (data not shown). Because histone acetylation is generally associated with an open chromatin state (53), we suggest the possibility that this downstream region represents a unique chromatin domain whose overall structure undergoes significant change in response to 1,25(OH)₂D₃. How this domain directly influences the expression of CYP24A1 in response to 1,25(OH)₂D₃, however, remains unclear. As expected, 1,25(OH)₂D₃ also induced the recruitment of RNA pol II to the CYP24A1 gene locus as well. The up-regulation of RNA pol II density at the promoter and particularly across the transcription unit itself likely reflects the increased transcription of the CYP24A1 gene that is induced by 1,25(OH)₂D₃ and measured at the RNA level. Surprisingly, a small increase in RNA pol II density was also observed at several of the downstream enhancers, including those at +41, +50, and +60 kb. This observation suggests the possibility that these regions may function as recruitment centers for RNA pol II or that they may direct the transcription of unannotated, non-coding RNA (54), perhaps of a regulatory nature (55). Indeed, we have observed the induction of transcription across this region in response to 1,25(OH)₂D₃ using double-stranded cDNA and our custom tiled microarrays (data not shown). Regardless of the role of these downstream regions, the ability of 1,25(OH)₂D₃ to induce both VDR/RXR binding and the recruitment of coregulators and to initiate changes in H4ac and RNA pol II activity all support the idea that these regions contribute to CYP24A1 regulation.

In conclusion, we have shown that the induction of CYP24A1 gene expression by 1,25(OH)₂D₃ is mediated by not only regulatory elements at the proximal promoter, as defined previously, but also by a newly defined cluster of at least four or more additional enhancers located significantly downstream of both the mouse and human genes. The discovery of this novel regulatory region in the CYP24A1 gene highlights the power of the unbiased ChIP-chip/ChIP-seq approach to gene analysis and suggests that the emerging concept that genes are frequently regulated by multiple distal elements is also applicable to CYP24A1, a prototypical vitamin D-responsive target gene. It will be interesting to explore this downstream region in the future for single nucleotide polymorphisms associated with altered levels of CYP24A1 gene expression.

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