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Large-Scale *in Silico* and Microarray-Based Identification of Direct 1,25-Dihydroxyvitamin D_3 Target Genes

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1α,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] regulates calcium homeostasis and controls cellular differentiation and proliferation. The vitamin D receptor (VDR) is a ligand-regulated transcription factor that recognizes cognate vitamin D response elements (VDREs) formed by direct or everted repeats of PuG(G/T)TCA motifs separated by 3 or 6 bp (DR3 or ER6). Here, we have identified direct 1,25$(OH)_2D_3$ target genes by combining 35,000+ gene microarrays and genome-wide screens for consensus DR3 and ER6 elements, and DR3 elements containing single nucleotide substitutions. We find that the effect of a nucleotide substitution on VDR binding *in vitro* does not predict VDRE function *in vivo*, because substitutions that disrupted binding *in vitro* were found in several functional elements. Hu133A microarray analyses, performed with RNA from human SCC25 cells treated with 1,25$(OH)_2D_3$ and protein synthesis inhibitor cycloheximide, identified more than 900 regulated genes. VDREs lying within −10 to +5 kb of 5′-ends were assigned to 65% of these genes, and VDR binding was confirmed to several elements *in vivo*. A screen of the mouse genome identified more than 3000 conserved VDREs, and 158 human genes containing conserved elements were 1,25$(OH_2)D_3$-regulated on Hu133A microarrays. These experiments also revealed 16 VDREs in 11 of 12 genes induced more than 10-fold in our previous microarray study, five elements in the human gene encoding the epithelial calcium channel TRPV6, as well as novel 1,25$(OH_2)D_3$ target genes implicated in regulation of cell cycle progression. The combined approaches used here thus provide numerous insights into the direct target genes underlying the broad physiological actions of 1,25$(OH)_2D_3$. (*Molecular Endocrinology* 19: 2685–2695, 2005)

APART FROM A limited number of dietary sources, naturally occurring vitamin D_3 is obtained by the UV light-induced conversion of cutaneous 7-dehydrocholesterol (1). It is a component of the skin's homeostatic system, which provides a protective barrier against the environment, and communicates directly with the body's immune and neuroendocrine functions (2). The biologically active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], has a broad range of physiological effects (2–4). It is primarily known for its critical role in calcium homeostasis, as 1,25$(OH)_2D_3$ is a critical regulator of calcium transport in intestinal epithelia, and modulates bone resorption. However, 1,25$(OH)_2D_3$ also has widespread effects on cellular proliferation and differentiation (Refs. 3 and 4 and references therein). It blocks cell proliferation in several cancer models, including myeloid leukemia, melanoma, and carcinomas of the breast, prostate, colon, and head and neck (3, 4). Moreover, epidemiological data have provided a correlation between the prevalence of certain cancers, particularly prostate and colon cancers, and exposure to sunlight, consistent with chemopreventive effects of 1,25$(OH)_2D_3$ (5). Support for these data is provided by the chemopreventive actions of 1,25$(OH)_2D_3$ and its analogs in animal models of colon, hamster cheek pouch, hepatocellular, gastrointestinal, and skin carcinogenesis (Ref. 5 and references therein).

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Abbreviations: ChIP, Chromatin immunoprecipitation; CHX, cycloheximide; DR3, direct repeat with 3 bp spacing; ER6, everted repeat with 6 bp spacing; FBS, fetal bovine serum; FOXO1A, F box 01A; LBD, ligand binding domain; MAD, MAX dimerization protein; MYC, avian myelocytomatosis viral oncogene homolog; 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; RXR, retinoid X receptor; SAM, significance analysis of microarrays; VDR, vitamin D receptor; VDRE, vitamin D response element.

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$1,25(OH)_2D_3$ is also a modulator of the immune responses, consistent with broad expression of the vitamin D receptor (VDR) in cells of the immune system and the capacity of $1,25(OH)_2D_3$ to regulate cellular differentiation. Indeed, mice in which the VDR gene had been ablated displayed abnormal proinflammatory T helper 1 cell development (6), and mice rendered $1,25(OH)_2D_3$ deficient by knockout of the gene encoding 25-hydroxyvitamin D3 1α-hydroxylase were deficient in peripheral T lymphocytes (7). Moreover, $1,25(OH)_2D_3$ inhibits dendritic cell maturation, which is critical for T cell-mediated immune responses (8–10), and reduces expression of the cytokine IL-12, the signaling of which is critical for T helper 1 cell maturation.

$1,25(OH)_2D_3$ signaling occurs through its cognate nuclear VDR (11), which is a member of the nuclear receptor family and a direct regulator of gene transcription. Nuclear receptors regulate transcription of target genes by ligand-dependent recruitment of accessory proteins known collectively as coregulators (12–14). The domain structure of the VDR is typical of nuclear receptors, with highly conserved DNA-binding and ligand-binding domains (LBDs) (3, 4). Similar to several nuclear receptors, the VDR functions as a heterodimer with members of the retinoid X receptor (RXR) family of receptors. Strong interactions between VDR and RXR LBDs are essential for stable dimerization and high-affinity DNA binding. Nuclear receptors regulate transcription, in part, by binding specific DNA sequences known collectively as hormone response elements, which are generally composed of tandem hexameric motifs and normally located in the 5′-flanking region of target genes (15). Vitamin D response elements (VDREs) are composed of tandem motifs with the consensus PuG(G/T)TCA, which are often arranged as direct repeats separated by 3 bp (DR3-type). VDR/RXR heterodimers can also recognize everted repeats of hexameric motifs spaced by 6 bp (16–18), in which the upstream motif is flipped through 180° (4).

Classically, investigations into physiological responses have tended to move from studies of the whole organism to the molecular. However, with the advent of near genome-wide microarrays and large-scale genome sequencing, genomic approaches have become increasingly powerful tools for probing physiological mechanisms (19). In many respects, $1,25(OH)_2D_3$ signaling is ideally suited to genomic analyses because the VDR is a direct regulator of gene transcription with a well-characterized binding site. Here, we have used combined approaches of microarray analyses and genome-wide screens for VDREs to identify $1,25(OH)_2D_3$ target genes. Microarray analyses and response element screens are complementary. Microarrays will identify both up- and down-regulated genes with a range of different fold regulations (*e.g*. Ref. 20). However, the genes identified will be limited to those regulated in the model system under investigation. Assignment of response elements to genes identified by microarray provides both a level of validation as well as a mechanism of regulation. Moreover, identification of putative response elements using *in silico* screens does not have the limitation of microarrays because it will identify potential target genes independent of their tissue of expression. The findings presented here provide numerous insights into the range of molecular genetic events underlying the broad physiological actions of $1,25(OH)_2D_3$, including its effects on calcium homeostasis and immune system function, as well as its anticancer actions.

RESULTS AND DISCUSSION

Experimental Approach

We are interested in determining the molecular genetic events underlying the broad physiological activities of $1,25(OH)_2D_3$ by identifying, on a large scale, the primary target genes of $1,25(OH)_2D_3$ signaling through its cognate VDR. Previously developed algorithms for identification of nuclear receptor binding sites such as NUBIScan (21) are based on weighted nucleotide distribution matrices and combined scores from both response element half-sites. However, importantly, most matrices developed to date reflect limited data derived from functional binding sites for a given receptor and pool data from binding sites of several nuclear receptors, and therefore do not account for receptor-specific sequence preferences and differing polarities of different RXR heterodimeric pairs. In addition, many hormone response elements identified to date by deletion analysis of promoters are highly degenerate and are often derived from functional analyses in the presence of overexpressed VDRs of limited proximal regions, which may have missed higher affinity distal elements. Recently developed algorithms used a limited number of such highly degenerate sequences to derive an information weight matrices for VDREs (*e.g*. Refs. 22 and 23). We have previously developed an algorithm for genome-wide screening of high-affinity response elements of the related estrogen receptor (24). This approach, although not exhaustive, functions on a genome-wide scale. Significantly, the screen identified several consensus or near-consensus elements in promoters of genes with previously characterized more degenerate promoter-proximal elements (24), suggesting that the latter may not be of primary importance in driving the hormonal response. Here, we have combined our genome-wide screen for response elements with Affymetrix Hu133A microarray analyses and have identified several hundred consensus or near-consensus VDREs in $1,25(OH)_2D_3$-responsive genes.

Identification of Direct $1,25(OH)_2D_3$ Target Genes by Screening Affymetrix Hu133A Oligonucleotide Microarrays

Our previous microarray screens of $1,25(OH)_2D_3$-regulated genes were performed in human SCC25 cells using 6,800+ gene Affymetrix HuGene FL oligonucleotide chips (20). SCC25 cells were isolated from a floor of the mouth/base of the tongue squamous tumor but retain many characteristics of more differentiated squamous epithelia (25, 26). Their growth is arrested in G$_0$/G$_1$ by 1,25(OH)$_2$D$_3$ (26). We were interested here in performing a substantially expanded study of direct 1,25(OH$_2$)D$_3$ target genes using the 35,000+ gene Affymetrix Hu133A chip. Our previous time course analysis revealed several kinetic profiles of 1,25(OH)$_2$D$_3$-regulated gene expression over a 48-h period (20). However, whether rapidly or more slowly affected, regulation of the vast majority of target genes was evident after 12 h of 1,25(OH$_2$)D$_3$ treatment (20). Therefore, to identify direct 1,25(OH)$_2$D$_3$ target genes, quadruplicate cultures of SCC25 cells were treated with the protein synthesis inhibitor cycloheximide (CHX) in the absence or the presence of 1,25(OH)$_2$D$_3$ for 12 h. A similar approach has been used to identify direct estrogen target genes (27). RNA samples were tested for consistency of expression of *gapdh* as an internal control, and for induction 24-hydroxylase (*cyp24*) transcripts, which were absent in cells treated with CHX alone, and uniformly strongly induced in CHX/1,25(OH)$_2$D$_3$-treated samples (data not shown).

Although Affymetrix's algorithm MAS 5 is widely used to analyze fold changes in gene expression, it is biased toward weakly expressed genes, tends to select similar numbers of up- and down-regulated genes, and lacks accuracy (28, 29) when compared with other algorithms such as dChip and robust multichip average. A more rigorous approach uses dChip and robust multichip average to create gene expression values, followed by significance analysis of microarrays (SAM) (30) to select differentially expressed genes. Instead of being based on fold changes, SAM creates a type of *P* value for genes that are differentially expressed, enabling one to better control false discovery rates (28) and select genes from the gamut of expression levels in an unbiased manner. We found this approach to be more reliable than MAS 5 at identifying known modestly regulated target genes of 1,25(OH)$_2$D$_3$. For example, the gene encoding the transcription factor MAD1, a regulator of c-MYC function, was not identified as a regulated gene using the MAS 5 algorithm (see below, and data not shown). Results of the SAM-based analysis of 1,25(OH)$_2$D$_3$-regulated genes in SCC25 cells are presented in supplemental Table 1 published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org. The 1409 entries correspond to 913 unique genes (note that several genes are represented by multiple series of oligonucleotides on Hu133A microarrays, and some genes have more than one annotation), of which 746 are named genes and 167 are less well characterized (expressed sequence tags, hypothetical genes, *etc*). Of the 913 genes, 734 are induced and 179 are repressed. The list contains a number of 1,25(OH)$_2$D$_3$-regulated genes identified in our previous microarray studies (Refs. 20 and 26, and see below), further confirming the 1,25(OH)$_2$D$_3$ responsiveness of SCC25 cells.

Genome-Wide Analysis of DR3 and ER6 VDREs

Similar to our previous estrogen response element screen (24), we mapped VDREs in the human genome lying within −10 kb to +5 kb regions of genes. We screened for consensus DR3 and consensus lower affinity ER6 elements (supplemental Tables 2 and 3 published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). Genome-wide scanning for highly degenerate elements (*e.g*. see Ref. 31) is impractical, and it is difficult to predict the functionality of such elements. Similar to our previous study (24), we therefore limited our screen to nonconsensus DR3 elements with a single nucleotide substitution in one of the two half-sites. Gel mobility shift assays with radiolabeled DR3 elements were used to assess the effects of all possible single-nucleotide substitutions in either half-site on VDR binding *in vitro* (Fig. 1) using the VDRE from the mouse osteopontin gene as a consensus (32). Specific complexes composed of VDR/RXR heterodimers are formed on this element in extracts of COS7 cells transfected with a VDR expression vector, as confirmed by coincubation of extracts with either an anti-VDR or anti-pan-RXR antibody (Fig. 1A).

Single-nucleotide substitutions had markedly varying effects on formation of specific complexes, with some substitutions diminishing binding by more than 80% (Fig. 1B). Significantly, however, such an approach did not appear to be a reliable indicator of potential response element function. A preliminary gene-by-gene analysis of previously identified highly regulated genes revealed three putative DR3 elements containing A to C substitutions at position 6 of the 5′-half-site that severely disrupted DNA binding *in vitro* in genes encoding IL1RL1 (a decoy receptor for IL-1; also known as T1/ST2; −5767, −1889) and COL13A1 (type XIII collagen; −1252). To test the function of one of these putative elements, the regulatory regions of the *col13A1* gene were cloned and inserted upstream of a promoterless *luciferase* reporter gene (Fig. 2). Analysis of reporter gene expression in cells transfected with plasmids containing promoters with intact or deleted VDRE sequences showed that 1,25(OH)$_2$D$_3$-responsiveness was fully dependent on the integrity of the VDRE in the *col13A1* promoter (Fig. 2A). These results suggested that the effects of nucleotide substitutions on DNA binding *in vitro* are not a reliable indicator of response element function *in vivo*. Therefore, we screened the human genome for DR3 elements with all possible single-nucleotide substitutions (supplemental Table 4 published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org).

We also screened for DR3 elements lying on both strands of DNA in either orientation relative to adjacent genes. To confirm that a DR3-type VDRE could func-

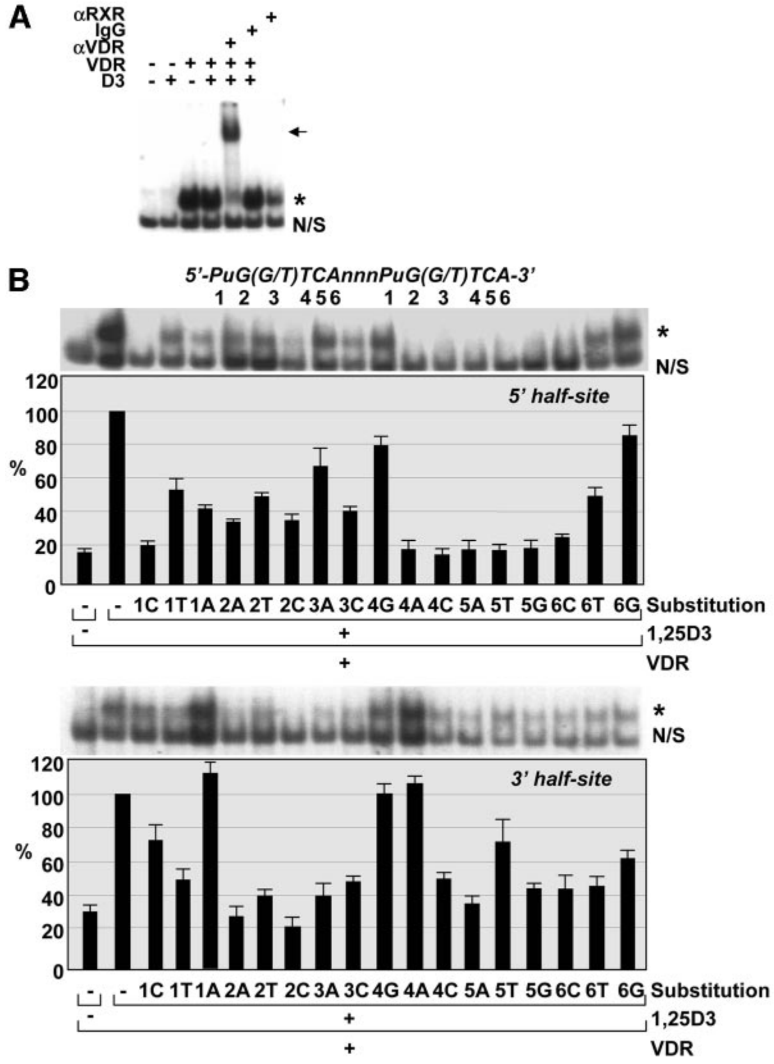

Fig. 1. VDR/RXR Binding to VDREs Containing Single-Nucleotide Substitutions

A, Binding of VDR/RXR heterodimers to the mouse osteopontin VDRE used for scanning mutagenesis studies. COS 7 cell were transfected with either empty vector (−) or a VDR expression vector (+), as indicated. The presence of the VDR and RXRs in the specific complex was detected by coincubation with control IgG or specific antibodies against the VDR or RXRs, as indicated. B, Quantification of results from EMSAs of VDR/RXR binding DR3 elements containing single-nucleotide substitutions in either the 5′- or 3′-half-site that deviate from the consensus half-site PuG(G/T)TCA. The base sequence used in these studies was that of the consensus DR3 element of the mouse osteopontin gene (32). Specific (*) and nonspecific (N/S) retarded complexes are shown. Densities of specific retarded complexes and free oligonucleotide (data not shown) were determined using an Alpha Innotech FluorChem and Alpha Ease FC (San Leandro, CA) software. Percentage of radioactivity in specific complexes was normalized to that of the wild-type sequence. Sequence of the consensus DR3 element, along with nucleotide numbering, is shown above.

tion in the reverse orientation, we cloned the proximal promoter region of the *trpv6* gene, which is induced by $1,25(OH)_2D_3$ in SCC25 cells (see supplemental Table 1). The *trpv6* promoter contains a consensus DR3 element at −1269 that is in the reverse orientation with respect to gene transcription (see below). Gene transfer experiments confirmed that a promoter fragment containing the VDRE conferred $1,25(OH)_2D_3$-dependent luciferase expression, whereas a fragment lacking the element was unresponsive (Fig. 2B).

The data generated from the *in silico* VDRE screens was then integrated into the results from our Hu133A microarray analysis. Of all of the $1,25(OH)_2D_3$ target genes identified by microarray analyses, 64.6% (590/913; see supplemental Table 1) contained 916 nonconsensus DR3 elements, 31 consensus D3, and 19 consensus ER6 elements (966 in total) lying within −10 kb and +5 kb of transcription start sites. Elements were found in both induced and repressed genes, with no enrichment of VDREs in induced genes observed. It

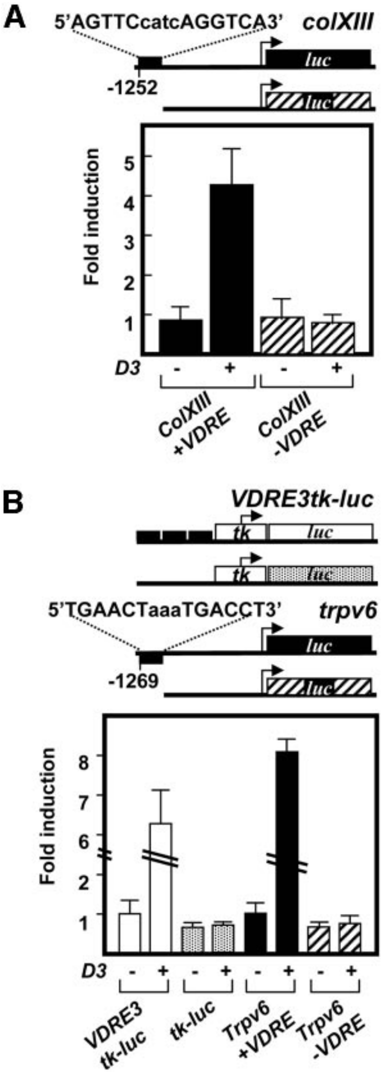

Fig. 2. Function of the Nonconsensus VDREs in Driving 1,25(OH$_2$)D$_3$-Dependent Reporter Gene Expression

A, Sequences containing or lacking the nonconsensus DR3 element at −1252 in the *col13A1* were cloned upstream of a promoterless *luciferase* gene, and 1,25(OH)$_2$D$_3$-inducible reporter gene was analyzed in transiently transfected COS-7 cells. B, Sequences containing or lacking the consensus DR3 element at −1269 in the *trpv6* gene were cloned upstream of a promoterless *luciferase* gene, and 1,25(OH)$_2$D$_3$-inducible reporter gene was analyzed in transiently transfected COS-7 cells. A promoter composed of three DR3 VDREs from the mouse ostropontin gene (32) inserted upstream of a truncated thymidine kinase promoter was used as a positive control (52). tk-luc, Thymidine kinase-luciferase.

should be noted here that, although the presence of a VDRE in a promoter is generally associated with transcriptional activation, there are several examples in the literature of VDR/RXR binding to VDREs that are required for 1,25(OH)$_2$D$_3$-dependent transcriptional repression (*e.g.* Refs. 33–35). Thus, the presence of VDREs in repressed genes is to be expected.

VDR Binding to Response Elements *in Vivo*

The VDRE screen identified known DR3 and ER6 elements in characterized 1,25(OH)$_2$D$_3$-responsive genes [*e.g.* the proximal ER6 (−151) and distal DR3 (−7769) of the *cyp3A4* gene (18)]. More importantly, the screen, coupled with the results of microarray analyses, identified numerous novel response elements and target genes (see supplemental Tables 1–4, and see below). For example, 16 VDREs were identified in the promoters of 11 of the 12 genes whose expression was induced 10-fold or greater in our previous microarray study (20), with the notable exception being the most highly induced of all genes, that encoding the 24-hydroxylase enzyme catalyzing 1,25(OH)$_2$D$_3$ metabolism (Fig. 3). Note that induction of all of these genes was also detected on the Hu133A chip (supplemental Table 1). All of the elements identified were nonconsensus sequences with a variety of either 5′- or 3′-substitutions. Binding of the VDR *in vivo* to promoter regions containing these elements was confirmed by chromatin immunoprecipitation (ChIP) assay (Fig. 3). Binding to the element of the *cst6* (cystatin M) gene that contains a disruptive G to A substitution at position 2 of the 3′-half-site was detected *in vivo*. Similarly, binding of the VDR to the three DR3 elements of the *il1rl1* and *col13A1* genes containing the disruptive A to C substitution at position 6 of the 5′-half-site was observed *in vivo*. We confirmed that the A to C substitution was disruptive *in vitro* in the context of the *col13a1* DR3 element and flanking sequence by gel mobility shift assay (Fig. 3B), consistent with its effect *in vitro* in the context of the sequence of the mouse osteopontin VDRE. Whereas subsaturating binding of the VDR was readily detected on the consensus *mop* element, no such complex was observed on the *col13a1* oligonucleotide. These results support our findings above (Figs. 1 and 2) that affinity of an element determined by gel mobility shift assay *in vitro* is not an accurate predictor of potential VDRE function *in vivo*. These discrepancies may arise because of differences in conformation of oligonucleotides and nucleosomal DNA, or association of RXR/VDRs with other transcription factors that stabilize binding. For example, the association of an estrogen response element half-site, which binds ERs poorly *in vitro*, with an Sp1 site generates a functional response element *in vivo* (36).

The *in silico*/microarray screening results also provide insights into molecular genetic events underlying the calcemic activity of 1,25(OH)$_2$D$_3$. Notably, multiple novel VDREs were identified in the regulatory region of the 1,25(OH$_2$)D$_3$-regulated *trpv6/ecac2* gene (transient receptor potential cation channel 6/epithelial calcium channel 2), which encodes an apical epithelial calcium transporter that is a critical element of 1,25(OH)$_2$D$_3$-stimulated intestinal calcium transport (37). Consistent with the results of microarray analysis (supplemental Table 1), induction of *trpv6* by 1,25(OH)$_2$D$_3$ was confirmed by RT-PCR in both SCC25 and intestinal epi-

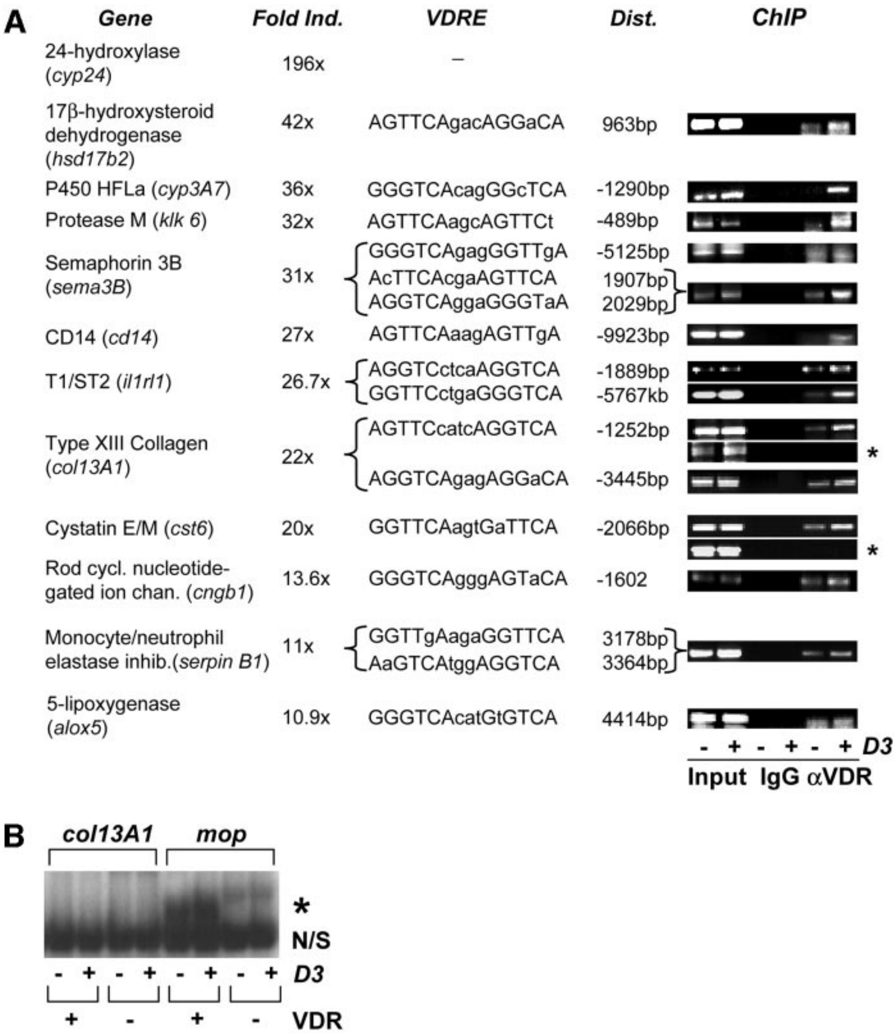

Fig. 3. Identification of VDREs in Highly Induced 1,25(OH)$_2$D$_3$ Target Genes

A, VDREs were identified by genome-wide screening in 11 of the 12 genes induced by 1,25(OH)$_2$D$_3$ over 10-fold in SCC25 cells in the microarray study of Lin *et al.* (20). Binding of the VDR to response elements was confirmed by ChIP assay. Note that ChIP assays could not resolve binding of closely spaced elements in the promoters of the *sema3B* and *serpinB1* promoters. Immunoprecipitation of fragments (*asterisks*) of the *cst6* and *col13A1* genes lying approximately 2 kb from putative VDREs was performed to control for specificity of immunoprecipitation and degree of DNA shearing. B, Gel mobility shift assays performed with extracts of COS7 cells transiently transfected with pSG5 (−) or pSG5-VDR (+) expression vectors comparing VDR binding to the DR3 element of the mouse osteopontin (*mop*) and *col13A1* gene (element at −1252). The section of the autoradiogram containing specific (*) and nonspecific (N/S) retarded bands is shown. Dist., Distance; Ind., indirect; cycl., cyclic; chan., channel; inhib., inhibitor.

thelial CaCo2 cells (Fig. 4A), and binding of the VDR *in vivo* to fragments encompassing the five VDREs was confirmed by ChIP assay in SCC25 cells. The list of consensus DR3 elements also contains VDREs in two genes *defb4 (defB2*; defensin β 2) and *camp* (cathelicidin antimicrobial peptide), which encode antimicrobial peptides. We have shown that expression of both of these genes is induced by 1,25(OH)$_2$D$_3$ in cells of the immune system, consistent with the enhanced secretion of antimicrobial activity by 1,25(OH)$_2$D$_3$-treated cells (38).

The combination of microarray analyses and response element screens also identified novel target genes that may underlie the anticancer properties of 1,25(OH)$_2$D$_3$ (Fig. 4B). For example, 1,25(OH)$_2$D$_3$ induced expression of the gene encoding MAD, which heterodimerizes with MYC cofactor MAX, thereby blocking MYC activity (39). It is also noteworthy that MAD expression is elevated during epithelial wound healing (40), a process that is stimulated by 1,25(OH)$_2$D$_3$ (41). 1,25(OH$_2$)D$_3$ also enhanced expression of the gene encoding the transcription factor FOXO1 (F box 01A). FOXO1 activity is controlled by phosphorylation by the kinase AKT, which induces FOXO1 nuclear export. Its function is inhibited in a number of epithelial cancers where expression of the

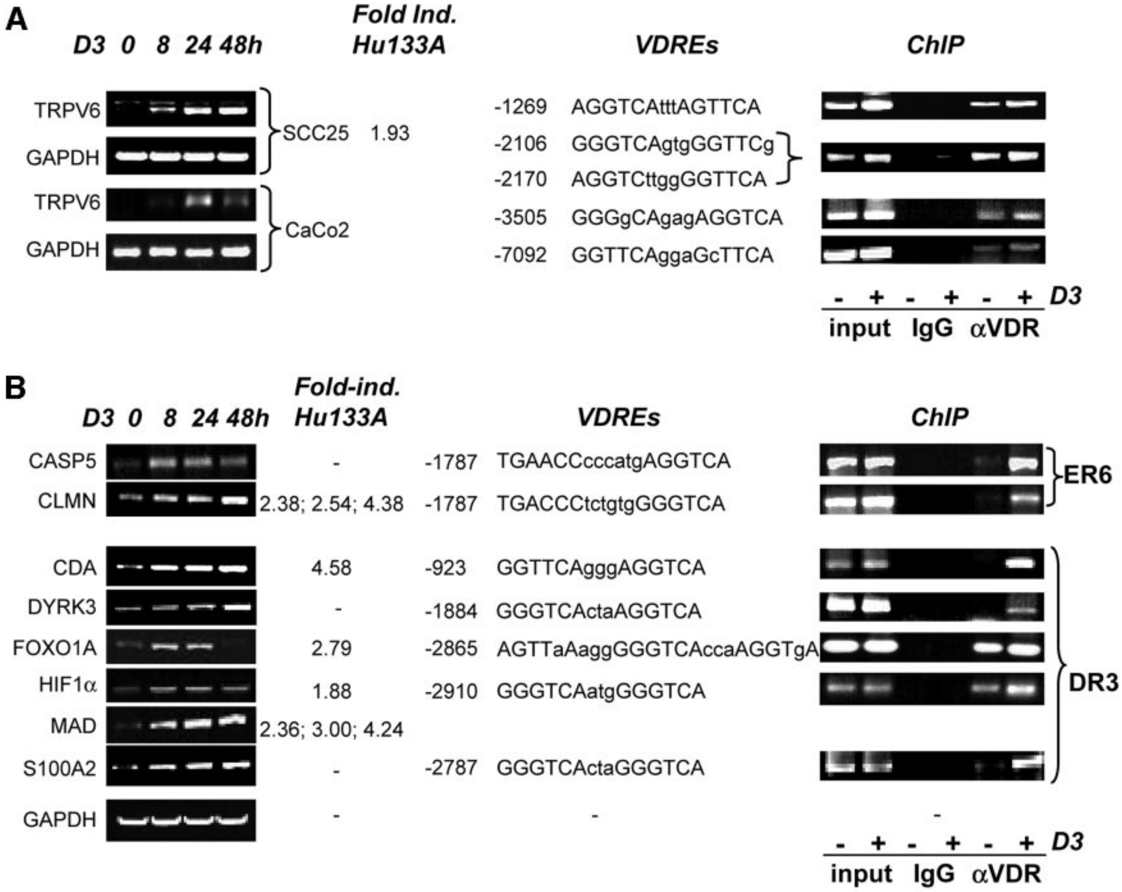

Fig. 4. Validation of Regulation of Target Genes of 1,25(OH)$_2$D$_3$ Identified by Microarray Analysis and Response Element Screening

Genes containing both consensus and nonconsensus DR3 elements and ER6 elements were chosen for further analysis. A, Identification of multiple VDREs in the promoter of the 1,25(OH)$_2$D$_3$-regulated gene encoding the epithelial calcium channel TRPV6/ECAC2. Results from microarray analysis, RT-PCR analysis in 1,25(OH$_2$)D$_3$-treated SCC25 and CaCo2 cells, and analysis of VDR binding to VDREs by ChIP assay are presented. B, Validation of 1,25(OH)$_2$D$_3$-regulated expression of selected target genes identified from microarray and/or *in silico* VDRE analysis in SCC25 cells. Note that the element in the *foxo1* promoter contains three direct repeats, each separated by 3 bp. Note also that no VDREs were identified in the gene encoding MAD. Ind., Indirect.

AKT inhibitor, phosphatase and tensin analog, is lost (42). FOXO1 activity controls cell cycle progression (42), in part by stimulating expression of the cyclin-dependent kinase inhibitor p27^{KIP1} and repressing that of cyclin D1 (43, 44).

Identification of VDREs Conserved between Human and Mouse

Apart from combining genome-wide screens with microarray analyses, another way to assess the potential functional relevance of response elements is to determine whether they are conserved between species. We screened the most recent build of the mouse genome and used the data to identify VDREs that were conserved between human and mouse (supplemental Table 5 published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). Similar to our previous study (24), we were more concerned with assessing conserved gene regulation rather than simply VDRE sequence and used a relatively relaxed definition of conservation. We therefore screened for consensus VDREs or DR3 elements with single-nucleotide substitutions and included elements as being conserved between species even if they differed in VDRE sequence. As in our previous analysis of conserved estrogen response elements (24), we screened for elements that differed by less than 2 kb in their positions relative to the 5′-ends of genes. This approach identified a total of 3537 elements in the human and mouse genomes that were conserved in 3062 genes. The human homologs of 157 of these genes were found to be regulated in our Hu133A microarray study (supplemental Table 5), of which 126 were induced and 31 were repressed (4.06:1), which is essentially the same ratio as the total numbers of induced and repressed genes identified by microarray analysis (734/179; 4.10: 1). Conserved elements include the ER6 sequence in the *clmn* gene, and (multiple) DR3 elements in the *foxo1a*, *hsd17b2*, *hif1a*, *klk6, sema3B*, *serpinB1*, and *trpv6* genes characterized in Figs. 3 and 4. The screen also identified conserved downstream DR3 elements in mouse and human *gadd45*, a gene that we found to be regulated by 1,25(OH)$_2$D$_3$ in human and mouse squamous carcinoma cells (supplemental Table 1 and Refs. 26 and 45).

Although conservation is an indicator of function, we note that there are several instances in which 1,25(OH)$_2$D$_3$ regulation of target gene expression is not conserved between human and mouse. For example, the gene encoding the noncollagen Ca^{++} binding matrix protein osteocalcin is robustly induced by 1,25(OH)$_2$D$_3$ in human but not in mouse (46, 47). Similarly, we identified consensus promoter-proximal DR3-type VDREs in the promoters of the *defB2* and *camp* antimicrobial peptide genes, which mediated their induction by 1,25(OH)$_2$D$_3$ (38). However, neither the elements nor the regulation by 1,25(OH)$_2$D$_3$ appears to be conserved in mouse (data not shown).

Complementarity of *in Silico* Response Element Screens and Microarray Analyses

Microarray analysis and *in silico* response element screens are complementary in many respects. Identification of 1,25(OH)$_2$D$_3$-regulated genes on microarrays depends on the source of RNA under study, the number of genes represented on the microarray and the sensitivity and accuracy of hybridization and data analysis protocols. *In silico* response element screens have the potential of identification of target genes independent of their tissue of expression. Importantly, they also provide a mechanism of regulation and a form of validation of microarray data. In this regard, we reiterate that we have not tried to be exhaustive; *i.e.* by screening the genome for all potential degenerate response elements. For example, the screens did not identify any VDREs in the promoter of the 1,25(OH)$_2$D$_3$-responsive *mad* gene. However, analysis of the regulatory region of the *mad* gene revealed several putative VDREs with two nucleotide substitutions (data not shown). Similarly, no consensus or near-consensus VDREs were identified in the promoter of the highly inducible *cyp24* gene, which contains a proximal element containing more than one substitution (48). Thus, more degenerate elements are likely to be present in several other regulated genes. Alternatively, the VDR may associate with target gene promoters by interacting with other classes of transcription factors (*e.g*. Ref. 49).

Our results do show that *in silico* screens can reveal target genes not identified by microarray analysis. For example, expression of *defb4* and *camp*, which encode antimicrobial peptides, is induced by 1,25(OH)$_2$D$_3$ in SCC25 cells, as well as cells of the immune system, consistent with the enhanced secretion of antimicrobial activity by 1,25(OH)$_2$D$_3$-treated cells (38). However, neither gene was identified in the Hu133A analysis. Similarly, genes encoding CASP5, dual specificity tyrosine phosphorylation-regulated kinase 3, and S100 calcium binding protein A2 were identified in screens for consensus DR3 or ER6 elements (supplemental Tables 2 and 4), but were not picked up by microarray analysis. They were found to be 1,25(OH)$_2$D$_3$ regulated by RT-PCR analysis, and binding of the VDR to VDREs *in vivo* was confirmed by ChIP assay (Fig. 3B). Regulation of S100 calcium binding protein A2 is of interest in understanding the anticancer properties of 1,25(OH)$_2$D$_3$ because it is a marker of epithelial cell differentiation, and its expression is predictive of survival in esophageal cancer (50).

One caveat that must be taken into account with the use of *in silico* screens alone for identification of transcription factor target genes is the possible identification of false positives; *i.e.* nonregulated genes containing binding sites. However, it should be noted that lack of regulation of a gene in a given cell type is not sufficient to eliminate it as a potential 1,25(OH)$_2$D$_3$ target. Although the VDR is widely expressed, many of its target genes are expressed in a cell-specific manner. For example, whereas expression of *defB4* and *camp* by 1,25(OH$_2$)D$_3$ was induced in SCC25 cells, only *camp* expression was regulated in cells of nonepithelial origin such as monocytes and neutrophils (38) in spite of the presence of a consensus DR3 element in the *defB4* promoter. An analysis of *defB4* regulation in monocytes, for example, would therefore have concluded that the *defB4* gene represented a false positive. Similarly, in this regard, expression of target several genes identified in this study was only modestly affected by 1,25(OH)$_2$D$_3$ treatment. However, because expression of many genes is modified by multiple signal transduction pathways, the effect of 1,25(OH)$_2$D$_3$ could be magnified in the presence of other transducers. For example, whereas expression of *defB4* was induced only modestly (2-fold) by 1,25(OH$_2$)D$_3$ alone in epithelial cells, the magnitude of the effect of 1,25(OH$_2$)D$_3$ was amplified in the presence of IL-1, another inducer of *defB4* expression (38).

In summary, we have used a combined approach of microarray analysis and *in silico* genome-wide screens for DR3 and ER6-type VDREs to identify direct 1,25(OH)$_2$D$_3$ target genes on a large scale. This approach identified VDREs in several known 1,25(OH)$_2$D$_3$-responsive genes and identified several novel 1,25(OH)$_2$D$_3$ target genes. The finding will help provide a molecular genetic basis for the broad physiological actions of 1,25(OH)$_2$D$_3$.

MATERIALS AND METHODS

Bioinformatics

The algorithms developed (24) were used to search the NCBI fasta and gbs files of the Human genome reference assembly (Build 35 version 1; August 26, 2004) and Mouse genome (Build 34 version 1; May 19, 2005) for a specified group of sequences and extract the positions of matching motifs in the genome contigs as well as the coordinates of the surrounding genes, mRNAs, and coding sequence within a preset cutoff distance of each motif. Homologous genes between human and mouse were identified with the NCBI HomoloGene database (Build 41 version 1; May 26, 2005). The algorithms were implemented with the Bioperl toolkit (51) and run on the bioinformatics cluster of The Quebec Bioinformatics Network (BioneQ; http://www.bioneq.qc.ca/). Results presented in this article were generated using a cutoff of -10 to $+5$ kb of the gene 5′-ends.

Recombinant Plasmids

Sequences of the *col13A1* promoter containing between -1273 or -1220 and $+23$ were generated by PCR amplification using primers described in supplemental Table 6 published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org. Fragments were cloned directly into PCR2.1 (Invitrogen, Burlington, Ontario, Canada) and then digested with *Hin*dIII and *Xho*I and subcloned into luciferase reporter plasmid pXP2 to make col13A1-p/pXP2 and col13A1-p(-V)/pXP2. *Trpv6* promoter sequences between -1590 or -1490 and $+223$ were cloned by PCR amplification of genomic DNA using primers described in supplemental Table 6. Fragments were cloned directly into PCR2.1 (Invitrogen), and then digested with *Hin*dIII and *Xho*I and subcloned into luciferase reporter plasmid pXP2 to make trpv6-p/pXP2 and trpv6-p(-V)/pXP2. The VDRE3-thymidine kinase promoter of the positive control plasmid, composed of three mouse osteopontin VDREs inserted upstream of a truncated thymidine kinase promoter, has been described (52).

Tissue Culture and Transfection

All lines were cultured under recommended conditions. SCC25, Calu-3, and U937 were obtained from American Type Culture Collection (Manassas, VA). Effects of 1,25(OH)$_2$D$_3$ on cell growth were analyzed by seeding cells in 100-mm petri dishes at 60–70% confluence in 10 ml of culture medium containing 10% fetal bovine serum (FBS). Media were changed after 24 h to charcoal-stripped medium containing 0.1 μM EB1089. Media were changed every 48 h, and fresh ligand was added. COS-7 cells grown in 6-cm wells in DMEM, supplemented with 10% FBS, were transfected in medium without serum with Lipofectamine 2000 (Invitrogen) with 100 ng of nuclear receptor expression vector pSG5/VDR, 300 ng of trpv6-p/pXP2, or trpv6-p(-V)/pXP2, and 100 ng of internal control vector pCMV-β-gal. Medium was replaced 6 h after transfection by DMEM, supplemented with 10% FBS. After 24 h, medium was replaced by a medium containing charcoal-stripped serum and ligand (100 nM) for 24 h. Cells were harvested in 200 μl of luciferase reporter lysis buffer (Promega Corp., Madison, WI).

RNA Isolation

Cells were grown in 100-mm dishes. Media were replaced with charcoal-stripped medium containing ligand. Total RNA was extracted with TRIZOL (GIBCO/BRL, Burlington, Ontario, Canada).

Microarray Analysis

Affymetrix Hu133A oligonucleotide microarray analyses were performed at the McGill University and Genome Quebec Innovation Centre. RNA integrity was verified using an Agilent 2100 Bioanalyzer. Probe was prepared from 10 μg of total RNA using the Affymetrix one-step protocol (20).

RT-PCR

Total RNA (3–5 μg) was subjected to oligo dT priming first-strand cDNA synthesis by SuperScript II (Invitrogen). For RT-PCR analysis of mRNA expression using primers described in supplemental Table 6, 3 μl of reverse transcription (RT) reactions was analyzed by PCR amplification as follows: 30 sec denaturation at 94 C, 45 sec elongation at 72 C, and 30 sec annealing starting at 60 C, down 1 C per cycle to 55 C, and continuing 20 cycles amplification (94 C for 30 sec, 57.5 C for 30 sec, 72 C for 45 sec). cDNAs were amplified using 5′-primer and 3′-primer. For amplification of glyceraldehyde 3-phosphate dehydrogenase, 1 μl of reverse transcription reaction was subjected to 18 cycles amplification (95 C for 30 sec, 56 C for 1 min, 72 C for 25 sec) using 5′-primer 5′-GGTGAAGGTCGGTGTCAACG-3′, and 3′-primer 5′-CAAAGTTGTCATGGATGACC-3′. All of the above reactions were performed in 50 μl of 1.5 mM MgCl$_2$, 50 mM KCl, and 10 mM Tris-HCl (pH 9.0) using 2.5 U of *Taq* DNA polymerase (Pharmacia, Baie d'Urfe, Quebec, Canada). PCR reactions were loaded on 0.8% agarose gel and analyzed. All experiments were repeated at least three times.

ChIP Assays

ChIP assays were performed essentially as described (52). SCC25 cells were propagated in charcoal-stripped serum and treated with 1,25(OH)$_2$D$_3$ or vehicle for 3 h before lysis. Lysates were immunoprecipitated with either normal rabbit IgG or anti-VDR (C-20) rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). PCRs were performed with primers listed in supplemental Table 6.

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