Transcriptional Synergy between Vitamin D-responsive Elements in the Rat 25-Hydroxyvitamin D₃ 24-Hydroxylase (CYP24) Promoter*  

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Transcription of the CYP24 gene is induced by 1,25-(OH)₂D₃ through a vitamin D receptor-dependent process. The functional activities of three possible vitamin D response elements (VDREs), located on the antisense strand of the rat CYP24 promoter, were investigated by transient expression of native and mutant promoter constructs in COS-1, JTC-12, and ROS 17/2.8 cells. A putative VDRE with a half-site spacing of 6 base pairs at −249/−255 (VDRE-3) did not contribute to 1,25-(OH)₂D₃ induced expression in the native promoter, although its activity has been reported when the element was fused to the heterologous thymidine kinase promoter. Two VDREs with half-site spacings of 3 base pairs at −150/−153 and −258/−244 (VDRE-1 and VDRE-2, respectively) showed transcriptional synergism in COS-1 cells when treated with 1,25-(OH)₂D₃ (10⁻⁷ to 10⁻¹¹ M). The contribution of both VDREs was hormone-concentration dependent from 10⁻¹⁰ to 10⁻¹² M, with VDRE-1 demonstrating greatest sensitivity to 1,25-(OH)₂D₃. Transactivation by VDRE-1 was always greater than VDRE-2, but the converse was observed for the binding of vitamin D receptor-retinoid X receptor complex by each VDRE in gel mobility shift assays. The synergy observed between VDRE-1 and VDRE-2 may have important implications in cellular responses to different circulating levels of 1,25-(OH)₂D₃.

The hormone 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃, or calcitriol) is a pleiotropic secosteroid that functions in the regulation of calcium homeostasis, cellular differentiation and proliferation, and immune function (1-5). Nuclear actions of 1,25-(OH)₂D₃ involve the transcriptional regulation of gene expression, which is mediated by the vitamin D receptor (VDR), a ligand-activated transcription factor that belongs to the nuclear receptor superfamily (1, 6-10). Activated VDR can bind as either a homodimer or a heterodimeric complex to a DNA sequence known as the vitamin D-responsive element (VDRE) present in the promoter of target genes (11-13). Heterodimers consisting of VDR and retinoid X receptor (RXR) are widely documented (12), although VDR heterodimeric complexes have also been demonstrated for both the retinoic acid receptor (14, 15) and the thyroid hormone receptor (16). Vitamin D-responsive elements generally display a binding motif that consists of two imperfect, direct repeat hexameric sequences (i.e. half-sites) that are separated by 3 bp or, more rarely, by 6 bp; these VDREs are referred to as DR-3 and DR-6, respectively (12).

Metabolic inactivation of 1,25-(OH)₂D₃ and conversion to water-soluble calcitroic acid occurs through the C-24 oxidation pathway. The initial step in this pathway involves the 24-hydroxylation of 1,25-(OH)₂D₃ by the mitochondrial enzyme 25-hydroxyvitamin D₃ 24-hydroxylase (CYP24) (17, 18). Rats fed a normal diet express a low level of CYP24, predominantly in the kidney. However, the enzyme can be substantially induced in kidney and intestine (19-23) and various other cells (24-29) by 1,25-(OH)₂D₃ treatment. Up-regulation of CYP24 expression (19-23) increases the metabolic clearance of 1,25-(OH)₂D₃, and, thereby, feedback regulates the hormone’s ambient and cellular levels (17, 18). The mechanism whereby 1,25-(OH)₂D₃ acts to modulate cellular CYP24 expression is of fundamental importance to understanding the hormone’s role in health and disease.

Molecular regulatory studies of CYP24 gene expression by 1,25-(OH)₂D₃ are in progress and promoter analysis data for rat (14, 30-33) and human (34) have been reported. In the rat CYP24 gene promoter, three VDREs on the antisense strand have been identified. We have previously defined the proximal VDRE (30) in its native promoter context, while the two more distal VDREs have been tested by fusing to a heterologous promoter (14, 31). To date, however, the functionality of the VDREs has not been verified in the context of the native CYP24 promoter, and there is no direct information available regarding the contribution of each VDRE to vitamin D induction or whether there is a cooperative interaction between the response elements. These issues are addressed in the current investigation, in which mutagenic constructs of the rat CYP24 promoter have been used in transient gene expression and gel mobility shift analysis.

EXPERIMENTAL PROCEDURES

Materials—Hoffmann La Roche (Nutley, NJ) generously supplied the 1,25-(OH)₂D₃. A Sequenase version 2.0 sequencing kit was obtained from U. S. Biochemical Corp. Synthetic oligonucleotides were synthesized by Bresatec (Adelaide, Australia). Luciferin was from Promega Corp. (Madison, WI).

Construction of Mutant Clones—A 365-bp PvuII/StuI fragment containing 298 bp of 5'-flanking sequence and encompassing the putative VDREs together with 74 bp of 5'-untranslated region was isolated from a rat CYP24 genomic clone (30) and used as template for site-directed
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mutagenesis. The fragment was cloned into the Hincl site of pBlue- 
script KS⁺ in the antisense orientation to generate pBKS-WT, and
single-stranded DNA purified as described by Kunkel et al. (35). Briefly, Escherichia coli CJ236 (dut⁺, ung-) was transformed with pBKS-WT
and superinfected with helper phage M13K07. Single-stranded DNA
was purified by NaCl/PEG precipitation and used as template in the
mutagenesis reactions. The primers employed (P1, P2, P4, and P5)
was purified by NaCl/PEG precipitation and used as template in the
and superinfected with helper phage M13K07. Single-stranded DNA
supplemented with 10% FCS. In preparation for electroporation, cells
supplemented with 10% fetal calf serum (FCS). Rat osteosarcoma-
fragments were excised from pBKS-WT by digestion with
site was introduced to produce the mutated nucleotides.
maintenance and Transfection of Tissue Culture Cells—
proximal tubular JTC-12 cells (kindly supplied by
were maintained in Dulbecco's modified Eagle's medium (DMEM)
were synthesized that encompassed the VDRE-1, VDRE-2, or VDRE-3
were transfected, either individually or in combination, by site-directed
for 10 min and divided (1.5 × 10⁶ cells) into two wells of a six-well plate
Gel Mobility Shift Assays—Double-stranded oligonucleotide probes
were synthesized that encompassed the VDRE-1, VDRE-2, or VDRE-3
in the rat CYP24 promoter. An oligonucleotide that encompassed a
site at molar excess concentrations in the bind-
for 10 h and then the media replaced with RPMI medium
(without phenol red) supplemented with 12% charcoal-stripped FCS.
Ethanol carrier or 1,25-(OH)₂D₃, was added at the indicated concentra-
tions and the cells incubated for 24 h prior to harvesting.
nuclease reporter gene (Promega). Plasmid DNA was prepared by
alkaline lysis and CsCl/ethidium bromide equilibrium density gradi-
ents (37). All plasmid DNA was quantified by spectrophotometry, and
supercoll was formed by 1% aagarose gel analysis to ensure
Maintenance and Transfection of Tissue Culture Cells—COS-1 cells
and monkey kidney proximal tubular JTC-12 cells (kindly supplied by
at the 5’ or 3’ ends, as shown below.

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| VDRE-1: | 5'-TCGAGGGGCCGCTTAAGTACGCGGACGCGGAGCT-3' |
| VDRE-2: | 3'-GGCGCCGCGCGACGCGCGGAGCT-5' |
| VDRE-3: | 5'-TCGACGCAGCCGCTGACCGACCGCGCGCGGAGCT-3' |
| mSpp1:  | 3'-CCACCGAGCCACCGGACGGACGCGGAGCT-5' |
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Each double-stranded oligonucleotide was labeled by end-filling with
[α-³²P]dCTP using Klenow enzyme and purified by 12% polyacrylamide
gel electrophoresis. Nuclear extracts from 1,25-(OH)₂D₃-treated COS-1
cells were prepared with or without co-transfection of pRSV-hVDR
expression vector, as described previously (30). Binding reactions for
each assay contained 5 μg of nuclear protein, 1 μg of poly(dI-dC) to a
final volume of 12 μl in binding buffer (25 mM Tris-HCl, pH 7.6, 100 mM
KCl, 0.5 mM dithiothreitol, 5 mM MgCl₂, 0.5 mM EDTA, and 10%
glycerol) and were incubated on ice for 10 min. Radiolabeled probe
(200,000 cpm) was added and samples incubated on ice for another 20
min. For gel shift inhibition assays, a VDR monoclonal antibody desig-
nated IgG2b (Affinity BioReagents Inc., Neshanic Station, NJ) was
employed. For supershift assays, an RXX monoclonal antibody (4X-
ID12, Ref. 41), kindly provided by Dr Pierre Chambon (Strasbourg
Cedex, France), was employed. These antibodies were included in the
binding reactions and incubated on ice for 10 min prior to addition of
probe. Gel shift competition assays were performed with unlabeled
competitor oligonucleotide at molar excess concentrations in the bind-
ing reaction. Retarded DNA nuclear protein complexes were resolved on
a 4% nondenaturing polyacrylamide gel in a low ionic strength running
buffer (0.5 × TBE) at 4 °C. The gel was dried and exposed to Kodak
X-Omat AR film with an intensifying screen at ~70 °C.

RESULTS

Transient Expression of Promoter Constructs in COS-1, JTC-
12, and ROS 17/2.8 Cells—Mutational analysis was used to de-
termine the functionality and cooperative interaction of
three putative VDREs in the rat CYP24 gene promoter. The three
VDREs (i.e. VDRE-1, VDRE-2, and VDRE-3) were altered,
either individually or in combination, by site-directed
mutagenesis (Figs. 1 and 2). The proximal half-site of VDRE-1

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Dr T. Matsumoto, University of Tokyo School of Medicine, Japan; Ref. 38) were maintained in Dulbecco's modified Eagle's medium (DMEM)
supplemented with 10% fetal calf serum (FCS). Rat osteosarcoma-
derived ROS 17/2.8 cells were maintained in DMEM/Ham's F-12 (1:1)
supplemented with 10% FCS. In preparation for electroporation, cells
were grown to 80–90% confluence, removed by trypsinization, washed
once in phosphate-buffered saline and resuspended at (6 × 10⁶ cells/ml)
in 20 mM Hepes (pH 7.05) containing 137 mM NaCl, 5 mM KCl, 0.7 mM
Na₂HPO₄, 6 mM dextrose (39), and 500 μM sheared salmon sperm
DNA (New England Biolabs) and 1 pmol of construct DNA
were electroporated at 280 V and 960 microfarads, while ROS 17/2.8
cells were electroporated at 200 V and 960 microfarads using a Bio-Rad
Gene Pulser. COS-1 cells were also co-transfected with VDR expression
clon pRSV-hVDR, generated by cloning the human VDR cDNA se-
quence (kindly supplied by Nigel Morrison, Garvan Institute for Med-
ical Research Sydney, Australia) downstream of the Rous sarcoma virus
promoter. Following electroporation, the samples were placed on ice
for 10 min and divided (1.5 × 10⁶ cells) into two wells of a six-well plate
containing DMEM, 10% FCS (for COS-1 and JTC-12 cells) or DMEM/
Ham's F-12, 10% FCS (for ROS 17/2.8 cells). Cells were allowed to
recover for 20 h and then the media replaced with RPMI medium
(without phenol red) supplemented with 12% charcoal-stripped FCS.

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Fig. 1. Mutations introduced into the VDREs located in the rat CYP24 gene promoter. A, sequence of the native promoter encom-
compassing the three VDREs. The numbers above the lines indicate the
distance relative to the transcription initiation site. Arrows indicate
VDRE hexameric half-sites. B, different mutations (shown in bold type)
introduced into VDRE-1 (M1), VDRE-2 (M2), VDRE-2 and VDRE-3
(M3), all three VDREs (M4), VDRE-3 (M5), and VDRE-1 and VDRE-2
(M6).

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A

![Image of alignment showing differences in VDRE sequences]

B

![Diagram of mutations introduced into VDREs]
and the distal half-site of VDRE-2 were each mutated in the constructs pG3-M1 and pG3-M2, respectively. Both half-sites of VDRE-2 (which included a common half-site with VDRE-3) were mutated in construct pG3-M3. Mutations were introduced into all three VDREs in construct pG3-M4, while only VDRE-3 was mutated in construct pG3-M5. In pG3-M6, half-sites in both VDRE-1 and VDRE-2 were mutated, leaving VDRE-3 intact. The impact of the half-site mutations on 1,25-(OH)2D3-directed transcriptional activation of the gene promoter was evaluated in COS-1, JTC-12, and ROS 17/2.8 cells. In the absence of added 1,25-(OH)2D3, basal expression of each of the mutant constructs in the different cell lines was the same as the wild-type construct, demonstrating that these VDREs do not contribute to basal expression (results not shown).

Transient transfection experiments were performed initially in COS-1 cells that were co-transfected with CYP24 promoter/luciferase constructs and VDR expression vector (pRSV-hVDR) to compensate for the deficiency of VDR in the COS-1 cell line. In response to 1,25-(OH)2D3 treatment, the wild-type construct (pG3-WT) gave a 17.8-fold level of induction in these cells (Fig. 2). When VDRE-1 alone was mutated (pG3-M1), leaving the distal VDRE-2 and VDRE-3 intact, the level of 1,25-(OH)2D3 induction was reduced to 2.8-fold, which demonstrated the substantial contribution of VDRE-1 to promoter activity. A 6.6-fold level of induction was observed with the pG3-M3 construct in which both VDRE-2 and VDRE-3 were mutated, and essentially the same level was observed when both VDRE-2 and VDRE-3 were mutated in pG3-M3 (Fig. 2). These findings established that both VDRE-1 and VDRE-2 are functional and suggested that VDRE-3 does not significantly contribute to induction even when the overlapping VDRE-2 is inactivated. To confirm this latter finding, the expression of pG3-M5, in which the proximal half-site in VDRE-3 was altered, was investigated. The hormone induction observed with this construct was similar to the wild type. The possible role of VDRE-3 was examined further by mutating both VDRE-1 and VDRE-2 and leaving VDRE-3 intact. This construct (pG3-M6) was inactive (Fig. 2). There was no response to 1,25-(OH)2D3 by the construct pG3-M4, in which all three VDREs were altered. Thus, when evaluated in all three cell types, VDRE-1 and VDRE-2 were responsible for the 1,25-(OH)2D3-mediated induction of the wild-type promoter region. The induction seen for the wild-type construct (17.8-fold) was greater than the sum of the individual contributions of VDRE-1 (6.6-fold) and VDRE-2 (2.8-fold), thus demonstrating transcriptional synergism.

Cell specificity for the synergism between VDRE-1 and VDRE-2 was also evaluated in JTC-12 and ROS 17/2.8 cells. Both cell lines express endogenous VDR and respond to 1,25-(OH)2D3-mediated gene induction (38, 42). Expression of the wild-type and mutant CYP24 promoter constructs in JTC-12 and ROS 17/2.8 cells produced transactivation results that were similar to those obtained in COS-1 cells (Fig. 2). A nearly 2-fold synergistic action between VDRE-1 and VDRE-2 was demonstrated in all three cell lines, in which a greater contribution was observed for VDRE-1 compared with VDRE-2 (Fig. 2).

VDREs Bind Nuclear Protein Complexes—Binding of nuclear proteins to the VDREs was investigated by gel mobility shift analysis using extracts from COS-1 cells transfected with pRSV-hVDR expression vector and treated with 10−7 M 1,25-(OH)2D3 (30). An oligonucleotide encompassing the mouse osteopontin VDRE (i.e. mSpp1-VDRE) was employed as a control probe and contained a functionally active VDRE known to bind strongly the VDR-RXR complex (40). A major protein complex of the same mobility as the mSpp1-VDRE was detected with VDRE-1 and VDRE-2, but the bands were not evident when probes were incubated with nuclear extract prepared from COS-1 cells not transfected with pRSV-hVDR (Fig. 3A). This finding indicated that VDR is present in the protein complex that binds to VDRE-1 and VDRE-2. Competition experiments using a 10-fold molar excess of mSpp1-VDRE completely prevented formation of the protein complex observed with either VDRE-1 or VDRE-2 (results not shown). To further characterize the protein complex, we employed both a monoclonal antibody to VDR that interfered with DNA binding of VDR and a supershifting RXR monoclonal antibody. The VDR monoclonal antibody prevented formation of the major protein complex detected when radiolabeled VDRE-1, VDRE-2, or mSpp1-VDRE were used as probes (Fig. 3B). The RXR monoclonal antibody supershifted the VDR-containing complex obtained with each of these probes (Fig. 3C). It can be concluded from these data that the major protein complex that binds to VDRE-1 and VDRE-2 contains both VDR and RXR. Other gel mobility shift experiments with mutant oligonucleotides established that the mutations introduced into VDRE-1 and VDRE-2 inhibited completely the binding of the VDR-RXR complex (data not shown).

Based upon the intensity of the protein-VDRE complexes, it appeared that VDRE-2 had a greater binding affinity than
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Fig. 3. Gel shift analysis using oligomers to VDRE-1 and VDRE-2. A, double-stranded oligomers to VDRE-1, VDRE-2, and mouse osteopontin VDRE (mSpp1-VDRE) were labeled by end-filling with \([\alpha-32P]dCTP\) and incubated with nuclear extracts of COS-1 cells either mock-transfected (−) or transfected with 5 μg of pRSV-hVDR (+). The major retarded complex (arrowed) is unique to pRSV-hVDR-transfected cells. B, for neutralization assays, nuclear extracts (from COS-1 cells transfected with pRSV-hVDR) were incubated with (+) or without (−) VDR monoclonal antibody (VDR mAb) prior to addition of labeled probes for VDRE-1, VDRE-2, and mSpp1-VDRE. C, for supershift assays, as in B but using RXR monoclonal antibody (RXR mAb). The upper arrow (*) indicates the supershifted complex.

VDRE-1 while the mSpp1-VDRE had a higher binding affinity than either VDRE-1 or VDRE-2 (Fig. 3). To investigate the binding affinity of VDRE-1 and VDRE-2 more directly, competition of the radiolabeled mSpp1-VDRE probe was undertaken with either unlabeled VDRE-1 or VDRE-2 at 10-, 25-, 50-, 100-, and 200-fold molar excess. The results showed that the protein complex was efficiently competed by a 10-fold excess of the competitor VDRE-2, but a 50-fold excess of VDRE-1 was required for significant competition (Fig. 4A). These results confirmed that the complex containing VDR and RXR has a stronger binding affinity for VDRE-2 than VDRE-1. Competition experiments were also carried out using radiolabeled VDRE-1 or VDRE-2 as probes and each of these oligonucleotides as competitors. With self-competition, the binding of the VDR or VDRE-2 as probes and each of these oligonucleotides as competitors. With self-competition, the binding of the VDR

Studies in COS-1 Cells Treated with Different Amounts of 1,25-(OH)\(_2\)D\(_3\) or VDR—Having used experimental conditions in which 1,25-(OH)\(_2\)D\(_3\) was non-limiting (10\(^{-7}\) M), it was decided to evaluate promoter activity in COS-1 cells under more physiological conditions by incrementally decreasing the level of hormone challenge to 10\(^{-9}\) M, 10\(^{-10}\) M, and 7.7 \(\times\) 10\(^{-11}\) M (Fig. 5A). However, when cells were treated with 7.7 \(\times\) 10\(^{-11}\) M 1,25-(OH)\(_2\)D\(_3\), the induction was reduced to 8.3-fold and was further lowered to 3.7-fold with 1.25-(OH)\(_2\)D\(_3\) at 10\(^{-12}\) M (Fig. 5A). Mutant constructs of VDRE-1 (pGL3-M1) and VDRE-2 (pGL3-M2) were active at 10\(^{-11}\) M 1,25-(OH)\(_2\)D\(_3\), giving 2.1-fold and 3.3-fold levels of induction, and they retained their transcriptional synergism. At the lowest level of 1,25-(OH)\(_2\)D\(_3\) tested (10\(^{-12}\) M), only VDRE-1 was found to be responsive (Fig. 5A). Separate experiments confirmed that VDRE-3 did not contribute to the induction response (when VDRE-1 and VDRE-2 were functional) under the decreasing concentrations of 1,25-(OH)\(_2\)D\(_3\) employed (data not shown).

The possible impact of cellular VDR concentration on the synergistic interaction between VDRE-1 and VDRE-2 was evaluated in COS-1 cells receiving one-tenth the amount of the transfected pRSV-hVDR. Under these conditions, the wild-type promoter construct gave an 8.9-fold level of induction compared with 16.4-fold when cells were co-transfected with the full amount of pRSV-hVDR (Fig. 5B). Studies with the mutant constructs pGL3-M1 and pGL3-M2 also showed a decreased level of induction, but the cooperative interaction between VDRE-1 and VDRE-2 was retained. Upon comparing results at different 1,25-(OH)\(_2\)D\(_3\) and VDR levels, it was noted that VDRE-1 makes a greater contribution to the hormone’s cellular induction than VDRE-2. Such results are diametric to the VDR-RXR binding affinities for the two VDREs, as measured by gel mobility shift analysis.

DISCUSSION

Transcription of the rat cytochrome P450\(_{24}\) (CYP24) gene is induced by 1,25-(OH)\(_2\)D\(_3\) (19–23). Three VDREs in the first 298 bp of the 5′-flanking sequence have been reported to participate in regulation of the rat CYP24 gene (14, 30–33). To date, only the proximal VDRE (VDRE-1) has been evaluated for function in the context of its native promoter (30). The other putative VDREs (VDRE-2 and VDRE-3) were characterized when linked to the heterologous thymidine kinase promoter (14, 31), which does not reflect native promoter architecture and function. Using constructs containing the first 298 bp of the CYP24 gene promoter, we have now characterized VDRE-1 and VDRE-2 as functional hormone response elements. Upregulation of the CYP24 promoter regulation in three different cell lines is consistent with the operation of a general modulatory loop that functions in 1,25-(OH)\(_2\)D\(_3\)-responsive cells expressing the CYP24 gene. A very similar regulatory pathway also appears to be present in the human CYP24 promoter, which has been verified to contain two VDREs of the DR-3 type at about the same position as the VDRE-1 and VDRE-2 in the rat (34).

VDRE-3 with a 6-bp spacing was not responsive to 1,25-(OH)\(_2\)D\(_3\) when tested in three cell lines following selected mutation of VDRE half-sites. The data from these cellular promoter expression studies demonstrate convincingly that VDRE-3 is not functional. Our findings with VDRE-3 are in contrast to those of Kahlen and Carlberg (14), who reported that this sequence responds to 1,25-(OH)\(_2\)D\(_3\) (4–6-fold) when fused to the thymidine kinase promoter and expressed transiently in ROS 17/2.8 cells. We conclude that the environment of the VDRE-3 in the rat CYP24 promoter is not conducive to 1,25-(OH)\(_2\)D\(_3\) responsiveness, at least in the cell types examined, and our study emphasizes the importance of investigating the functional role of a VDRE in the context of its native promoter architecture. Nevertheless, it is possible that VDRE-3 may be active in an appropriate cellular environment, since the sequence has been shown in gel mobility shift assays to bind VDR homodimers, heterodimers of VDR, and retinoic acid receptors (14) and, in the present work, to bind weakly a complex of VDR-RXR (data not shown). In the human CYP24 promoter (34), the sequence 5′-ATGCGAaagccgGAGTTC-3′ corresponds to the rat VDRE-3, but its function has not been investigated. Other DR-6 type VDREs have been identified in...
the promoters for osteocalcin and fibronectin (12). The human osteocalcin DR-6 motif confers 1,25-(OH)2D3 inducibility when fused to the thymidine kinase promoter (13). However, as far as we are aware, it has not been established whether the osteocalcin DR-6 or fibronectin DR-6 motifs contribute to 1,25-(OH)2D3 responsiveness in their natural promoters.

Gel mobility shift analysis was used to investigate the protein complexes in COS-1 cell nuclear extracts that bind to VDRE-1 and VDRE-2. A major retarded band was identified with each VDRE, and antibody studies established that this complex contained both VDR and RXR. This result agrees with other studies, where different DR-3 type VDRE sequences have been shown to bind this heterodimer (12). Gel shift competition data indicated that the VDR-RXR protein complex bound more tightly to VDRE-2 than VDRE-1, a somewhat unexpected result, since the contribution of VDRE-1 to the 1,25-(OH)2D3 inductive response was always greater than that of VDRE-2. Liu and Freedman (43) have demonstrated substantial tran-

FIG. 4. Competition studies with VDRE-1 and VDRE-2. A, a double-stranded oligomer to mSpp1-VDRE was labeled by end-filling with [α-32P]dCTP, incubated with nuclear extracts from COS-1 cells transfected with 5 μg of pRSV-hVDR, and competed with unlabeled VDRE-1 or VDRE-2 at 10-, 25-, 50-, 100-, or 200-fold molar excess. The major protein complex containing VDR-RXR is arrowed. The reduced intensity of bands in the 10× lane for VDRE-2 is due to a loading variation. B, double-stranded oligomers for VDRE-1 and VDRE-2 were labeled by end-filling, incubated with nuclear extracts as in A, and competed with either VDRE at 5-, 10-, and 20-fold molar excess for self-competition and 10-, 50-, and 100-fold excess for the cross-competition. The VDR-RXR complex is arrowed.

FIG. 5. Transient expression of promoter-luciferase constructs in COS-1 cells in the presence of different concentrations of 1,25-(OH)2D3 and pRSV-hVDR. A, induction of luciferase expression in COS-1 cells treated with 1,25-(OH)2D3 at a concentration range of 10-10 to 10-7 M and co-transfected with 1 μg of pRSV-hVDR. Values shown (†) are for 10-10 M 1,25(OH)2D3; essentially identical values were seen with 10-7, 10-8, and 10-9 M. The levels of luciferase expression are shown as the ratio of luciferase activity from 1,25-(OH)2D3-treated cells to that from untreated cells. Data presented are the average of three experiments ± S.D. B, 6-fold induction of luciferase activity in COS-1 cells co-transfected with 100 ng or 1 μg of pRSV-hVDR and treated with 10-7 M 1,25-(OH)2D3. Arrows indicate VDRE hexameric half-sites, with X indicating a mutation site.
criptional synergism between VDR and various classes of non-receptor transcription factors when the DNA binding sites for these proteins are positioned closely in a reporter plasmid. Such a situation may explain the greater transactivation of VDRE-1, in which its affinity for VDR-RXR (compared with VDRE-2) is enhanced through a cooperative interaction with nearby, and as yet unidentified, promoter-bound transcription factor(s). It is also possible that the low 1,25-(OH)_{2}D_{3} transactivation contributed by VDRE-2 arises from the greater distance of this VDRE from the transcriptional machinery. This seems unlikely, however, since deleting the promoter region between the two VDREs does not alter transactivation by 1,25-(OH)_{2}D_{3} (31).

A significant finding in the present work is the transcriptional synergistic response between VDRE-1 and VDRE-2, which resulted in an 18-fold level of induction in COS-1 cells. Transient studies of CYP24 promoter constructs in COS-1 cells under non-limiting conditions for VDR or 1,25-(OH)_{2}D_{3} showed that the transactivation was about twice the sum of the hormone-dependent transactivations for VDRE-1 and VDRE-2 when evaluated separately. A similar level of synergism between these VDREs was also seen in JTC-12 and ROS 17/2.8 cells in which VDR is expressed endogenously. The mutations introduced into VDRE-1 or VDRE-2 inhibited completely the response of each to 1,25-(OH)_{2}D_{3} induction. In previous work (30), mutated VDRE-1 prevented 1,25-(OH)_{2}D_{3} induction from a CYP24 promoter that contained this VDRE but not VDRE-2. Combined mutagenesis of VDRE-1 and VDRE-2, in the current work, prevented induction and demonstrated the effectiveness of both mutations. Hence, the coordinated interaction between the two VDREs appears to be the sole basis for the observed synergism.

Synergistic induction of the rat CYP24 promoter constitutes one of the highest stimulations observed so far for any 1,25-(OH)_{2}D_{3}-responsive promoter and would appear to be the only regulatory region in which two VDREs have been shown to be functional in their native promoter. The synergism observed between the two VDREs may be at the level of enhanced cooperative DNA binding of the VDR-RXR protein complexes. Liu and Freedman (43) have demonstrated cooperative binding of dimeric VDR complexes to two VDRE sites separated by 50 bp in an artificial promoter. However, Zierold et al. (31) were unable to show cooperative binding between protein complexes bound to sequences encompassing VDRE-1 and VDRE-2 in gel shift experiments. Another possible mechanism could involve independent binding of the protein complexes to each VDRE, with synergism resulting from an interaction of these complexes with the basal transcription machinery (43, 44). Of possible relevance to this latter mechanism is the demonstration that VDR can interact with the general transcription factor TFIIH (45).

Maximal induction of the CYP24 promoter in COS-1 cells was observed over a 1000-fold range of 1,25-(OH)_{2}D_{3} concentrations (i.e. 10^{-7} m to 10^{-10} m). Therefore, it would appear that the VDREs can function maximally in a synergistic manner at physiological concentrations of 1,25-(OH)_{2}D_{3}. Continued decline in hormone concentration to 10^{-11} m resulted in VDRE-1 being the dominant response element, although synergism was retained between the two VDREs. The same effect was observed with similar 1,25-(OH)_{2}D_{3} concentrations in ROS 17/2.8 cells, but a 10-fold higher concentration of hormone was required in JTC-12 cells (results not shown). The reason for the reduced sensitivity in these cells is under investigation. Nevertheless, the collective results establish synergism occurrence over a physiological range of 1,25-(OH)_{2}D_{3} concentration in which VDRE-1 is always the major contributor to induction, particularly at low hormone concentrations.

The transcriptional synergism between the two VDREs in the promoter of CYP24 may have important biological consequences. The induction of CYP24 by 1,25-(OH)_{2}D_{3} constitutes an interesting feedback mechanism whereby 1,25-(OH)_{2}D_{3} acts to regulate its metabolic clearance rate and, thereby, influence its ambient concentration. Increased levels of CYP24 activity will result in elevated side-chain oxidation of 1,25-(OH)_{2}D_{3} and ultimately conversion to the water-soluble calcitriol acid and subsequent excretion (17, 18). In the normal situation, CYP24 is expressed predominantly in the kidney but can be induced by 1,25-(OH)_{2}D_{3} in this tissue, in the intestine (19–23), and also in a variety of other cell types (24–29). It seems probable, therefore, that CYP24 expression in different tissues not only protects the tissue from the effects of excessive 1,25-(OH)_{2}D_{3} but also regulates serum hormone levels by increasing its metabolic clearance rate. A synergistic response would ensure rapid removal of hormone when levels are sufficiently high to cause hypercalcemia and accelerated bone resorption (46). It can be predicted that VDRE-1 is preferentially utilized at lower 1,25-(OH)_{2}D_{3} levels, but at higher hormone levels both VDREs are activated and transcriptional synergism facilitates the efficient inactivation of 1,25-(OH)_{2}D_{3}.

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34. Chen, K.-S., and DeLuca, H. F. (1995) Biochim. Biophys. Acta 1263, 1–9
35. Kunkel, T. A., Bebenek, K., and McClary, J. (1991) Methods Enzymol. 204, 125–139
36. Buluwela, L., Forster, A., Boehm, T., and Rabbitts, T. H. (1989) Nucleic Acids Res. 17, 452
37. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
38. Matsumoto, T., Kawanobe, Y., and Ogata, E. (1985) Biochim. Biophys. Acta 845, 358–365
39. Graham, F. L., and Van Der Eb, A. J. (1973) Virology 52, 456–467
40. Noda, M., Vogel, R. L., Craig, A. M., Prahl, J., DeLuca, H. F., and Denhardt, D. T. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9995–9999
41. Rochette-Egly, C., Lutz, Y., Pfister, V., Heyberger, S., Scheuer, I., Chambon, P., and Gaub, M. P. (1994) Biochim. Biophys. Res. Commun. 204, 525–536
42. Morrison, N. A., Shime, J., Fragonas, J.-C., Verkest, V., McMenemy, M. L., and Eisman, J. A. (1989) Science 246, 1158–1161
43. Liu, M., and Freedman, L. P. (1994) Mol. Endocrinol. 8, 1553–1604
44. Chi, T., Lieberman, P., Ellwood, K., and Carey, M. (1995) Nature 377, 254–257
45. MacDonald, P. N., Sherman, D. R., Dowd, D. R., Jefcoat, S. C., Jr., and DeLisle, R. K. (1995) J. Biol. Chem. 270, 4748–4752
46. Smith, R. (1993) in Metabolic Bone & Stone Disease (Nordin, B. E. C., Need, A. G., and Morris, H. A., eds) 3rd Ed., pp. 213–248, Churchill Livingston, Edinburgh