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Analysis of Regulatory Regions in the COL1A1 Gene Responsible for 1,25-Dihydroxyvitamin D_3-Mediated Transcriptional Repression in Osteoblastic Cells

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Abstract The synthesis of type I collagen in bone cells is inhibited by the calcium-regulating hormone 1,25-dihydroxyvitamin D_3. Earlier work from our laboratories has indicated that vitamin D regulation is at the level of transcription, based on results from both nuclear run-off assays and functional promoter analysis of a hybrid gene consisting of a 3.6 kb COL1A1 promoter fragment fused to the chloramphenicol acetyltransferase reporter gene. In the present study, we investigated the molecular basis for vitamin D-mediated transcriptional repression of the COL1A1 gene and report the identification of a region within the COL1A1 upstream promoter (the HindIII-PstI restriction fragment between nucleotides −2295 and −1670) which is necessary for 1,25-dihydroxyvitamin D_3 responsiveness in osteoblastic cells. This hormone-mediated inhibitory effect on the marker gene parallels the inhibition of the endogenous collagen gene. A 41 bp fragment from this region (between nucleotides −2256 and −2216) contains a sequence which is very similar to vitamin D-responsive elements identified in the osteocalcin gene. Extracts from cultured cells which express a high level of vitamin D receptor contain a hormone:receptor complex that binds specifically to this 41 bp fragment, as demonstrated by bandshift analysis. However, deletion of this vitamin D receptor binding region from either a −3.5 kb or a −2.3 kb promoter fragment did not abolish vitamin D responsiveness. These results indicate that a vitamin D response element similar to that described for other vitamin D responsive genes (osteocalcin and osteopontin) does not alone mediate the repression of COL1A1 by 1,25-dihydroxyvitamin D_3.

Key words: type I collagen, gene regulation by steroid hormone, bone cells in culture, vitamin D, nucleotides

A prominent physiological role of 1,25-dihydroxyvitamin D_3 (vitamin D) is the regulation of calcium homeostasis, which is achieved through the coordinated action of the hormone on the cells of three different organs: intestine, kidney, and bone [DeLuca and Schnoes, 1983; Haussler and McCain, 1977]. Vitamin D regulates transcription of several genes including calbindins [Minghetti et al., 1988; Perret et al., 1988], matrix-Gla protein [Fraser et al., 1988], *c-myc* oncogene [Simpson et al., 1987], and parathyroid hormone [Okazaki et al., 1988]. In bone cells, the effect of vitamin D on the synthesis of osteopontin [Noda et al., 1990], osteocalcin [Price and Baukol, 1980], and α1(I) collagen (COL1A1) [Rowe and Kream, 1982] has been studied. In contrast to its stimulatory effect on osteopontin and osteocalcin genes, vitamin D suppresses the synthesis of type I collagen in rat calvariae [Bringhurst and Potts, 1981; Kream et al., 1980; Rowe and Kream, 1982], osteoblastic cell lines [Rosen and Luben, 1983], and osteosarcoma cells [Kream et al., 1986], but not in the periosteal

Abbreviations used: CAT, chloramphenicol acetyltransferase; COL1A1, α1(I) collagen; COL1A2, α2(I) collagen; HRE, hormone-response element; PTH, parathyroid hormone; SEM, standard error of the mean; VDR, vitamin D receptor; VDRE, vitamin D-response element; VDRBR, vitamin D receptor binding region; vitamin D, 1,25-dihydroxyvitamin D_3.

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fibroblasts of the calvariae [Genovese et al., 1984]. The inhibitory effect of vitamin D on collagen synthesis is associated with a decrease in steady state levels of procollagen mRNA in fetal rat calvariae and osteosarcoma cells [Genovese et al., 1984; Kream et al., 1986]. Furthermore, nuclear run-off assays showed that vitamin D decreased COL1A1 and COL1A2 gene transcription in nuclei isolated from hormone treated ROS 17/2.8 cells [Harrison et al., 1989].

A vitamin D response element (VDRE) has been identified in the rat [Demay et al., 1990; Markose et al., 1990; Yoon et al., 1988] and human [Kerner et al., 1989; Morrison et al., 1989; Ozono et al., 1990] osteocalcin genes and in the mouse osteopontin gene [Noda et al., 1990]. The critical sequence in the rat OC gene is *GGGTGA*ATG*AGGACA;* imperfect direct repeats are italicized. Mutations in both repeats as well as the three base pair "spacer" affected the vitamin D response. The human osteocalcin and mouse osteopontin VDREs also consist of direct repeats of sequences which are identical or related to the rat OC VDRE. A tentative consensus sequence for VDRE which mediates stimulation of transcription by vitamin D ($^{A}/_{G}GGNN^{A}/_{C}A^{T}/_{C}$ $G^{A}/_{G}GGNN^{A}/_{C}$) has been derived by sequence comparison of VDREs from different genes as well as from mutational analysis of the human and rat OC VDREs [Demay et al., 1992]. In addition, in cotransfection experiments using vitamin D receptor expression vectors and articifical response element-reporter gene constructs, it was found that the sequences GGTTCA, AGGTCA, or GGGTGA, arranged as direct or inverted repeats of varying spacing, can act as VDREs [Carlberg et al., 1993].

These studies, together with others showing that the transcriptional stimulation of the osteocalcin gene by vitamin D requires a functional vitamin D receptor [McDonnell et al., 1989], provided direct evidence that the hormone-occupied vitamin D receptor acts as a transcriptional activator, similar to the other members of the steroid/thyroid hormone receptor superfamily. To analyze the molecular basis for transcriptional regulation of the type I collagen gene in osteoblasts, we previously isolated a 3.6 kb rat COL1A1 promoter fragment and fused it to the CAT reporter gene [Lichtler et al., 1989]. When this hybrid gene was transiently transfected into ROS 17/2.8 cells, its transcription was inhibited by vitamin D. In the present study, osteoblastic and fibroblastic cell lines were stably transfected with deletion mutants of the 3.6 kb rat COL1A1 promoter fragment. We identified a 625 bp DNA region which mediates transcriptional inhibition by vitamin D. A 41 nucleotide fragment from this region shares a high degree of sequence homology with VDREs from other genes and provides a high affinity binding site for the occupied vitamin D receptor. However, deletion analysis of this fragment indicated that it is not required for hormonal responsiveness.

MATERIALS AND METHODS

Production and Hormonal Treatment of Stably Transfected Cells

The osteoblastic cell line ROS 17/2.8 [Majeska et al., 1978; Price and Baukol, 1980] and two fibroblastic lines NIH3T3 and EL2 [Liboi et al., 1984] were maintained in F-12 medium as described [Pavlin et al., 1992]. Stably transfected cell populations derived from pooled colonies of neomycin resistant cells were produced as described earlier [Pavlin et al., 1992]. For treatment with vitamin D, stably transfected ROS 17/2.8, NIH3T3 and EL2 cells were grown 3–5 days past confluency. At the start of the hormone treatment, medium was supplemented with 50 μg/ml ascorbic acid and the fetal calf serum was lowered from 10% to 2%. Vitamin D (kindly provided by Dr. Milan Uskokovic, Hoffman-LaRoche, Nutley, NJ) dissolved in ethanol to 10 nM or an ethanol vehicle was added to the medium (0.1% final ethanol concentration) 24 h before the cells were harvested.

DNA Constructs of the COL1A1 Promoter

Construction of ColCAT3.6, ColCAT2.3, ColCAT1.7, and ColCAT0.9, which contain sequences of the COL1A1 promoter between −3518, −2295, −1670, and −944 bp and +116 bp fused to the chloramphenicol acethyltransferase (CAT) reporter gene and the SV40 small t antigen splice site and polyadenylation signal derived from pSV2CAT, has been previously described [Lichtler et al., 1989; Pavlin et al., 1992]. The previously reported 5′ end points of ColCAT3.6, ColCAT2.3, and ColCAT1.7 were −3521, −2295, and −1672 bp, respectively. These end points have been changed to correct a mistake in the published sequence [Lichtler et al., 1989] originally pointed out to us by Dr. Barbara Smith. Our published sequence between positions −1620 and −1611 bp, GCCAAGGCGA, should be changed to GCCAGCGA. For

site-directed mutagenesis of the VDRBR, the overlap extension polymerase chain reaction (PCR) method was used [Ho et al., 1989]. The following four synthetic oligonucleotides were used in this procedure: 1) 5′-CACATTC-TTCCTCTCGC-3′, 2) 5′-CTCACATTACAC-*CTCGAG*TTTGCCCCAGGAGTTGTGG-3′, 3) 5′-*CTCGAG*GTGTAATGTGAGCAGACACGT-CAAGCACC-3′, 4) 5′-GTGCAACGAAGGCAA-AG-3′. The 3′ ends of oligonucleotides 2 and 3 were complementary to template target sequences immediately upstream and downstream of the region to be deleted, while 18 bp at the 5′ ends were complementary and contained an inserted XhoI restriction site and 12 randomly selected nucleotides. Two overlapping DNA fragments, which immediately flanked the sequence between −2256 bp and −2216 bp, were first produced in two independent PCR reactions using primer pairs 1/2 and 3/4. Primers 1 and 4 were selected to include the HindIII and PstI restriction sites in the corresponding amplified products. The bands from the first PCR reaction were isolated from a polyacrylamide gel by electroelution. Approximately 1/10 of the original two bands was used in a second PCR reaction which also included the original concentration of oligonucleotides 1 and 4. Annealing of overlapping extended sequences allowed one strand from each fragment to act as a primer, so that polymerization of each strand to the full length of the template resulted in production of a mutated fragment with preserved HindIII and PstI restriction sites and a new XhoI site substituted into the deleted region. A HindIII-PstI restriction fragment from this mutation product was used for the final mutant constructions as follows. To produce ColCAT2.3ΔVD, the mutagenized HindIII-PstI fragment was cloned into the ColCAT2.3 construct cut with HindIII and PstI. To clone ColCAT3.6ΔVD, a HindIII-HindIII fragment from ColCAT3.6, was ligated into the ColCAT2.3ΔVD mutants linearized with HindIII. Successfully mutagenized constructs were identified by restriction cuts and gel electrophoresis, utilizing the unique XhoI site built into the deleted COL1A1 sequence.

Preparation of Cell Extracts and Bandshift Analysis

Cos-1 cells were transiently transfected with a eukaryotic expression vector pAD-hVDR containing the human vitamin D receptor cDNA [McDonnell et al., 1989] by the calcium phosphate precipitation method. Two hours prior to harvesting, medium containing 2% fetal calf serum and 10 nM vitamin D or ethanol vehicle was added to the cells. Forty-eight to seventy-two hours after transfection cells were scraped into cold PBS and disrupted by Dounce homogenization in buffer containing 10 mM Tris-HCl, pH 7.6, and 5 mM DTT. After homogenization, KCl was added to the buffer so that the final concentration was 0.3 M (TDK-0.3 buffer). A supernatant containing cytosolic extract was obtained by centrifugation of the homogenate at 50,000*g* and stored as 20 μl aliquots at −70°C until assayed.

Two different types of radiolabeled probes were used for bandshift assays. The first was a 51 bp synthetic oligonucleotide (VD51) which includes nucleotides −2256 to −2216 of the COL1A1 upstream promoter (see Fig. 4) to which ClaI restriction sites were added on both the 3′ and 5′ ends. This ClaI-ClaI synthetic DNA fragment was subcloned into pBS$^+$SKII (Stratagene, La Jolla, CA). The second probe was a 201 bp genomic fragment between the HindIII (−2294) and BglI (−2094) restriction sites of the COL1A1 upstream promoter which was subcloned into the pBS$^+$SKII vector. Both probes were 3′ end-labeled with [^{32}P] to 10^8 cpm/μg DNA [Sambrook et al., 1989] following restriction digestion from the Bluescript vector, and subsequently isolated by gel electrophoresis and electroelution of radiolabeled fragments from an 8% polyacrylamide gel.

For the bandshift analysis, a DNA probe (2 to 7×10^4 cpm/reaction) was incubated with 6 μg of cell extract protein in a reaction buffer containing 10 mM Tris/HCl, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 1 mM EDTA, 1 mM DTT, and 2 μg of poly(dI-dC) for 20 min at room temperature [Schneider et al., 1986]. In some experiments an excess of unlabeled specific competitor DNA was preincubated with nuclear extract for 10 min at room temperature. In antibody inhibition experiments, nuclear extracts were preincubated for 20 min at room temperature with monoclonal antibody 9A7 [Pike, 1984] or nonspecific purified antibodies to mouse immunoglobulins, as indicated. Samples were electrophoresed on a 5% polyacrylamide gel prepared in Tris/glycine (TGE) or Tris/borate (TBE) buffer [Sambrook et al., 1989] at 150 V

constant. Gels were dried and exposed to Kodak XAR-5 film at −70°C.

RESULTS

Initial Mapping of the VDRE Within the 3.6 kb COL1A1 Promoter Fragment

To identify regions within the 3.6 kb COL1A1 promoter which confer vitamin D responsiveness, we initially used four promoter constructs: ColCAT3.6, ColCAT2.3, ColCAT1.7, and ColCAT0.9, which extend from −3518 bp, −2295 bp, −1670 bp, and −944 bp, respectively, and end at +116 bp downstream from the transcription start site (see map in Fig. 3). These promoter fragments were fused to the CAT marker gene [Lichtler et al., 1989] and stably transfected into the osteoblastic cell line ROS 17/2.8 and the two fibroblastic cell lines NIH3T3 and EL2. The mRNAs from all chimeric genes were initiated from the correct transcription start site, as shown earlier using an RNase protection assay [Pavlin et al., 1992].

The effect of vitamin D on the activity of the promoter constructs and the endogenous collagen synthesis in ROS 17/2.8 cells is shown in a representative experiment in Figure 1. The effect of vitamin D on the endogenous collagen synthesis was measured to ensure that the transfection and selection protocol did not alter the phenotype of the cells. Activity of the ColCAT3.6 and ColCAT2.3 constructs was inhibited by vitamin D to the same extent as the endogenous gene. This inhibitory effect was lost with the ColCAT1.7 and ColCAT0.9 constructs, while the inhibition of the endogenous gene was maintained. In EL2 and NIH3T3 fibroblasts, the expression of either the full length construct, or any of the deletion mutants, was not significantly affected by vitamin D (Fig. 2). Figure 3 summarizes the effect of vitamin D on the stably transfected cell populations each carrying one of the ColCAT constructs. Repression of ColCAT3.6 activity in ROS 17/2.8 cells averaged 39%, which is comparable to the effect on the endogenous gene. This level of inhibition was maintained with the ColCAT2.3 construct. The inhibition of both of these constructs was statistically significant. However, further deletion to −1670 bp resulted in the loss of most or all of the vitamin D inhibitory effect on CAT activity. The small degree of inhibition seen with ColCAT1.7, and the stimulation seen with ColCAT0.9, were not significant. These data suggest that the COL1A1 upstream promoter sequence between nucleotides −2295 and −1670 contains a vitamin D response element (VDRE), which is

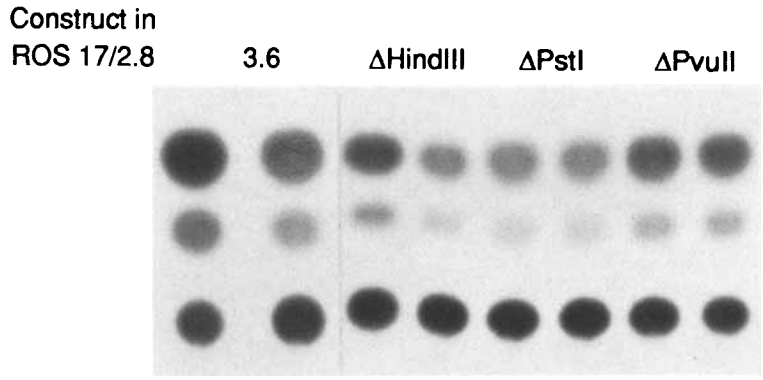

Fig. 1. A representative experiment showing the effect of vitamin D on COL1A1 promoter constructs in ROS 17/2.8 cells. CAT activity is expressed as a percentage of acetylated chloramphenicol products. For each construct an equal amount of protein was assayed for the C (control) and D (vitamin D-treated) samples. The amount of protein assayed was over 20-fold larger for the COLCAT2.3 (ΔHindIII), ColCAT1.7 (ΔPstI), and ColCAT0.9 (ΔPvuII) cells, compared to the ColCAT3.6 cells, because of differences in level of basal expression. Percent collagen synthesis represents the percent radiolabeled collagen secreted into the culture medium.

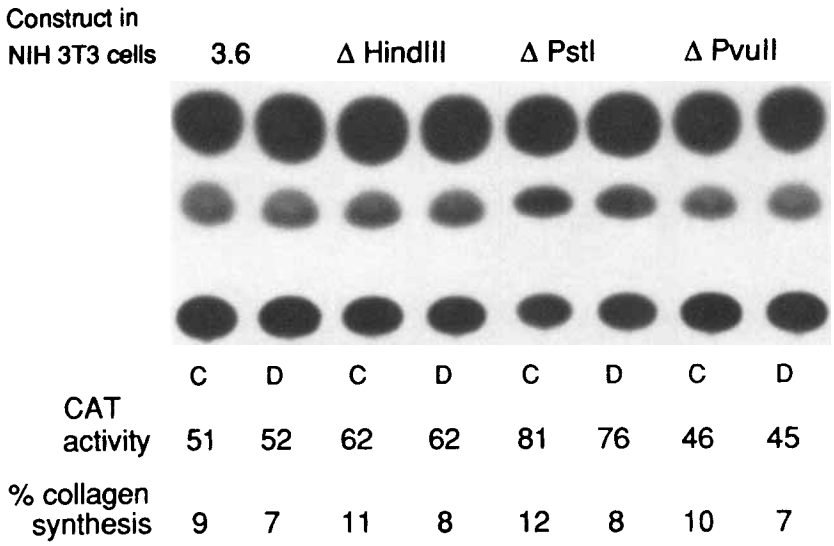

Fig. 2. A representative experiment showing the effect of vitamin D on COL1A1 promoter constructs in NIH 3T3 cells. Assay conditions and expression of CAT activity and endogenous collagen synthesis were as described in Figure 1.

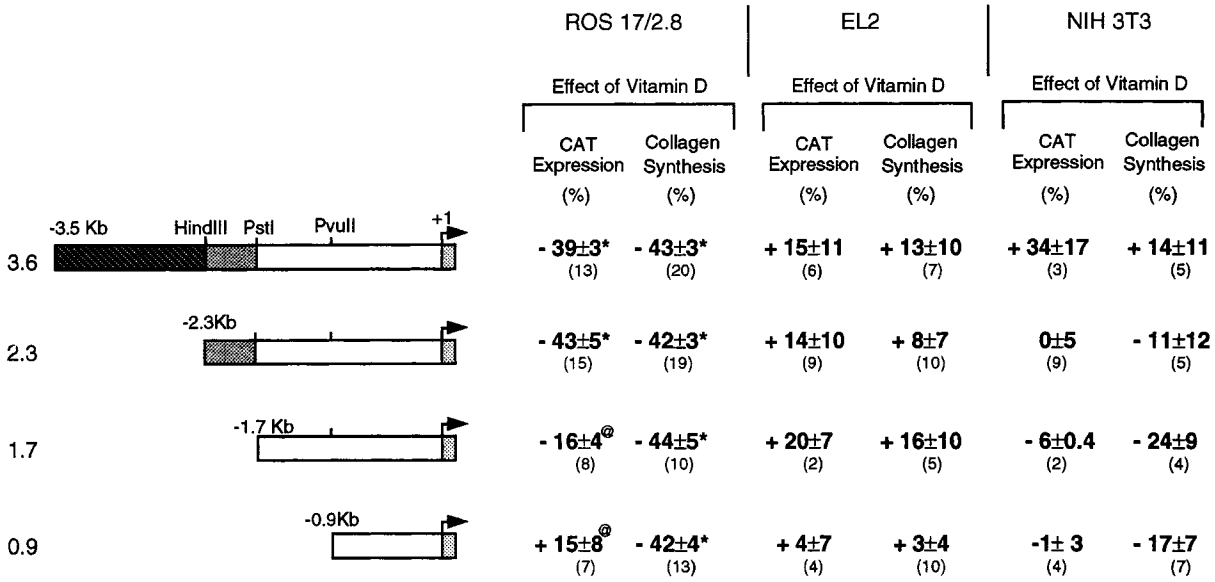

Fig. 3. The effect of vitamin D on CAT expression and endogenous collagen production in cell lines carrying four ColCAT constructs. Each value is the mean of multiple assays (number of assays indicated in parentheses) using cells from one to four different stably transfected cell populations. CAT activity and percent collagen synthesis are expressed as percent change ± standard error of the mean (SEM). Plus sign indicates stimulation, minus sign inhibition. *, Values that are significantly different from zero (zero would indicate no inhibition). @, Values not significantly different from zero, but significantly different from values for ColCAT3.6 and ColCAT2.3. Statistical significance analyzed by one way analysis of variance with post hoc analysis using Bonferoni's *t* test.

necessary for hormonal repression in osteoblastic cells, but is not active in fibroblastic cells.

Binding of the VDR to a Putative VDRE

We examined the sequence between −2.3 and −1.7 kb for potential VDRE's and found a 7 bp element at −2240 to −2234 which is present in both the rat and human osteocalcin VDREs. This element, GGGTGAA, is the 5′ repeat and the first base of the spacer in the VDRE of both genes. Comparison of the region immediately downstream of this domain with the consensus

VDRE derived by Demay et al. [1992] revealed considerable conservation in critical positions. There were no other sites with significant similarity to either this consensus sequence or the combinations of half sites described in [Carlberg et al., 1993] within the 625 bp region which is necessary for the vitamin D response. Therefore, we determined whether this site is necessary for the vitamin D inhibition of the α1(1) collagen gene.

To determine if the vitamin D receptor interacts with the putative COL1A1 VDRE, we used a gel mobility shift (bandshift) assay. A 51 bp synthetic oligonucleotide (VD51) and a 201 bp genomic fragment (HB204), which include the putative VDRE, were used as radiolabelled probes (Fig. 4). The probes were incubated with cytosolic or nuclear extracts from two different cell lines overexpressing the VDR. The first type of extract was prepared from the simian Cos-1 cell line transiently transfected with the eukaryotic construct pAD-hVDR, which directs the expression of the full length human VDR [McDonnell et al., 1989], as described in Materials and Methods. The second, a CV4 nuclear extract rich in VDR, was obtained from the human cell line 293 infected with the adenovirus vector CV4-hVDR1 [Smith et al., 1991]. Both types of vitamin D receptor-expressing cells were treated with 10 nM vitamin D for 2 h prior to harvesting. For control extracts, 293 cells were infected with the parental vector dl309 and Cos-1 cells were mock transfected.

A representative bandshift assay utilizing Cos-1 nuclear extract and the VD51 probe is shown in Figure 5. Two prominent protein DNA complexes (1 and 2) were formed with the extract from Cos-1 cells transfected with the VDR expression vector (lane 1). However, with the extract from the cells overexpressing the VDR

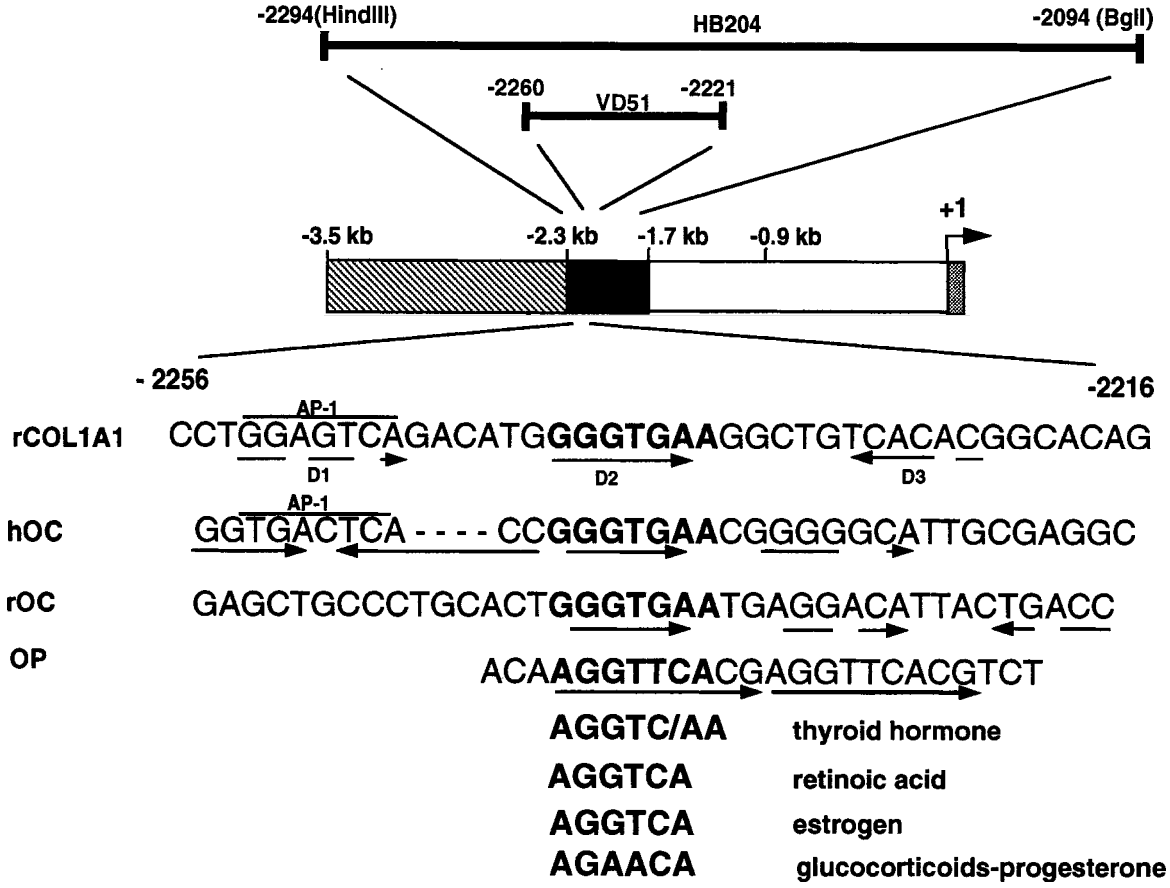

Fig. 4. Probes used in bandshift experiments and sequence similarity between the rat COL1A1 promoter fragment and VDREs from human (hOC) and rat (rOC) osteocalcin genes and the mouse osteopontin gene. Half-palindromic DNA binding sites for four other steroid hormone receptors which display a high homology to the hOC and rOC VDREs and to a rCOL1A1 putative VDR binding site.

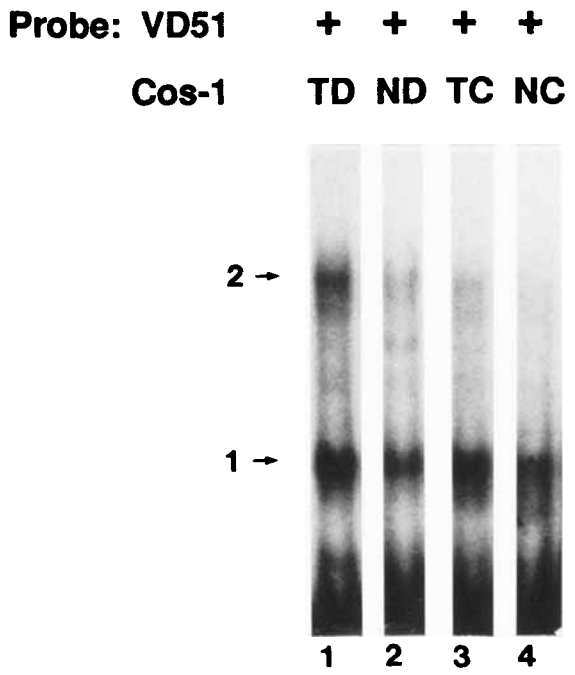

Fig. 5. A representative bandshift experiment utilizing radiolabeled probe VD51 and nuclear extract from either Cos-1 cells transfected with the human VDR expression vector pAD-hVDR (lanes 1 and 3) and treated with vitamin D (*TD*) or vehicle (*TC*), or mock-transfected Cos-1 cells (lanes 2 and 4) treated with vitamin D (*ND*) or vehicle (*NC*).

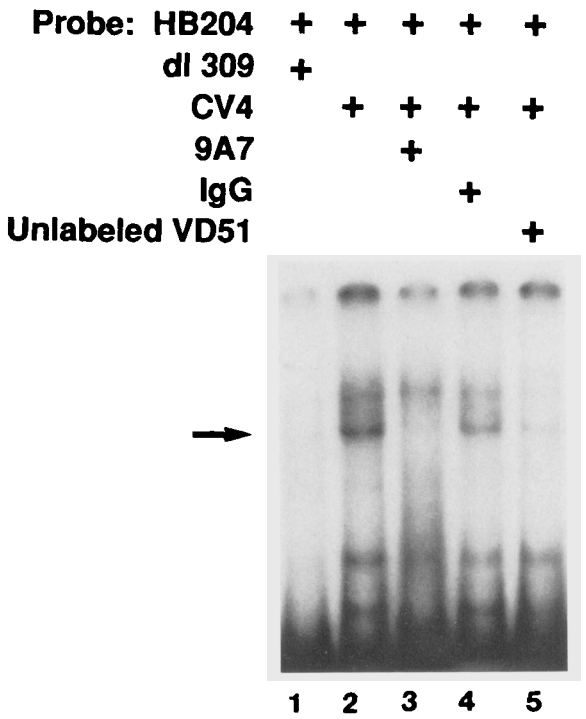

Fig. 6. A representative experiment showing protein:DNA interactions with nuclear extracts from 293 cells overexpressing the VDR (CV4) (lanes 2–5) or expressing only a few thousand copies of the VDR (dl 309) (lane 1) [Smith et al., 1991]. Concentrations of antibody and unlabeled DNA competitor are indicated in the text.

but not treated with vitamin D (lane 3), or with extracts from mock-transfected cells either treated (lane 2) or not treated (lane 4) with vitamin D, complex 2 was very weak or undetectable. This suggests that the formation of complex 2 is dependent on or enhanced by the presence of a high concentration of hormone:receptor complexes. Figure 6 shows the protein-DNA complexes formed after incubation of the 201 bp genomic probe HB204 with nuclear extracts from 293 cells infected with the adenovirus expression vector CV4hVDR1 which overexpresses the VDR (lane 2). These complexes were not detected when the probe was incubated with the extract from 293 cells infected with the parental adenovirus expression vector dl309 [Smith et al., 1991]. These cells contain only a very small number of VDRs (lane 1). The addition of 1 μg of the anti-VDR monoclonal antibody 9A7, which hinders DNA binding of the VDR by interacting with its DNA-binding domain [Pike, 1984], specifically blocked the formation of only one of the complexes (compare lane 3 with lane 2). However, an unrelated antibody of the same class did not affect the formation of any particular complex (lane 4). A 200-fold molar excess of unlabelled probe VD51 interfered with complex formation less specifically, by blocking both upper protein DNA complexes (lane 5). These data strongly suggest that one of the complexes formed with CV4 extract (arrow) represents a specific interaction of vitamin D receptor with the DNA probe.

We next examined the interactions of proteins from the CV4 extract with the shorter synthetic probe VD51 (Fig. 7). The strongest of the three interactions (complex 1 in lane 2) was specifically inhibited with 9A7 antibody (lane 3), whereas this interaction was not affected by an IgG (lane 4). Since the distal domain (D1) of the COL1A1 VDRE contains an imperfect consensus site for the nuclear oncogene heterodimer *c-Jun/c-Fos*, we asked whether this transcription activator protein could bind to the VD51 probe along with the hormone-occupied VDR. As shown in Figure 8, the addition of a molar excess of an oligonucleotide containing the consensus sequence for either the AP-1 DNA binding site (lane 3) or the glucocorticoid receptor binding site (lane 4) did not affect the formation of any of the complexes formed with VD51.

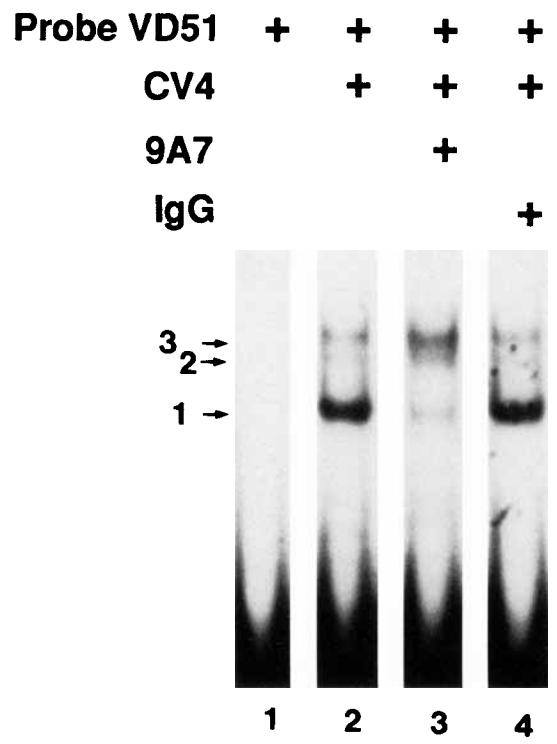

Fig. 7. A bandshift experiment utilizing synthetic oligonucleotide probe VD51 with VDR-rich nuclear extract CV4. The formation of complex 1 was specifically blocked with 1 μg of monoclonal antibody 9A7.

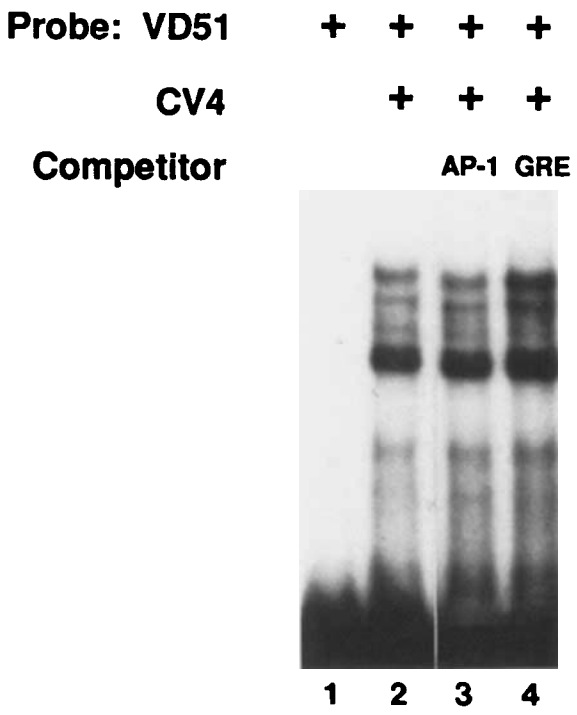

Fig. 8. A bandshift assay used to assess the competition of protein:DNA complexes with a 500-fold molar excess of synthetic oligonucleotide containing the consensus sequence of the DNA binding site for complexed c-Fos/c-Jun cellular oncogene products (AP-1), or the glucocorticoid receptor (GRE).

Altogether, the results from the bandshift experiments provide evidence that the activated vitamin D receptor binds to the COL1A1 upstream promoter between bases −2260 and −2221 of the COL1A1 gene. Furthermore, the nuclear factor AP-1 is not likely to compete with the VDR for binding to this putative vitamin D response element.

Site-Directed Mutagenesis of the Vitamin D Receptor Binding Region

To determine if the vitamin D receptor binding region (VDRBR) between nucleotides −2256 and −2216 confers vitamin D responsiveness, we analyzed two mutant DNA constructions, shown in Figure 9, which contain a deletion and a partial substitution of the entire VDRBR between bases −2256 and −2216, within the context of the full length (ColCAT3.6ΔVDRBR construct) and the truncated (ColCAT2.3ΔVDRBR construct) promoters. Mutant DNA constructs were transfected into ROS 17/2.8 cells and stably transfected cell populations, each containing one of the constructs, were produced. Following treatment with 10 nM vitamin D for 48 h, confluent cells were assayed for CAT activity and endogenous collagen synthesis. Vitamin D inhibited CAT activity of ColCAT3.6 or ColCAT2.3 constructs lacking the VDRBR (Fig. 9). This suggests that the region of the COL1A1 upstream promoter between nucleotides −2256 and −2216, which provides a binding site for occupied vitamin D receptor, does not alone confer an inhibitory response to vitamin D in ROS 17/2.8 cells.

DISCUSSION

Vitamin D regulates several genes involved in mineral metabolism including matrix-Gla protein, the calbindin gene family, osteocalcin, osteopontin, and type I collagen. The stimulatory effect of vitamin D on many of these genes is exerted through a steroid hormone mechanism involving a specific interaction of the VDR with DNA sequences within the target genes [Ozono et al., 1991]. Our inital mapping of the vitamin D response element within the HindIII-PstI fragment of the COL1A1 upstream promoter [Pavlin et al., 1989] coincided with identification of a vitamin D transactivation region in the osteocalcin gene [Demay et al., 1990; Kerner et al., 1989; Markose et al., 1990; Morrison et al., 1989]. We

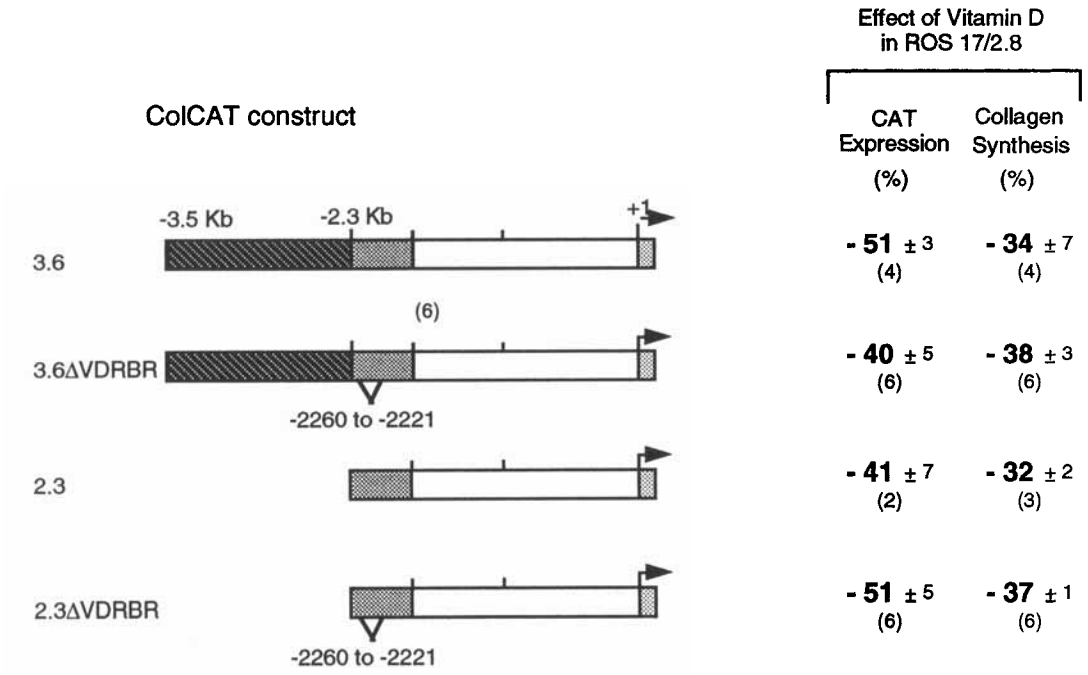

Fig. 9. Vitamin D inhibition of CAT and endogenous collagen synthesis after site-directed deletion of the VDRBR. Three stably expressing populations (produced by separate transfections) carrying the ColCAT3.6ΔVDRBR construct and three populations carrying the ColCAT2.3ΔVDRBR construct were analyzed in triplicate. Each value is the mean ± SEM of multiple assays (number of assays indicated in parentheses) from 3 (ColCAT3.6, ColCAT3.6ΔVDRBR, and ColCAT2.3ΔVDRBR) or 2 (ColCAT2.3) different cell populations analyzed in two experiments. CAT activity and percent collagen synthesis are expressed as described in Figure 1. Minus sign indicates inhibition. In all cases, vitamin D inhibition was statistically significant; statistical analysis was carried out as described in Figure 3.

focused our attention on a 41 nucleotide region within the COL1A1 HindIII-PST fragment, which contains the greatest similarity to known VDRE's of any site within the Hind III-PST fragment, a 7 bp sequence conserved in the human and rat osteocalcin VDRE. The results of the mobility shift experiments showed that the occupied VDR indeed specifically interacts with the DNA sequence between −2256 bp and −2216 bp. However, functional analyses of both the full length and the truncated promoter constructs with and without this 41 bp region containing the putative vitamin D response element showed that this locus does not function as a vitamin D responsive region in the COL1A1 gene. These results did not support our hypothesis that a DNA element with considerable homology to a positive VDRE could mediate a negative response in a different gene.

This finding, however, does not exclude the possibility that vitamin D represses transcription of COL1A1 by directly binding to other cis-active elements between −2.3 kb and −1.7 kb, which do not resemble the osteocalcin VDRE. Alternatively, an inhibitory hormone effect could be exerted via multiple low affinity VDR binding sites located downstream of −2.3 kb. A steroid hormone response element consisting of several widely spaced binding sites has been identified in the estrogen-induced DNase I-hypersensitive region of the chicken ovalbumin gene located between −3.7 and −3.1 kb [Kato, 1992, number 216]. In this case, a deletion of one of the VDR DNA binding sites would not necessarily abrogate the hormone response, especially considering the level of inhibition (50%) of the COL1A1 gene by vitamin D. This possibility seems unlikely since there are no sites with high similarity to known VDREs within the region between −2.3 kb and −1.7 kb other than the one we have analyzed, however it cannot be eliminated.

Another regulatory mechanism which could account for transcriptional inhibition of COL1A1 by vitamin D is interaction of the occupied VDR with various transactivator nuclear regulatory proteins. This mechanism would not require a direct binding of vitamin D receptor to a DNA element, such as the one identified in this study. In light of rapidly accumulating evidence on various mechanisms for gene repression [Foul-

kers and Sassone-Corsi, 1992; Jackson, 1991; Levine and Manley, 1989; Lewin, 1990; Renkawitz, 1990; Schweers et al., 1990], the action of the VDR could hypothetically take place at any of three major levels. The first level is the basal transcription initiation complex, a group of proteins which bind in close proximity to the transcription start site and direct the initiation of basal transcription [Buratowski et al., 1989; Workman and Roeder, 1987]. An example of one key constituent factor in the basal transcriptional aparatus, which has been shown to be a target in the regulation by other upstream transactivators, is transcription factor IID [Hoey et al., 1990; Horikoshi et al., 1988]. Second, VDR could interact with factors which connect upstream regulatory elements with the basal transcription initiation complex. These bridging molecules, termed coactivators [Pugh and Tjian, 1990] or adaptors [Berger et al., 1990], provide a means by which upstream transactivators and the basal transcription complex can interact and have been proposed as target sites for several transcriptional regulators [Berger et al., 1990; Lewin, 1990]. The role of such an adaptor molecule has been suggested in the transcriptional repression by the yeast GAL4 protein [Berger et al., 1990]. Conceivably, a similar inhibitory mechanism could operate in osteoblasts, wherein VDR stimulates promoters containing a VDR binding site (osteocalcin, osteopontin) and represses promoters which lack functional receptor binding sites (COL1A1). The third level at which the VDR could interact with transactivating machinery is by an association with nuclear factors which bind to upstream regulatory elements (enhancers) and mediate positive effects on genes.

Another possibility is that vitamin D may inhibit the production of an autocrine factor which stimulates type I collagen synthesis. A candidate for such an action is insulin-like growth factor I (IGF-I). It has been postulated that the effect of vitamin D on differentiation and proliferation in many cell types, including bone cells, is mediated via a mechanism which utilizes the IGF regulatory system [Scharla et al., 1991]. In bone cells, IGF-I stimulates type I collagen synthesis [Canalis, 1980], while vitamin D reduces IGF-I at both the mRNA and protein levels [Scharla et al., 1991]. One could hypothesize that modifications in the IGF regulatory system could be involved in the regulation of COL1A1 in osteoblasts. The possibility of such an indirect mechanism of action of vitamin D on bone cells is further supported by recent evidence that anabolic effect of estradiol on type I collagen in bone may be mediated by IGF-I [Ernst et al., 1988, 1989].

Regardless of the level at which the activated VDR interacts with nuclear transcription regulatory proteins (basal initiation complex, coactivator/adaptor molecules, or the upstream enhancer-binding transactivators), it could affect transcription either by direct binding to any of these molecules, or indirectly, by sequestration of positive transcriptional regulators [Ptashne, 1988]. Examples of transcriptional repression by overexpression of activating proteins include regulation of the prolactin gene by estrogen [Adler et al., 1988] and repression by the yeast GAL4 protein [Berger et al., 1990]. This possibility is particularly attractive since direct binding of the VDR to DNA is responsible for the transcriptional stimulation of other bone proteins such as osteocalcin [Ozono et al., 1991] and osteopontin [Noda et al., 1990]. High concentration of occupied receptor could sequester nuclear proteins which mediate transactivation of the COL1A1 gene, resulting in a net inhibition of transcription.

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