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Identification of a DNA sequence responsible for binding of the 1,25-dihydroxyvitamin D_3 receptor and 1,25-dihydroxyvitamin D_3 enhancement of mouse secreted phosphoprotein 1 (*Spp-1* or osteopontin) gene expression

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ABSTRACT **Secreted phosphoprotein 1 (Spp-1; osteopontin) is one of the abundant noncollagenous proteins in bone matrix and is produced by osteoblasts. We examined the promoter region of the mouse *Spp-1* gene and identified a sequence responsible for 1,25-dihydroxyvitamin D_3 enhancement of the *Spp-1* gene expression. This 24-base-pair (bp) sequence (vitamin D response element) is located 761 bp upstream of the transcription start site and consists of two direct repeats of a unique 9-bp motif, AGGTTCACG. The vitamin D response element confers responsiveness of a heterologous promoter to 1,25-dihydroxyvitamin D_3 in a position- and orientation-independent and copy-number-dependent manner. The basal level of expression of the reporter constructs containing this sequence and its response to 1,25-dihydroxyvitamin D_3 were not affected by cotreatment with transforming growth factor β or the tumor promoter phorbol 12-myristate 13-acetate or by cotransfection with a JUN expression vector. The vitamin D response element forms DNA–protein complexes, as indicated by gel-retardation assays. The addition of a monoclonal antibody raised against the vitamin D receptor further retarded the mobility of the DNA–protein complex. Another antibody that recognizes the DNA binding region of the vitamin D receptor attenuated its binding to the sequence. These results indicate that this 24-bp sequence containing two 9-bp motifs binds to the vitamin D receptor and mediates the vitamin D_3 enhancement of murine *Spp-1* gene expression.**

Spp-1 (osteopontin) is a 44-kDa glycoprotein produced most abundantly by osteoblasts and kidney cells and in lesser quantities by cells of several other tissues (1). Spp-1 is stored in bone matrix as one of the abundant noncollagenous bone matrix proteins. Its amino acid sequence deduced from a cDNA revealed the presence of a Arg-Gly-Asp-Ser motif, previously described (1, 2) in fibronectin and other cell adhesion molecules as a site responsible for cell attachment to substrates through receptors on the cell surface, suggesting that Spp-1 may play a role in bone-cell attachment.

In cultured osteoblasts or osteoblast-like cells, *Spp-1* expression is regulated by calcitropic hormones and cytokines. Transforming growth factor β, fibroblast growth factor, leukemia inhibitory factor, tumor necrosis factors α and β, and interleukin 1 enhance the *Spp-1* gene expression, whereas dexamethasone and parathyroid hormone inhibit expression in osteoblast-like cells (3–7).

Spp-1 gene expression is markedly induced by the tumor promoter phorbol 12-myristate 13-acetate in JB6 fibroblasts and in normal murine epidermis (8–10). There is also a strong correlation between oncogenic transformation, particularly by *ras*, and the level of *Spp-1* expression (10–13). *Spp-1* expression in fibroblasts is enhanced by fibroblast growth factor and inhibited by retinoic acid and dexamethasone (9, 14).

1,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] is a major calcitropic hormone and one of its functions in bone is to regulate the synthesis of bone matrix proteins including Spp-1, collagen, and osteocalcin. 1,25$(OH)_2D_3$ modulates the expression of these genes through transcriptional control (1, 15).

Steroid hormones exert their effect by the binding of receptor–ligand complexes to short DNA sequences called hormone response elements in the regulatory regions of the genes (16, 17). Recently, vitamin D response elements (VDREs) that are 20–30 base pairs (bp) long and are located 700 and 400 bp upstream from the transcription start sites have been identified in human and rat osteocalcin genes, respectively (18–20, 27).

Here we report that the VDRE of the mouse *Spp-1* gene has a unique 9-bp direct repeat that confers responsiveness of a heterologous promoter to 1,25$(OH)_2D_3$. This VDRE was shown to bind to the vitamin D receptor.

METHODS

Plasmid Construction. Plasmids were constructed according to standard methods (21). 5′-Deletion mutants were generated from a *Pst* I–*Sty* I fragment (positions −910 to +79) of the mouse *Spp-1* gene and subcloned into pSVOCAT *Sma* I (ATCC 37154), as described (ref. 22, A.M.C. and D.T.D., unpublished results). Further deletion fragments were generated by polymerase chain reaction techniques and were subcloned into the Promega basic CAT (chloramphenicol acetyltransferase) vector. All constructs containing inserted sequences were verified by sequencing using dideoxynucleotides (21).

Cell Cultures. ROS 17/2.8 cells were cultured in modified F12 medium, supplemented with 5% (vol/vol) fetal bovine serum and MC3T3E1 cells were cultured in the α modification of minimum essential medium supplemented with 10% (vol/vol) fetal bovine serum as described (3).

DNA Transfection and CAT Assay. Cells were transfected with 10 μg of DNA by the DEAE transfection method (21). The CAT assay was performed as described (21, 23).

Labeling DNAs. The probes for gel-retardation assay were labeled by a filling reaction using the Klenow fragment of DNA polymerase I and appropriate radionucleotides (21).

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Abbreviations: 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; VDRE, vitamin D response element; CAT, chloramphenicol acetyltransferase.

Preparation of Crude Nuclear Extracts. Nuclear extracts were prepared as described by Dignam *et al.* (24) with some modifications. Protein concentration was adjusted to 5 $\mu g/\mu l$.

Gel-Mobility-Retardation Assays. Gel-mobility-retardation assays were performed essentially as described (21) using probes of the indicated promoter regions. Monoclonal antibodies raised against the vitamin D receptor (25) were added to incubation mixtures containing crude pig intestinal nuclear extracts and radiolabeled probes as described (20).

RESULTS

The first series of deletion mutants, including pSV2AR777-CAT, pSV2AR543CAT, and pSV2AR253CAT constructs (Fig. 1*A*) were transfected into rat osteosarcoma ROS17/2.8 cells, which were subsequently cultured for 48 hr in the presence or absence of 10 nM $1,25(OH)_2D_3$ in medium supplemented with 5% fetal bovine serum. As shown in Table 1 and Fig. 2*A*, $1,25(OH)_2D_3$ enhanced the expression of the pSV2AR777CAT construct only, increasing CAT levels by 3- to 5-fold. The vitamin D effect on this construct was time- and dose-dependent starting at 0.1 nM within 24 hr (Fig. 2 *B* and *C*). A similar response was observed when the construct was transfected into MC3T3E1 cells (data not shown).

To further analyze the region between positions −777 and −543, we made three additional deletion mutants using the polymerase chain reaction and cloned them into CAT expression vectors (pSV2AR740CAT, pSV2AR670CAT, and pSV2AR600CAT; Fig. 1*A*). The second series of the CAT assays indicated that only the expression of the pSV2AR-777CAT construct, and none of the constructs with sequences downstream to the position −740, was enhanced by $1,25(OH)_2D_3$ (Table 1).

The 38-bp sequence corresponding to the region between positions −777 to −740 was synthesized and designated 2S (Fig. 1*B*). 2S was cloned into the *Sal* I site in the Promega promoter CAT vector, at its multiple cloning site 3′ to the CAT gene, driven by the simian virus 40 early promoter. As shown in Table 1, the P3-2 construct, which contains four tandem copies of 2S in the same direction (5′ to 3′) downstream of the coding region, confers to the simian virus 40 early promoter responsiveness to vitamin D_3. Another construct, P3-4, which has two copies of 2S in opposite directions, showed increased basal activity but its expression could no longer be enhanced by $1,25(OH)_2D_3$ (Table 1). The response to $1,25(OH)_2D_3$ of the third construct, P3-1, which differs from P3-2 by one copy of 2S in the opposite (3′ to 5′) direction, was intermediate between the other two (Table 1).

The specificity of the response of the 2S sequence to $1,25(OH)_2D_3$ was demonstrated by showing that reagents, such as transforming growth factor β, fibroblast growth factor, dexamethasone, parathyroid hormone-(1–34), and phorbol 12-myristate 13-acetate, that are known to affect *Spp-1* gene transcription do not alter the CAT response to $1,25(OH)_2D_3$. Additionally, neither sense nor antisense JUN expression vectors affected the basal level or the vitamin D-induced level of CAT expression by the P3-2 containing construct (data not shown).

To define further the response element in 2S, the 3′ half of the 2S region (designated as 2.5S, Fig. 1*B*), which corre-

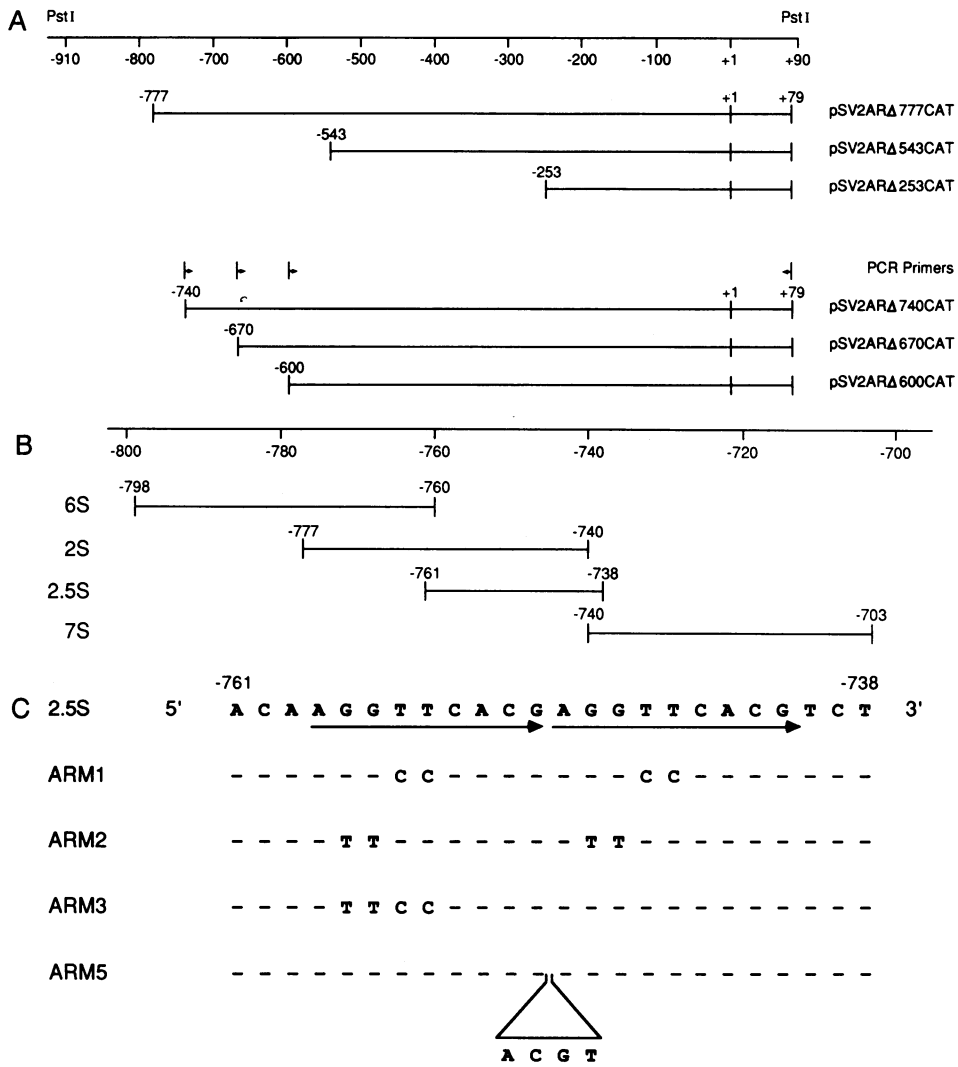

FIG. 1. Regions of the mouse *Spp-1*/osteopontin gene promoter used to construct CAT expression vectors. (*A*) Deletion mutants of the mouse *Spp-1*/osteopontin gene. (*B*) Synthetic oligonucleotides corresponding to the region between positions −798 and −703. (*C*) Oligonucleotides with substitution or insertion mutations in the direct repeat.

Table 1. Summary of the effects of mutations of *Spp-1*/osteopontin promoter or enhancer on CAT expression

Plasmid	Sequence	Upstream positions	CAT activity, fold induction
pSV2AR777CAT			4.2
pSV2AR543CAT			0.8
pSV2AR253CAT			1.0
pSV2AR740CAT			1.1
pSV2AR670CAT			0.9
pSV2AR600CAT			0.9
—	6S(-)	798/760	0.9
P3-1	2S(----)	740/777, $(777/740)_3$	1.5
P3-2	2S(----)	$(777/740)_4$	10.6
P3-4	2S(--)	740/777, 777/740	1.0
P6-13	2.5S(-)	761/738	1.8
P5-11	2.5S(--)	$(761/738)_2$	3.6
P5-15	2.5S(-)	738/761	1.7
—	7S(-)	740/703	1.1
—	ARM1(-)	761/738	0.7
—	ARM2(-)	761/738	0.4
—	ARM3(-)	761/738	1.0
—	ARM5(-)	761/738	1.1

ROS17/2.8 cells at 6×10^4 cells per 9.5-cm^2 well were transfected in duplicate with 10 μg of plasmid DNA as described (22). Cells were harvested 48 hr later and CAT activity was measured as described (22). Data are from one of at least two experiments with similar results. Dashes indicate number of copies.

sponds to the region between positions −761 and −738, was synthesized and cloned into the *Sal* I site of the Promega promoter CAT vector. This 24-bp (2.5S) sequence also conferred $1,25(OH)_2D_3$ response in a position- and orientation-independent and copy-number-dependent manner (Table 1). The 24-bp sequence contains two direct repeats of 9-bp sequence, AGGTTCACG. Substitution mutations of either GG (second and third) to TT or TT (fourth and fifth) to CC in each of the two repeats (ARM1 and ARM2, respectively; Fig. 1*C*) abolished the response to $1,25(OH)_2D_3$ treatment (Table 1). Similarly, the response was also blocked (Table 1) when the GGTT sequence (second to fifth) was converted to TTCC (ARM3, Fig. 1*C*) in the first repeat only and not in the second repeat. Insertion of 4 bp, between the two direct repeats (ARM5, Fig. 1*C*) also attenuated the response to $1,25(OH)_2D_3$ (Table 1). The DNA sequences flanking the direct repeat were also synthesized (6S, positions −810 to −760, and 7S, positions −740 to −703; Fig. 1*C*) and were cloned into the *Sal* I site of Promega promoter CAT vector. These two additional sequences, 6S and 7S, did not confer any responsiveness of the simian virus 40 early promoter to $1,25(OH)_2D_3$ (Table 1).

To examine whether these sequences bind to nuclear factors, gel-retardation assays were performed using crude nuclear extracts of rat osteosarcoma ROS 17/2.8 cells or mouse calvaria-derived MC3T3E1 cells. As shown in Fig. 3, 2.5S and 2S specifically bound to nuclear proteins. Similar results were obtained using the crude nuclear extracts from both types of cells (data not shown). Binding of the radiolabeled 2.5S or 2S sequences to the nuclear proteins was competed for by unlabeled 2.5S or 2S sequences but not by the addition of an excess (40-fold) of unrelated DNA sequences such as a 30-bp sequence from the rat osteocalcin

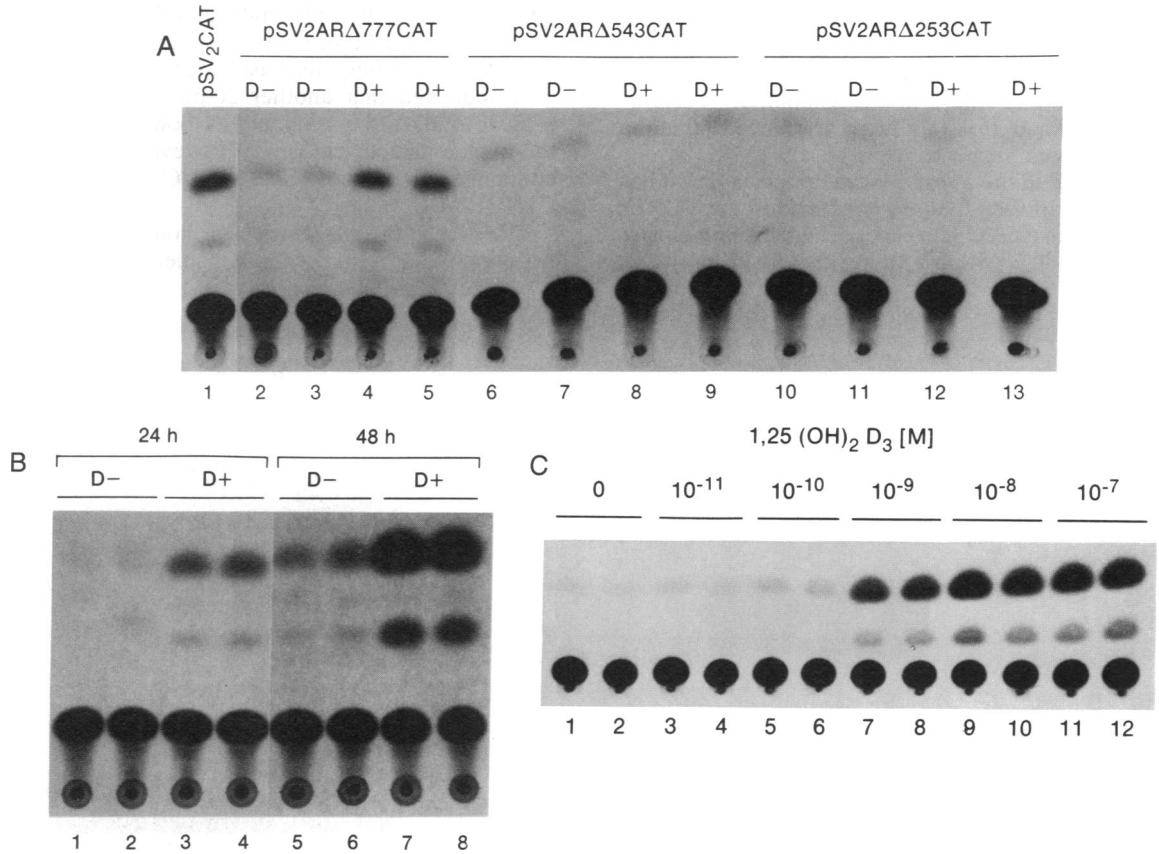

FIG. 2. Effects of $1,25(OH)_2D_3$ on the activity of the *Spp-1*/osteopontin promoter. (*A*) ROS17/2.8 cells were transfected with 10 μg of plasmid and were subsequently cultured for 48 hr in the absence (lanes D−) or the presence (lanes D+) of 10 nM $1,25(OH)_2D_3$, and CAT assays were carried out. (*B*) Time course of the $1,25(OH)_2D_3$ effect on *Spp-1*/osteopontin promoter. ROS17/2.8 cells were transfected with pSV2AR777CAT and cultured for the indicated periods of time in the absence (lanes D−) or the presence (lanes D+) of 10 nM $1,25(OH)_2D_3$. h, hr. (*C*) Dose dependence of the effect of $1,25(OH)_2D_3$ on the promoter of *Spp-1*/osteopontin gene. ROS17/2.8 cells were transfected with pSV2AR777CAT and were cultured for 48 hr in the presence of the indicated concentrations of $1,25(OH)_2D_3$.

gene (−198/−169) (Fig. 3) or salmon sperm DNA (data not shown). At least two bands were observed in the gel-retardation assay using 2.5S or 2S probes, a major upper band and a lesser lower band. Both disappeared when competed for with unlabeled 2.5S or 2S sequence.

Mutations in the direct repeats resulted in loss of competing ability for the formation of DNA–protein complex as with 2.5S or 2S sequences (Fig. 3*B*). The substitutions of four bases in 2.5S consisting of mutations of two bases each in the two repeats (ARM1 and -2, Fig. 1*C*), completely abolished the binding activity (data not shown), whereas another four-base substitution in only one of the two repeats (ARM3, Fig. 1*C*) still allowed binding (data not shown). However, the affinity of the latter mutant, ARM3, for binding appeared to be low since unlabeled ARM3 did not compete efficiently against the binding of 2.5S (Fig. 3*B*).

To examine the nature of the DNA-binding factor(s) in the nuclear extracts, the protein–DNA complex, which was formed by Spp-1–VDRE and nuclear extract was further incubated with monoclonal antibody against vitamin D_3 receptor. As shown in Fig. 4, the monoclonal antibody (XVIE10C6) raised against the vitamin D_3 receptor further retarded migration (lane 3). The other monoclonal antibody (VIIID8C12) raised against the DNA binding region of the vitamin D_3 receptor attenuated the binding (lane 4). Nonimmune IgG used as a control did not affect the intensity of the signals or the mobility of the bands (lane 5).

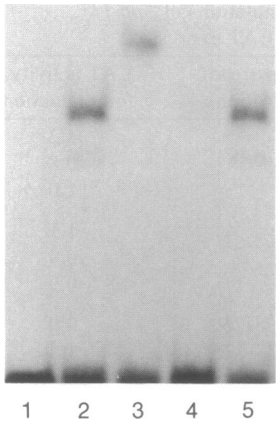

FIG. 4. Identification of vitamin D receptor as a component in the Spp-1/osteopontin–VDRE–nuclear protein complexes. The incubation mixture of ^{32}P-labeled 2.5S and crude pig intestinal nuclear extracts were further incubated in the absence (lane 2) or the presence (lane 3) of 5 μg of monoclonal antibody XVIE10C6 and monoclonal antibody VIIID8C12 (lane 4). Lanes 1 and 5 contained, respectively, only probe DNA or 5 μg of nonimmune IgG.

DISCUSSION

We have identified a 24-bp VDRE in the mouse *Spp-1* (osteopontin) gene promoter. This sequence is located 761 bases upstream of the transcription start site. It confers vitamin D responsiveness to a heterologous promoter in a position- and orientation-independent but copy-number-dependent manner and binds to the vitamin D receptor. These features indicate that this 24-bp sequence fulfills the criteria for a hormone response element.

The 24-bp VDRE in the *Spp-1* gene is unique in that it has two direct repeats of 9 bp that are not found in the VDREs of the rat and human osteocalcin genes. There are homologies of about 70% and 80% within a frame of 9 or 11 bp of this VDRE sequence with the rat and human osteocalcin genes, respectively, based on the University of Wisconsin Genetics Computer Group computer program. It should also be pointed out that in these three VDREs, at least two repeats are necessary for their function. However, no clear consensus sequence associated with functional activity was apparent between these elements, since core motifs indispensable for the response of each VDRE were not identical (Fig. 5). As expected by the sequence homology, the rat or human osteocalcin VDREs could compete with Spp-1–VDRE in binding to crude nuclear extracts shown by gel-retardation assay (data not shown), suggesting that these three VDREs bind to the same region (or species) of the vitamin D receptor. Attenuation of the signal of the DNA–protein complex by the addition of monoclonal antibody against the DNA binding region of the receptor also indicated that the common site of the vitamin D receptor is responsible for its binding to these three VDREs. Computer-based analysis of the mouse *Spp-1* gene indicated that another 20-bp sequence (at positions −280 to −261) also has about 80% homology to the VDRE of the human osteocalcin gene. However, the CAT construct containing this region, pSV2AR543CAT, did not respond to $1,25(OH)_2D_3$.

The human osteocalcin VDRE contains a complete transcription factor AP-1 response element in its core and in fact

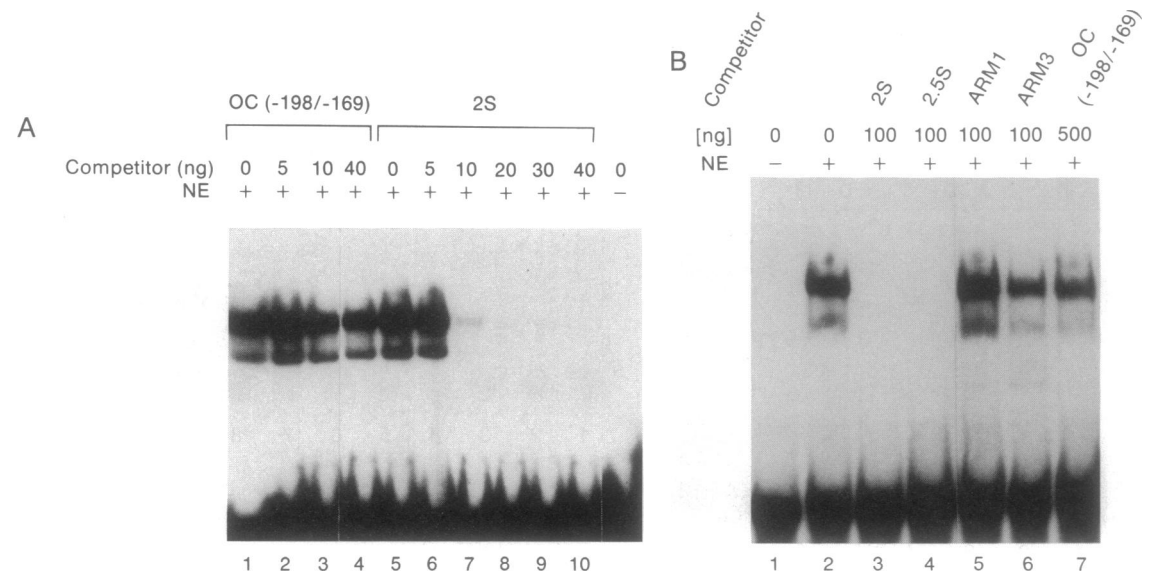

FIG. 3. Binding of *Spp-1*/osteopontin–VDRE to the nuclear extracts from ROS17/2.8 cells. (*A*) Crude nuclear extracts prepared from ROS17/2.8 cells treated with 10 nM $1,25(OH)_2D_3$ were incubated with 1 ng of ^{32}P-labeled 2S sequence in the presence or absence of the indicated amount of unlabeled 2S or an unrelated DNA sequence [30-bp sequence from rat osteocalcin (OC) promoter region at positions −198 and −169]. (*B*) ^{32}P-labeled 2.5S sequence (1 ng) was incubated with crude nuclear extracts prepared from ROS17/2.8 cells in the absence or presence of the indicated amounts of unlabeled competitor DNAs. NE, nuclear extract.

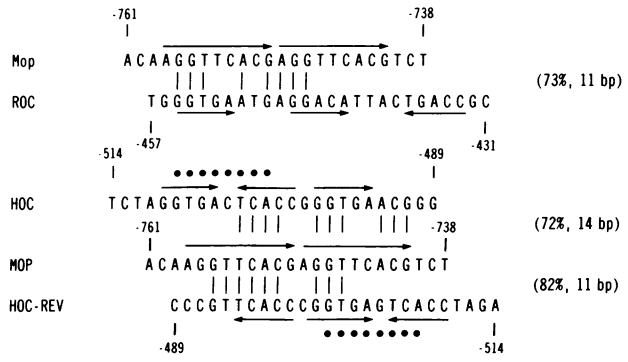

FIG. 5. Comparison of the sequences of VDREs from mouse *Spp-1*/osteopontin and rat and human osteocalcin genes. Arrows indicate each motif. Dots indicate internal AP-1 site. The ROC sequence is from refs. 20 and 27 and the HOC sequence is from refs. 18 and 19. MOP, mouse osteopontin; ROC and HOC, rat and human osteocalcin, respectively; Rev, reverse sequence.

the JUN–FOS complex was shown to be involved in the regulation of the VDRE function, whereas no such AP-1 consensus sequence was found in VDREs of rat or human osteocalcin and mouse *Spp-1* genes. Several hormone response elements are known to cross-respond to other steroids or hormones. Spp-1–VDRE, however, did not respond to other steroids or reagents (transforming growth factor β, phorbol 12-myristate 13-acetate, JUN expression vector, fibroblast growth factor, dexamethasone, and parathyroid hormones) known to affect the transcription of this gene, suggesting the relatively high specificity of the response of this sequence. Although limited homology was also found between Spp-1–VDRE and the retinoic acid response element found in retinoic acid receptor gene, treatment with retinoic acid did not affect the CAT expression of P3-2 construct transfected into ROS17/2.8 cells, which do possess receptors for retinoic acid.

The response to $1,25(OH)_2D_3$ of the 2S or 2.5S sequence is dependent on its copy number. As shown for other hormone responsive elements, the increase in the copy number of 2.5S or 2S in the same direction enhanced the responsiveness to vitamin D_3. On the other hand, if the two copies of 2S were in the opposite direction (P3-4), the basal expression of CAT was enhanced but $1,25(OH)_2D_3$ no longer affected expression. Furthermore, four direct tandem repeats of the 2S sequence in the same direction (P3-2) significantly reduced the basal level while increasing its ability to enhance promoter activity upon stimulation with vitamin D_3. The negative regulatory role of the nonliganded receptor by binding to the cognate hormone response element was also shown for the thyroid receptor. The findings described above may indicate a similar mechanism(s) in the action of the vitamin D receptor.

Substitution mutations on adjacent guanosines or thymidines in each of the two 9-bp direct repeats totally abolished the response to vitamin D as well as the DNA binding activity, indicating that these nucleotides are critical for DNA–receptor interactions. Changing both guanosines and thymidines in only the first of the two direct repeats (ARM3) effectively blocked the response to $1,25(OH)_2D_3$, suggesting that the vitamin D receptor binds to these DNA elements as a dimeric form. This ARM3 mutant can still bind weakly to a protein in nuclear extracts but cannot compete against the 2.5S or 2S sequences, suggesting that the remaining intact half of the repeat may bind to the monomeric form of the receptor or only one binding site of its dimeric form. The latter appears to be more likely because the extent of mobility shift for ARM3 is similar to that for 2.5S. This proposal was further supported by the attenuation of the binding of the other mutant in 2.5S (ARM5), where four nucleotides were inserted between the two direct repeats. As expected, this mutation also abolished the response to $1,25(OH)_2D_3$.

In conclusion, we have identified a 24-bp VDRE in the mouse *Spp-1* gene promoter region. The unique direct repeats of the sequence allowed us to analyze the interactions between the VDRE and multimeric form(s) of the vitamin D receptor. Further investigation on the VDREs in the genes responsive to vitamin D and their interactions with receptor are needed to understand the regulatory mechanism(s) and specificity of the vitamin D actions in a wide variety of tissues in a body.

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