Characterizing Early Events Associated with the Activation of Target Genes by 1,25-Dihydroxyvitamin D₃ in Mouse Kidney and Intestine in Vivo*S

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In this report, we explore the interaction of the vitamin D receptor (VDR) at regulatory sites within both the Cyp24a1 and the Trpv6 genes using chromatin immunoprecipitation techniques in a mouse model in vivo. We show that exogenous 1,25(OH)₂D₃ induces rapid VDR and RXR (retinoid X receptor) binding to the Cyp24a1 gene in both the kidney and the intestine and to the Trpv6 gene in the intestine. Separate studies of Trpv6 in vitro suggest that VDR binding occurs directly to VDR response elements located −2 and −4 kb upstream of the TSS. VDR binding is dose-dependent, demonstrating EC₅₀ values that are comparable with those for the induction of both Cyp24a1 and Trpv6 mRNA. Importantly, interaction of the VDR with these targets results in rapid changes in histone 4 acetylation as well as the recruitment of RNA polymerase II. The presence of both VDR and RNA polymerase II at these sites declines between 3–6 h, whereas the changes observed in acetylation decrease more slowly. Finally, we show that whereas mediator protein 1 is recruited to the Cyp24a1 promoter in the intestine, this coactivator is apparently not required for Trpv6 activation. These studies provide the first evidence for 1,25(OH)₂D₃-induced VDR interaction at key target genes in vivo, revealing the consequences of that interaction on the Cyp24a1 and Trpv6 genes.

Two sequential hydroxylations of vitamin D₃ in the liver and kidney lead to the formation of 1,25(OH)₂D₃, a steroid hormone with the hierarchical function of controlling mineral homeostasis in higher organisms (1–3). Like other hormones of this class, the biological effects of 1,25(OH)₂D₃ are achieved through the regulation of gene expression and mediated by the vitamin D receptor (VDR), a latent transcription factor that when activated by 1,25(OH)₂D₃ binds DNA, recruits coregulators, and modulates transcriptional output. Numerous VDR target genes have been identified and regulatory elements (VDRES) described (4–8). Cyp24a1, a gene in which the catabolic enzyme product controls intracellular levels of hormonal 1,25(OH)₂D₃, represents a particularly well characterized VDR target. It is induced in virtually all vitamin D₃-sensitive tissues via two VDRES located immediately proximal to the transcriptional start site (TSS) (6). This information, coupled with additional studies on the dynamics of Cyp24a1 activation by 1,25(OH)₂D₃ at the molecular level, makes it a particularly attractive candidate for further molecular analysis in vivo (9).

Experimentation over several decades has firmly established a role for the soluble calcium-binding proteins calbindins D9K and D28K, the basolateral calcium ATPase PMCA1b, and the sodium calcium exchanger NCX1 in vitamin D₃-regulated calcium uptake across the intestinal and renal epithelia (10–12). More recently, however, two additional proteins have also been discovered, transient receptor potential vanilloid (TRPV) type 5 (TRPV5) and type 6 (TRPV6) (13–15). The central role and importance of these ion channels in mediating calcium uptake at the apical surface of epithelial cells has resulted in their being described as the “gatekeepers” of transepithelial calcium transport (16). As with the calbindins and the basolateral transporters, both the TRPV5 and TRPV6 genes are also prime targets of 1,25(OH)₂D₃ action (17–21). Indeed, it is now widely believed that the regulatory activity of 1,25(OH)₂D₃ at these latter genes may be central to the ability of the hormone to orchestrate the multiple events associated with vertebrate calcium homeostasis.

Trpv6 is the most striking calcium-regulating ion channel target of 1,25(OH)₂D₃ action, undergoing a substantial down-regulation in the intestine during the course of vitamin D depletion or in the absence of a functional VDR and a dramatic up-regulation in response to supplemental 1,25(OH)₂D₃ (14, 22–24). Indeed, our most recent studies in vitro have shown that the human TRPV6 gene is induced in human colon cancer cell lines via at least five individual VDRES, each located within the first 6 kilobases of the TSS (23). This interaction is accompanied by co-localization of retinoid X receptor (RXR), the recruitment of coregulators such as steroid receptor coregulator-1 (SRC-1), and the induction of RNA polymerase II (RNA pol II). These studies provide unequivocal support for the idea that Trpv6 is a direct target of vitamin D action and is involved in mediating biological actions of 1,25(OH)₂D₃.

In the present study, we explored the molecular events asso-
associated with the activation of renal Cyp24a1 and both intestinal Cyp24a1 and Trpv6 by 1,25(OH)₂D₃ in the mouse in vivo. Using chromatin immunoprecipitation (ChIP), we show that 1,25(OH)₂D₃ rapidly induces localization of both VDR and RXR at these gene targets in a time- and dose-dependent manner. We also show that VDR localization at these sites leads to broad histone H4 (H4) acetylation and rapid recruitment of RNA pol II. Many of these early events associated with Cyp24a1 and Trpv6 up-regulation are similar. Surprisingly, unlike Cyp24a1, Trpv6 activation by 1,25(OH)₂D₃ does not require recruitment of MED1 (DRIP205).

**EXPERIMENTAL PROCEDURES**

**Reagents**—1,25(OH)₂D₃ was obtained from Solvay (da Weesp, The Netherlands). Minimum Eagle’s medium was purchased from Cellgro (Herndon, VA). Oligonucleotide primers were obtained from IDT (Coralville, IA). Anti-VDR (C-20, sc-1008), RXR (DN-197, sc-774), and MED1 (DRIP205/TRAP220) (M-255, sc-8998) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-acetyl lysine H4 (tetra, 06-866) antibody was obtained from Upstate (Charlottesville, VA). Anti-RNA pol II (8WG16) was obtained from Covance Research Products (Dover, PA). Lipofectamine Plus was obtained from Invitrogen.

**Plasmids**—The pCH110-β-gal β-galactosidase reporter plasmid was described previously (25). Construction of hTrpv6 (−7Kb/+160) was also documented earlier (23). All additional reporter plasmids were prepared by cloning Trpv6 DNA fragments obtained through amplification of mouse genomic or BAC clone DNA into the pGL3-basic vector as indicated. All reporter plasmid numbering begins with the most 5’ nucleotide found in the mRNA transcript (225 base pairs from the start site of translation based on the University of California at Santa Cruz mouse genome build (February 2006)). Mutagenesis was performed using the QuikChange mutagenesis kit from Stratagene (San Diego, CA). All inserts were sequenced for verification.

**Recombineering**—Methods have been described previously (26). Additional information regarding this methodology can be found online (recombineering.ncifcrf.gov). Briefly, primers were designed to amplify 300–500-bp fragments located 5’ and 3’ of the desired region of the mouse Trpv6 promoter. Amplified fragments were cloned into pG3L3-basic (Promega, Madison, WI) to create “retrieval vectors.” Bacterial Artificial Chromosome clone RP24-368G11 (CHORI) was authenticated via sequence and digestion analyses and then electroporated into EL350 Escherichia coli (26–28) using a MicroPulser Electroporator (Bio-Rad). BAC-containing EL350 were grown overnight at 32 °C, induced at 42 °C for 15 min, and then electroporated with linearized retrieval vector. Retrieved plasmids were digested and sequenced for verification.

**Cell Culture**—LS180 cells were cultured in minimum Eagle’s medium supplemented with 10% non-heat-inactivated fetal bovine serum, 1% non-essential amino acids, 1% sodium pyruvate, and 1% penicillin-streptomycin from Invitrogen.

**Transfection Analysis**—LS180 cells were seeded into 24-well plates by dilution (single cell suspensions are difficult to obtain) and transfected using Lipofectamine Plus as described by the manufacturer. Individual wells received 300 ng of DNA comprising 250 ng of specific TRPV6 promoter reporter plasmid and 50 ng of pCH110-β-gal. After transfection, the cells were cultured in medium supplemented with 10% fetal bovine serum with or without 1,25(OH)₂D₃. Cells were harvested 24 h after stimulation and the lysates assayed for luciferase and β-galactosidase activities as described (25). Luciferase activity was normalized in all cases using β-galactosidase activity.

**RNA Isolation and Analysis**—C57BL6 wild-type female mice, 8–9 weeks of age, were fed diet TD.06198 for 7 days (Harlan Teklad, Madison, WI). This diet is vitamin D-deficient and contains 0.8% Sr²⁺, 0.42% Phosphorus, and 0.5% Ca²⁺. Its application leads to modest reductions in serum 1,25(OH)₂D₃ and serum Ca²⁺ levels (data not shown). Mice were treated by intraperitoneal injection with either ethanol or doses of an increasing concentration of 1,25(OH)₂D₃ in propylene glycol (Gallipot, St. Paul, MN). Animals were anesthetized with ether, and 2 cm of the proximal duodenum and one kidney were isolated and snap-frozen in TRI reagent (MRC, Cincinnati, OH). Total RNA was isolated via the TRI reagent protocol, and 2 μg of the product was reverse-transcribed using a SuperScript III RNase H reverse transcriptase kit obtained from Invitrogen. The cDNA was subjected to amplification using standard methods (see also ChIP analysis). Primer sequences are available upon request. Animal experimental protocols were reviewed and approved by the Research Animals Resource Center (University of Wisconsin, Madison).

**In Vivo Chromatin Immunoprecipitation Assays**—ChIP was performed as described previously (9, 25, 29) with several modifications. C57BL6 wild-type female mice, 8–9 weeks of age, were treated by intraperitoneal injection with either ethanol vehicle or the indicated concentrations of 1,25(OH)₂D₃/g body weight (bw) as described above. 7 cm of proximal duodenum or the kidneys were isolated, rinsed with cold 1× phosphate-buffered saline, and subjected to a cross-linking reaction for 10 min with 1.5% formaldehyde. After the fixative was neutralized for 5 min with glycine (0.125 mM final concentration), the intestinal or kidney tissue was homogenized briefly in phosphate-buffered saline using a loose fitting Dounce pestle, and the cells were collected via 70-μm nylon cell strainers (BD Falcon). Cells were then subjected to ChIP analysis as described previously. DNA fragments were purified from chromatin using Qiagen QIAquick spin kits (Valencia, CA), and the precipitated DNA was then evaluated either by traditional PCR or by a quantitative real-time PCR (qPCR) method using primers designed to amplify individual fragments of the target gene promoters of interest. For traditional PCR, analyses for each primer set were carried out in a predetermined linear range of DNA amplification, and the PCR products were resolved on 2% agarose gels. DNA acquired prior to precipitation was used as input. Densitometric quantitation was carried out using Kodak One-dimensional Image Analysis software (version 3.5). qPCR was accomplished using an Applied Biosystems 7500 fast real-time PCR instrument and Power SYBR Green Master Mix. Primer sequences are available upon request. All samples were quantitated using an external standard curve and corrected for input variations. In all cases, each point represents the average ± S.E. evaluated statistically using unpaired t test analysis (*, p < 0.05).
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**RESULTS**

1,25(OH)₂D₃ Induces Cyp24a1 in the Kidney in a Time- and Dose-dependent Manner—1,25(OH)₂D₃ represents a primary inducer of *Cyp24a1* in virtually all vitamin D target tissues in *vivo* (2). This inductive activity is mediated by the VDR and its partner RXR, which bind to a regulatory region as a heterodimer and direct a series of events that facilitate enhanced transcriptional output. To establish a kinetic basis for exploring this induction in *vivo*, we first examined the ability of 1,25(OH)₂D₃ to induce *Cyp24a1* in the kidney following a single injection of the hormone into mice. As seen in Fig. 1A, 1,25(OH)₂D₃ induced a striking up-regulation of *Cyp24a1* mRNA and promoted VDR and RXR localization, RNA pol II recruitment, and H4 acetylation at the *Cyp24a1* gene locus in the kidney. Mice were treated with increasing concentrations of 1,25(OH)₂D₃ for a 6-h time period. Total RNA was prepared from isolated kidneys and subjected to quantitative reverse transcriptase PCR using primers to *Cyp24a1*. The EC₅₀ and R² values from statistical analyses are displayed. Each point represents the mean ± S.E. (n = 5). This study is representative of at least two similar experiments. B, time-dependent induction of *Cyp24a1* by 1,25(OH)₂D₃ in the kidney. Mice were treated with a single concentration of 1,25(OH)₂D₃ (10 ng/g bw) for the periods indicated, and total RNA was isolated and evaluated as indicated in A. Each point represents the mean ± S.E. (n = 5). This study is representative of at least two similar experiments. C, schematic depiction of *Cyp24a1* promoter region and primer sets used for ChIP analysis. All numbering is relative to the TSS. *Shaded boxes* refer to established VDREs. *CDS*, coding region control. D, localization of VDR and RXR, recruitment of RNA pol II, and acetylation of H4 at the *Cyp24a1* locus. Groups of mice (3/treatment) were injected with either vehicle or a single dose of 1,25(OH)₂D₃ (10 ng/g bw), and the kidneys were harvested 1 h later and subjected to ChIP analysis. Input samples were obtained prior to the immunoprecipitation. Precipitated DNA was subjected to qPCR analysis, quantitated using an external standard curve, and corrected for input variations. Each point represents the average quantity/input for the three samples ± S.E. with an unpaired t test analysis compared with vehicle (*, p < 0.05). These results are representative of at least six similar studies.

Having established an assay for the early events associated with *Cyp24a1* gene activation in the kidney in *vivo*, we next explored the kinetics of these events and their sensitivity to 1,25(OH)₂D₃. Mice were treated with increasing concentrations of 1,25(OH)₂D₃ analogous to those used to induce *Cyp24a1* mRNA, and the kidneys were harvested after 1 h and subjected to ChIP using antibodies to VDR, RXR, RNA pol II, and acetylated H4 as outlined in detail under “Experimental Procedures.” Immunoprecipitated fragments were evaluated for the enrichment of *Cyp24a1* using both conventional PCR and qPCR techniques with primer sets located at the mouse *Cyp24a1* locus (documented in Fig. 1C). Supplemental Fig. S1A illustrates the results of such an experiment utilizing direct PCR analysis wherein 1,25(OH)₂D₃ strongly induced accumulation of VDR at the *Cyp24a1* promoter but not at a downstream coding region. VDR accumulation at the promoter was accompanied by an equivalent increase in RXR and by the recruitment of RNA pol II, which was also detected within the coding region. We also observed a significant increase in the level of H4 acetylation (data not shown). No enrichment of DNA fragments was observed when the immunoprecipitations were carried out using IgG. Fig. 1D depicts a qPCR analysis of these data using related sets of DNA primers. This quantitative assessment of levels of DNA enrichment suggests that 1,25(OH)₂D₃ induced a 6–8-fold increase in VDR and RXR at the *Cyp24a1* promoter and a substantial increase in RNA pol II as well. A more modest 3-fold increase in H4 acetylation was observed. These findings indicate that *Cyp24a1* induction is mediated by the VDR, in which binding to the *Cyp24a1* promoter likely precedes changes in mRNA output.

Early Events Associated with Activation of *Cyp24a1* Are Dose- and Time-dependent and Differentially Sensitive to 1,25(OH)₂D₃—Having established an assay for the early events associated with *Cyp24a1* gene activation in the kidney in *vivo*, we next explored the kinetics of these events and their sensitivity to 1,25(OH)₂D₃. Mice were treated with increasing concentrations of 1,25(OH)₂D₃ analogous to those used to induce *Cyp24a1* mRNA, and the kidneys were harvested after 1 h and subjected to ChIP using antibodies to VDR, RNA pol II, and tetraacylated H4. Fig. 2 documents the results, revealing that the EC₅₀ for both VDR binding and RNA pol II recruitment at the *Cyp24a1* promoter are similar to that observed for RNA induction. Although VDR does not appear within the coding region, RNA pol II is strongly evident, suggesting enhanced transcriptional activity consistent with mRNA production. H4 acetylation is broad and is detectable at both the *Cyp24a1* promoter and within the coding region. Perhaps most interesting is the high level of sensitivity of acetylation to 1,25(OH)₂D₃. Indeed, maximal induction is achieved at the lowest dose of 1,25(OH)₂D₃ administered, suggesting that changes in chromatin structure initiated by this chemical change at the *Cyp24a1* promoter may require a substantially reduced level of...
VDR occupancy. The results in Fig. 3 document the time-sensitive nature of these changes with respect to both VDR and RXR binding as well as H4 acetylation. As can be seen, localization of VDR and RXR occur in parallel, peaking at 3 h and remaining partially elevated 24 h later. H4 acetylation at the Cyp24a1 promoter occurs within 1 h, however, possibly because of the increase insensitivity of the response to VDR DNA occupancy. Interestingly, this modification appears to remain fully intact for almost 24 h. These results suggest that VDR and RXR binding initiates a rapid and highly sensitive change in gene structure following treatment with 1,25(OH)₂D₃ in vivo that results in the recruitment of RNA pol II and the initiation of Cyp24a1 transcription.

1,25(OH)₂D₃, Induces Cyp24a1 and Trpv6 Expression in the Intestine in a Time- and Dose-dependent Manner—Based upon these studies of Cyp24a1 induction in the kidney, we turned next to an investigation of the properties of Cyp24a1 activation by 1,25(OH)₂D₃ in the intestine. We also explored the activation of Trpv6 by 1,25(OH)₂D₃ as well, as this gene is believed to be central to the regulation of calcium homeostasis by 1,25(OH)₂D₃ (17). Because the regulatory elements of this latter gene have not been precisely defined, we utilized both an in vivo ChIP scanning analysis and then an in vitro evaluation to identify the functional elements. We first established a kinetic basis for exploring Cyp24a1 and Trpv6 activation in the intestine by...
assessing induction profiles at the mRNA level in mice as described earlier for renal Cyp24a1. 1,25(OH)₂D₃ induces both Cyp24a1 and Trpv6 gene expression (Fig. 4, A and B) with EC₅₀ values slightly lower than that seen for Cyp24a1 in the kidney, although these differences are not statistically significant given the level of variability. The time course for the induction of both genes is similar, however, with both peaking at 6 h (Fig. 4, C and D). Interestingly, although the basal activity of Cyp24a1 was measurable and the activation of the gene sustained at 24 h in the kidney, basal levels of Cyp24a1 expression in the intestine were undetectable in the absence of 1,25(OH)₂D₃ and returned rapidly to base-line levels 24 h after induction. Trpv6 was also strongly induced by 1,25(OH)₂D₃. Its activity, unlike that of Cyp24a1, remained substantially up-regulated at 24 h.

1,25(OH)₂D₃ Promotes VDR Localization to a Proximal Promoter Site at the Cyp24a1 Gene and to a Broad Region Upstream of the Trpv6 Gene Promoter.—We next explored the localization of VDR at the Cyp24a1 and Trpv6 promoters following a single injection of 1,25(OH)₂D₃ into mice. The experimental protocol was similar to that described earlier. The locations of the primer sets used to identify DNA gene fragments are depicted schematically for Cyp24a1 and Trpv6 in Figs. 1C and 4E, respectively. Because the regulatory elements of the mouse Trpv6 gene remained unidentified, we employed a PCR scanning approach that enabled us to search for VDR binding activity up to 6 kilobases upstream of the Trpv6 TSS at a resolution of ∼1 kb. The direct PCR results, depicted in supplemental Fig. S1, B and C, reveal that 1,25(OH)₂D₃ induces VDR binding in the intestine to a single upstream region of the Cyp24a1 gene and to a broad stretch of DNA 2–4 kb upstream of the Trpv6 TSS. Neither gene demonstrated significant 1,25(OH)₂D₃-induced VDR binding within its coding region. Fig. 4, E and F, depicts qPCR quantitation of a similar experiment, demonstrating that 1,25(OH)₂D₃ induces an ∼4–5-fold increase in VDR at the Cyp24a1 promoter, and a 3–5-fold increase in VDR binding at potentially multiple sites upstream of the Trpv6 promoter. These observations suggest that early events associated with 1,25(OH)₂D₃ activity can be measured at target genes in the intestine and point to the location of possible VDR regulatory sites at the Trpv6 gene locus.

Several Upstream Regions of the Human Trpv6 Gene Are Highly Conserved at the Mouse Trpv6 Locus.—Previous studies have identified five regulatory regions containing seven VDREs that have the potential to mediate the actions of 1,25(OH)₂D₃ in the human Trpv6 gene (23). Five of these VDREs have been shown to retain functional activity. Of these five regions in the human gene, two (regions 2 and 4) are particularly well conserved, as illustrated in supplemental Fig. S2, and three (regions 1, 3, and 5) are not. Indeed, the primary 1,25(OH)₂D₃-inducing activity in the human gene is located in these two conserved regions and mediated by two pairs of VDREs for which the sequences are shown in the figure. Interestingly, whereas regions 2 and 4 are highly conserved overall in both rat and mouse, only two of the four VDREs located therein retain significant sequence identity, as shown in supplemental Fig. S2. Accordingly, VDRE a in region 2 of the mouse gene appears to contain a single base pair deletion that reduces the interval between the two key half-sites to 2 rather than 3 base pairs, thus reducing the likelihood that this element might mediate 1,25(OH)₂D₃ action. VDRE b in region 4, on the other hand, contains several base pair changes relative to the human gene that also have the potential of altering functionality. These dif-
demonstrate their activity are shown on the context of the 5′ deletions. Similarly, a mutation in VDRE a in region 4, whereas the Trpv6 construct −4.0kb/+213 contains only the proximal VDRE b of region 4. A single triplet mutation introduced into VDRE b at region 2 in each of the constructs, which prevents activity at VDRE b, allowed us to examine the role of this regulatory element within the context of the 5′ deletions. Similarly, a mutation in VDRE a in region 4 allowed us to explore its contribution as well. The results suggest that 1,25(OH)2D3-inducible activity is associated predominantly with region 2 and to a lesser extent with region 4. Thus, although activity was enhanced modestly when a sequence containing VDRE a in region 4 was added, mutation of this element in the context of the full-length construct had little consequence. Mutation of VDRE b in region 2, on the other hand, routinely eliminated 1,25(OH)2D3 response in all of the constructs tested. We conclude that a single VDRE in region 2 is essential for overall responsiveness to 1,25(OH)2D3 and that a single VDRE in region 4 can participate in, or at least support, the induction process. Collectively, these data indicate that elements that mediate the induction of the mouse Trpv6 gene are located at regions 2 and 4, consistent with the initial observations made by our in vivo ChIP analysis.

Temporal Characterization of the Early Events Associated with Activation of Cyp24a1 and Trpv6 by 1,25(OH)2D3 in the Intestine—The above described studies provide strong evidence that the VDR binding activity identified at the Trpv6 locus in the intestine in vivo correlates directly with the presence of functional regulatory elements (VDREs). Based upon this confirmation, we conducted a final kinetic experiment wherein we quantitated the appearance of VDR at Cyp24a1 and Trpv6 loci as a function of time following treatment with 1,25(OH)2D3. We also explored the temporal nature of chromatin modification at H4 and the recruitment of RNA pol II. Accordingly, mice were treated with a single dose of 1,25(OH)2D3 (10 ng/g bw), the intestines were isolated and fixed at the indicated time points, and the sample was subjected to ChIP analysis using antibodies to VDR, RNA pol II, and tetraacetylated H4. As seen in Fig. 6, 1,25(OH)2D3 induces rapid binding of VDR to both the Cyp24a1 and Trpv6 genes, with activity peaking at 3 h followed by a slow decline in activity as a

Identification of the Vitamin D Regulatory Elements Located in the Mouse Trpv6 Gene—To confirm the functionality of potential regulatory components in the mouse Trpv6 gene, we cloned several large fragments (−4.1, −5.5, and −10 kb) into a luciferase expression vector using a recombineering approach and assessed the activity of these constructs in response to increasing concentrations of 1,25(OH)2D3 following transfection into a human colon cancer cell line, LS180. We also introduced the human version of the Trpv6 (i) promoter (hTRPV6 (−7 kb/+160)) for comparison. As seen in Fig. 5A, 1,25(OH)2D3 induced a dose-dependent up-regulation of activity in all of the TRPV6 (i) promoter constructs examined whereas the parent vector was inactive. Interestingly, although the comparable human TRPV6 construct was inducible by 12−14-fold, the mouse versions of the Trpv6 gene were less active, manifesting a 4-fold induction at best. These differences appear inherent to the constructs and may reflect differing regulatory activities of 1,25(OH)2D3 in the two species. They also suggest that additional VDREs in the human TRPV6 (i) gene may be responsible for the enhanced overall activity. In Fig. 5B, a series of 5′ deletion constructs of the mouse Trpv6 gene promoter are depicted on the left of the panel; the results that demonstrate their activity are shown on the right. As can be seen, the Trpv6 construct −4.1kb/+213 contains both VDRE a and VDRE b located in region 4, whereas the Trpv6 construct −4.0kb/+213 contains only the proximal VDRE b of region 4. A single triplet mutation introduced into VDRE b at region 2 in each of the constructs, which prevents activity at VDRE b, allowed us to examine the role of this regulatory element within the context of the 5′ deletions. Similarly, a mutation in VDRE a in region 4 allowed us to explore its contribution as well. The results suggest that 1,25(OH)2D3-inducible activity is associated predominantly with region 2 and to a lesser extent with region 4. Thus, although activity was enhanced modestly when a sequence containing VDRE a in region 4 was added, mutation of this element in the context of the full-length construct had little consequence. Mutation of VDRE b in region 2, on the other hand, routinely eliminated 1,25(OH)2D3 response in all of the constructs tested. We conclude that a single VDRE in region 2 is essential for overall responsiveness to 1,25(OH)2D3 and that a single VDRE in region 4 can participate in, or at least support, the induction process. Collectively, these data indicate that elements that mediate the induction of the mouse Trpv6 gene are located at regions 2 and 4, consistent with the initial observations made by our in vivo ChIP analysis.

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function of time. This decline is rapid for Cyp24a1, returning to near base-line levels after 24 h, similar to that seen at the corresponding mRNA level. Activity at the Trpv6 locus is more gradual, however, remaining significantly elevated even at 24 h. This residual activity is equally reminiscent of the prolonged activity observed at the Trpv6 mRNA level. Recruitment of RNA pol II is similarly induced, rising to a peak at 3 h for both genes and then falling in a fashion similar to that of VDR. RNA pol II is also observed within the coding region of the Cyp24a1 gene and modestly within the Trpv6 gene as well. Finally, as observed previously in the kidney, 1,25(OH)2D3 induces a broad and extremely rapid increase in H4 acetylation at the Cyp24a1 locus that peaks within 1 h and remains generally sustained for up to 24 h. Induction of H4 acetylation at the Trpv6 gene appears to be similar to that observed for the Cyp24a1 gene, but less rapid, and remains fully elevated for at least 24 h post-treatment. These findings suggest both similarities and differences in the early events associated with the induction by 1,25(OH)2D3 of intestinal Cyp24a1 and Trpv6.

**MED1 Participates in Early Events Associated with Induction by 1,25(OH)2D3 of Cyp24a1 but Not Trpv6**—In a final experiment, we explored the participation of MED1 in the activation of intestinal Cyp24a1 and Trpv6. MED1 functions as a coactivator for several members of the nuclear receptor gene family and is believed to play a role in the recruitment and activity of RNA pol II (30, 31). To determine whether MED1 was involved in Cyp24a1 or Trpv6 induction, we treated mice with a single dose of 1,25(OH)2D3 as indicated, isolated the intestine after 1 h, and subjected the samples to ChIP analysis using antibodies to VDR and MED1. Immunoprecipitated DNA was then subjected to qPCR using the primers as indicated in Fig. 1C for Cyp24a1 and Fig. 4E for Trpv6. As seen in Fig. 7, 1,25(OH)2D3 again induced binding of the VDR to the relevant regulatory regions of both the Cyp24a1 and Trpv6 genes. Surprisingly, however, whereas MED1 antibodies enriched Cyp24a1 promoter DNA selectively following treatment with 1,25(OH)2D3, no such enrichment was detected at any of the regulatory regions of the Trpv6 gene or at the TSS in response to hormone. These data suggest that the recruitment of RNA pol II to the Trpv6 locus may require a coactivator distinct from that of MED1.

**DISCUSSION**

The activation of gene expression by the vitamin D hormone involves a series of complex events that begins with the local-
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ization of VDR at specific DNA binding sites on target genes and culminates in the recruitment and activation of the RNA pol II complex. In this report, we utilized ChIP to explore the nature of the early events that occur at the Cyp24a1 and the Trpv6 genes following treatment of mice with a single dose of 1,25(OH)2D3 in vivo. Our results suggest that 1,25(OH)2D3 induces rapid VDR localization to the VDREs of these two genes, an activity that is accompanied by co-localization of RXR and correlates temporally with the direct recruitment of RNA pol II. The features of these events are consistent with the subsequent induction of Cyp24a1 in the kidney and both Cyp24a1 and Trpv6 in the intestine. 1,25(OH)2D3 also induces broad H4 acetylation at these two gene loci. Surprisingly, our results suggest that chemical changes to chromatin initiated by 1,25(OH)2D3 at these sites may require lower levels of VDR occupancy. Thus, although histone acetylation may be necessary, it is unlikely to be sufficient for transcriptional activation, because additional VDR binding leads to higher levels of RNA pol II activity and higher levels of mRNA output. Collectively, our results provide an initial glimpse into the early events associated with 1,25(OH)2D3 action at target genes in an animal model.

We utilized ChIP analysis to obtain the bulk of the results described in this report. ChIP has been applied broadly to the analysis of the molecular events associated with gene regulation in cell culture but less routinely to in vivo models. The methodological approach we used here was not significantly different from that which we employed in cell culture. Accordingly, we established the kinetics of gene activation by first characterizing the parameters of Cyp24a1 and Trpv6 mRNA up-regulation and then focusing upon VDR and RXR localization, the impact of that binding on chromatin modification, and the recruitment of RNA pol II. A knowledge of these events is essential to understanding 1,25(OH)2D3 action in vivo and could well be key to elucidating the unusual activities of synthetic vitamin D analogues and other novel activators of the VDR.

Using the ChIP approach, our studies focused upon the nature of gene activation by 1,25(OH)2D3 in vivo, and contrasted this activity in two different tissues and two different genes. Our results suggest a course of molecular activity that culminates in the induction of Cyp24a1 and Trpv6 mRNA. In both the kidney and intestine, Cyp24a1 mRNA production is preceded by VDR and RXR binding, RNA pol II recruitment, and H4 acetylation. Interestingly, the dissociation of the VDR from the Cyp24a1 promoter is more rapid in the intestine than in the kidney. This loss of VDR activity across the two tissues correlates with Cyp24a1 mRNA levels, which return to base line in the intestine at 24 h and yet remain well above the base line in the kidney at that time point. A surprising finding is the rapidity with which H4 at the Cyp24a1 promoter is acetylated in both tissues. This finding suggests that acetylation at the Cyp24a1 locus, which we have established previously (23), is particularly sensitive to 1,25(OH)2D3 and occurs at subsaturating levels of VDR occupancy. Thus, although acetylated H4 levels do not continue to increase above treatment levels of 0.1 ng of 1,25(OH)2D3/g bw, levels of VDR and RXR binding and RNA pol II continue to increase in parallel. Finally, H4 acetylation is sustained at the Cyp24a1 locus well after VDR levels are depleted. These differences suggest that the molecular events associated with the up-regulation of gene expression by 1,25(OH)2D3 may require different levels of the VDR. Interestingly, Trpv6 is also regulated in the intestine in a fashion similar to that of Cyp24a1. The exquisitely rapid H4 acetylation that is seen at the Cyp24a1 locus is not, however, apparent. This difference may highlight major quantitative differences in the overall levels of basal expression of Cyp24a1 and Trpv6. Thus, whereas Cyp24a1 remains undetectable in the absence of exogenous 1,25(OH)2D3, levels of Trpv6 mRNA, although low, are clearly measurable. Future studies will be required to focus on these apparent differences at the two gene loci.

Studies of the human TRPV6 gene have revealed the presence of several regulatory regions and multiple VDREs that mediate the actions of 1,25(OH)2D3 and its receptor in vitro (23). The absence of a suitable cell line, however, prompted an in vivo approach to studying the mouse gene. We utilized a ChIP scanning approach initially to identify potential regions of VDR interaction at the mouse Trpv6 gene and then followed these studies with more traditional mapping experiments. Interestingly, activation of mouse Trpv6 is mediated by a subset of the five VDREs originally identified in the human gene. Surprisingly, although the ChIP scan suggested that the duplex VDREs in regions 2 and 4 of the mouse gene might be involved, only two of these elements were eventually shown to be active. The remaining elements were either not conserved or only weakly conserved. This dramatic difference between the genes of these two species may underlie the modest 1,25(OH)2D3-inducible activity that we observed following transfection of the large mouse promoter constructs into LS180 cells, although other explanations are equally possible. Despite this difference, the approach we have taken suggests that a reliance on cell culture models may not be essential for delineating key gene regulatory regions.

Interestingly, our studies revealed that although MED1 can be detected at the Cyp24a1 promoter, this factor is not apparent at the Trpv6 promoter. Although it is possible that our ChIP analysis simply failed to detect MED1 because of differences in complex formation at the Trpv6 locus (epitope masking), it seems more likely that the recruitment of Mediator complex to this gene by the VDR is facilitated by an alternative member of the complex. If this proves to be the case, it could provide additional support for both the unique requirements of individual promoters and the potential for the receptor to interact with different coregulators. These findings highlight the potentially diverse mechanisms that can be exploited during the selective gene activation that can occur at different genes and in different tissues by selective nuclear receptor modulators. Although these mechanisms are not well defined for vitamin D ligands, the approaches and observations made here might well facilitate future studies.

In conclusion, we have utilized both traditional and ChIP approaches to characterize the early events associated with the activation target genes by 1,25(OH)2D3 in mice. Our results define a temporal scheme whereby the activation and binding of the VDR to these target genes correlates directly with the recruitment of RNA pol II and precedes the induction of two important mRNAs. Collectively, these results provide the basis
for further studies of 1,25(OH)2D3 in vivo and are likely to be useful in assessing the unique and unusual activities of specific vitamin D analogues.

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