Identification of a Novel Suppressive Vitamin D Response Sequence in the 5′-Flanking Region of the Murine Id1 Gene*

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Vitamin D promotes differentiation of cells either by simply enhancing phenotypic gene expression and/or by suppressing expression of inhibitors of differentiation. Previously, we reported that expression of a gene encoding Id1, a negative type helix-loop-helix transcription factor, was transcriptionally suppressed by 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) (1). To identify the sequence required for the negative regulation by 1,25(OH)$_2$D$_3$, a 1.5-kilobase 5′-flanking region of murine Id1 gene was examined by transiently transfecting luciferase reporter constructs into ROS17/2.8 osteoblastic cells. The transcriptional activity of this construct was repressed by 10$^{-8}$–1.25(OH)$_2$D$_3$. Deletion analysis revealed that a 57-base pair (bp) upstream response sequence (URS) (~1146–1090) was required for the suppression by 1,25(OH)$_2$D$_3$. This sequence conferred negative responsiveness to 1,25(OH)$_2$D$_3$ to a heterologous SV40 promoter. The 57-bp URS contained not only Egr-1 consensus sequence (2) but also four direct repeats of a heptamer sequence (C/A)CAGCCC. Electrophoresis mobility shift assay revealed that the 57-bp URS formed specific nuclear protein-DNA complexes, which were neither competed by previously known positive and negative vitamin D response elements nor supershifted by anti-vitamin D receptor antibody, suggesting the absence of vitamin D receptor in these complexes. These results indicate the involvement of the novel 57-bp sequence in the vitamin D suppression of Id1 gene transcription.

The active form of vitamin D, 1,25-dihydroxyvitamin D3 (1,25(OH)$_2$D$_3$; calcitriol), is not only a major calcitrophic hormone that controls systemic calcium metabolism but also a potent modulator of differentiation in several types of cells including osteoblasts (2, 3). Many studies have revealed that the molecular mechanisms of vitamin D actions, including its promotion of cell differentiation, could be explained mainly by its genomic actions via the vitamin D receptor (VDR) as a ligand-dependent transcription factor (4–7). VDR binds to vitamin D response elements (VDREs) within the promoter regions of the target genes to activate or suppress their expression. Several types of differentiation-related genes are regulated through this type of vitamin D action during cell differentiation. In addition, recent studies also showed the involvement of the nongenomic action of vitamin D in regulation of cell differentiation (9–11). For instance, monocyte differentiation was reported to be mediated by vitamin D without requiring binding to VDR (9), and keratinocyte differentiation-related genes were shown to be stimulated by 1,25(OH)$_2$D$_3$ without the presence of VDRE (6, 10, 11). Therefore, vitamin D could promote cell differentiation via both genomic and nongenomic actions (3, 6).

We have been interested in the molecular mechanism of the differentiation of osteoblasts as one of the target cells of 1,25(OH)$_2$D$_3$ (3). Similarly to other types of cells, expression of various phenotype-related genes is enhanced by 1,25(OH)$_2$D$_3$ in osteoblasts (11–14). In parallel to its direct control of the genes encoding phenotype-related proteins in osteoblasts, we hypothesized that vitamin D may regulate higher order regulatory genes to modulate osteoblastic differentiation. We have shown that Id1, a dominant negative regulator of helix-loop-helix-type transcription factors (15), is expressed in osteoblasts and that its level is transcriptionally suppressed by 1,25(OH)$_2$D$_3$ (1). Because Id1 has been shown to be a negative modulator of positive regulatory transcription factor(s) that modulate cell differentiation, 1,25(OH)$_2$D$_3$ could exert its effects on osteoblasts by suppressing expression of Id1. In the previous study, we have also shown that the suppression was specific to 1,25(OH)$_2$D$_3$ and was mediated at the level of gene transcription without requiring new protein synthesis (1). However, the mechanism with which 1,25(OH)$_2$D$_3$ suppresses Id1 gene transcription was still unknown.

Ligand-dependent or -independent repression by nuclear hormone receptor superfamilies has been investigated (8, 16–23). However, the molecular mechanisms of transcriptional repression appear to be more complicated than those of transcriptional activation (22, 23), and the mechanisms for steroidal or nonsteroidal ligand-dependent repression have also been found to be variable. In some cases, to suppress expression of the target genes, hormones utilize the same or similar response elements as those used for transactivation (18), whereas in other cases, sequences different from the classical hormone response elements are utilized for negative regulation (16, 17, 19).

Although vitamin D receptor also acts as both a transcriptional activator and a repressor similar to other members of the nuclear hormone receptor superfamilies, little is known about the mechanisms of transcriptional repression by 1,25(OH)$_2$D$_3$. *This study was supported by Grants-in-aid from the Ministry of Education 07557096, 08044258, and 09307034, a grant from the Spina Bifida Foundation, Grant 0076 from the Japan Orthopedic and Traumatology Foundation, grants from the Core Research for Evolutional Science and Technology of Japan Science and Technology Corporation, and Grant 96090205 from the Research for the Future Program of the Japan Society for the Promotion of Science. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.‡ To whom correspondence should be addressed: 3-10 Kanda-Surugadai, 2-Chome, Chiyoda-ku, Tokyo 101, Japan. Tel.: 81-3-5280-8066; Fax: 81-3-5280-8066; E-mail: noda.mph@mri.tmd.ac.jp.

1 The abbreviations used are: 1,25(OH)$_2$D$_3$, 1α,25-dihydroxyvitamin D$_3$, LUC, luciferase; EMSA, electrophoresis mobility shift assays(s); URS, upstream regulatory sequence(s); OPN, osteopontin; VDR, vitamin D receptor; PTH, parathyroid hormone; BSP, bone sialoprotein; kb, kilobase; bp, base pair(s); PCR, polymerase chain reaction.
which is also capable of utilizing nongenomic action. Five cases of negative VDRE sequence were reported such as parathyroid hormone (PTH) gene, parathyroid hormone-related protein gene, interleukin-2 gene, bone sialoprotein (BSP) gene, and vitamin D receptor binding fragment-5, a negative vitamin D responsive gene isolated from rat genomic DNA (24–30). However, because these repressive sequences are quite variable, consensus sequences have not been defined yet. Furthermore, molecular mechanisms of vitamin D repression of the genes encoding key molecules such as transcription factors involved in cell differentiation has not yet been clarified. To understand the molecular mechanisms of the 1,25(OH)₂D₃ suppression of Id1 gene expression, which could be an important step in cell differentiation, we investigated the 1,25(OH)₂D₃ effect on the transcriptional activity of the 1.5-kb promoter region of the Id1 gene and identified a sequence that is required for the 1,25(OH)₂D₃ action.

**MATERIALS AND METHODS**

**Plasmid Construction**—The promoter region of the Id1 gene (−1574/+88; 1.5BV) or PCR-generated 5′ deletion sequences (−3527/+88; 5′del-2), (−3272/+88; 5′del-3), (−272/+88; 5′del-4), (−127/+88; 5′del-5), and (−52/+88; 5′del-6) were subcloned by Tournay and Benezra into HindIII site of pGL2-Basic vector (BV) (Promega Corp., Madison, WI) as described previously (2). We generated by PCR further deletion constructs, 5′del-1100, 5′del-1050, 5′del-1000 containing the 5′ upstream regions of the Id1 promoter corresponding to −1100/+88, −1050/+88, and −1000/+88 by using 20-mer oligonucleotides as primers. SacI (5′) sites and BglII (3′) sites were introduced on each end of the PCR products, respectively, and the fragments were inserted into SacI and BglII sites of the pGL2-Basic vector.

A double-stranded 63-bp oligonucleotide containing a 57-bp sequence corresponding to the −1146−1090 region (URS) of Id1 promoter and MluI sites (underlined below) on both its ends was synthesized. The top strand (5′-CGCGTCGACGGACTCTGGCCGTCTCCATGGCGACC-CCGGCCGCGCCAGCACCTGACTGCCCA-3′) and the bottom strand (5′-GGCCGGGCGCCGCCCTGAGGCGAAGCCGACACAACTGCCCCTGA-3′) were annealed and subcloned into the MluI site 11 bp upstream of SV40 promoter in PGV-P vector (Toyo Ink MFG. Co., Ltd., Tokyo, Japan) in a normal or a reverse orientation to generate 57-PGV, 57R/PGVP (R stands for reverse orientation), and 57″-PGVP (containing two copies of the 57-bp URS), respectively. Similarly, four types of double-stranded mutated oligonucleotides of the 57-bp URS were also inserted into the same vector to generate mutated URS constructs, 57M-PGVP, 16A16-PGVP, 57A-3′-PGVP, and 57A-3′-PGVP (see Table 1). These mutated URS constructs were each divided into two segments and the internal 37-bp sequence (5′-TGGCGACCGCAAAAGCGGCGCCAGCCTGACAGCCCA-3′) were stored at −80 °C. Pig intestine nuclear extracts were kindly provided by Dr. DeLuca. Aliquots of 20,000 cpm of the probes were incubated with nuclear proteins for 30 min at 30 °C in a 35-ml reaction mixture containing 3 mg of bovine serum albumin and 2 mg of poly(dI- dC) (Pharmacia Biotech Inc.). For the detection of vitamin D receptor, monoclonal antibodies (10C6 or 8C12) raised against porcine vitamin D receptor (57′) (kindly provided by Dr. DeLuca) were added to the incubation mixture containing nuclear proteins extracted from pig intestine.

**Statistical Analysis**—Statistical significance of the difference was evaluated by Dunnett’s test for multiple comparison or Student’s t test for per-comparison analysis. To test whether certain sequences respond to vitamin D treatment, mean values of the “relative luciferase activities’’ of the constructs (vitamin D+ versus −) were compared with those of the control construct, pGL2-Control, or to the noninserted construct, PGV-P. The data based on more than three independent experiments for each construct were put together, and statistical significance (p < 0.05) was evaluated by Dunnett’s test. In the case of comparison between 57′-PGVP and 57-PGV, per-comparison analysis was applied.

**RESULTS**

Vitamin D Suppresses Transcriptional Activity of the 1.5-kb Id1 Promoter—We first examined the effect of 1,25(OH)₂D₃ on the transcriptional activity of the 1.5-kb promoter region of Id1 gene (−1574/+88; 1.5BV) by LUC assay and found that 1,25(OH)₂D₃ treatment suppressed Id1 promoter activity by 50–70% (Fig. 1). The effect was first observed at 10⁻⁸ M and peaked at 10⁻⁸ M 1,25(OH)₂D₃ (data not shown). Although the level of the luciferase activity in the control cultures continuously increased up to 72 h, similar levels of vitamin D suppression were observed during this time period (Fig. 1). Because Id1 promoter activity has been shown to be activated by serum (2), we examined the effect of serum concentration on the 1,25(OH)₂D₃ suppression. Although the basal levels of Id1 transcriptional activity was correlated to serum concentrations as reported before (2), the magnitude of 1,25(OH)₂D₃ suppression was similar (about 50–70% suppression) regardless of the concentrations of fetal bovine serum at either 0.5 or 10% (data not shown). These results were consistent with our previous observation of the effect of 1,25(OH)₂D₃ on Id1 expression in...
Northern analysis and nuclear run-on assay (1), indicating that this 1.5-kb promoter fragment is necessary and sufficient for the 1,25(OH)₂D₃ suppression of Id1 gene.

Localization of the Suppressive Vitamin D Response Sequence in Id1 Promoter—To examine whether any particular regions in the 1.5-kb fragment mediate the vitamin D suppression of Id1 expression, deletion analysis was carried out by transfecting 1.5BV and seven types of deletion mutant constructs of the Id1 promoter into ROS17/2.8 cells. By this deletion analysis, the response region was located within a 221-bp fragment (−1147/−927) (Fig. 2A). The downstream promoter region located within −927/+88 did not significantly contribute to the 1,25(OH)₂D₃ suppression. Therefore, we concentrated on analyzing the 231-bp region by further deletion analysis. Three additional deletion mutants were made by PCR and were subcloned into pGL2-Basic vector (5’-del-1100, 5’-del-1050, and 5’-1000). This second series of deletion analysis showed that only the activity of 5’-del-2 (−1147/+88) construct but not that of any of the other constructs (5’-del-1100, 5’-del-1050, and 5’-1000) was repressed by 1,25(OH)₂D₃, indicating that the sequence between −1147 and −1100 was essential for the 1,25(OH)₂D₃ suppression (Fig. 2B).

A 57-bp Upstream Regulatory Sequence (URS) Confers Negative Response to Vitamin D to a Heterologous Promoter—To examine the negative 1,25(OH)₂D₃ regulation via the sequence between −1147 and −1100 identified above, we made three additional constructs, 57-PGVP, 57R-PGVP, and 57*2-PGVP. To generate these constructs, a double-stranded oligonucleotide of the 57-bp URS corresponding to the positions between −1147 and −1100 was made. Then, a single or a double copy of this sequence was inserted into the multiple cloning site immediately upstream from the SV40 early promoter in PGV-P vector in a normal (57-PGVP and 57*2-PGVP) or a reverse orientation (57R-PGVP). As shown in Fig. 3, the 57-bp URS conferred repressive response to 1,25(OH)₂D₃ to the SV40 early promoter in a position- and orientation-independent manner. There was a small but statistically significant difference between the levels of vitamin D suppression in 57-PGVP versus 57*2-PGVP constructs when per-comparison analysis was applied (Student’s t test, p < 0.01; Fig. 3). The activity of 1,25(OH)₂D₃ was confirmed by using a positive VDRE control vector, OPN*2-PGVP, which showed 5- to 6-fold enhancement of luciferase activity in response to 1,25(OH)₂D₃ treatment (Fig. 3).

The 57-bp fragment contained in its mid-portion an Egr-1 consensus sequence (5’-CGCCGGGC-3’) (37) at −1117 to −1109 and a YY-1 consensus sequence (5’-CCATGGCGA-3’) at −1127 to −1119 as reported before (2) (underlined in Fig. 4). In addition, it contains a sequence 5’-GGGGCC-3’ in −1118/−1113 (reverse direction), which matches the core region of Sp1 binding consensus sequence (38) (Fig. 4, dotted line), and an overlapping 12-bp sequence in almost the same region (−1120/−1109), which corresponds to the 10-bp sequence out of the 12-bp consensus sequence for WT1, a suppressor protein for
FIG. 3. The 57-bp URS confers vitamin D suppression to heterologous SV40 promoter. Four constructs, 57-PGVP, 57R-PGVP, 57′-2-PGVP, and OPN′-2-PGVP were generated as described under “Materials and Methods.” Responsiveness to the treatment with 10−8 M 1,25(OH)2D3 was examined by transfecting the plasmid into confluent ROS17/2.8 cells as described in the legend to Fig. 1. Relative luciferase activity was calculated as described in the legend to Fig. 2A. Vitamin D suppressed the luciferase activity in the cells transfected with the constructs, 57-PGVP and 57′-2-PGVP, but not that of the PGV-P vector transfected cells (Dunnett’s test; **, p < 0.01 against PGV-P). The relative luciferase activity in 57′-2-PGVP (per-comparison analysis; Student’s t test; **, p < 0.01 against PGV-P). The numbers of independent experiments (the numbers of independent experiments used for calculation are indicated in parentheses). The error bars indicate standard deviations.

Wilms’ tumor (5'-GCCGGGGCGGTG-3') (39) (Fig. 4, dashed line). We referred to this 21-bp portion of the 57-bp URS, as the GC-rich region (20 bases are G or C) (Fig. 4). Moreover, there are four direct repeats of a novel heptamer sequence (5'-(A/C)CAGCCC-3') separated by 1-, 8-, or 20-bp gaps within the 57-bp URS (Fig. 4, Hep1–Hep4), whereas no conserved VDR binding sequence that includes VDRE half-site consensus sequence RKKNSA (40) was found within the 57-bp URS.

The 57-bp URS Binds the Proteins in the Nuclear Extracts of ROS17/2.8 Cells—To examine whether the 57-bp sequence binds to nuclear factors in ROS17/2.8 cells, electrophoresis mobility shift assays (EMSA) were performed. As shown in Fig. 5A, the 57-bp URS bound nuclear proteins forming two complexes L and H (Fig. 5A, lane 2). The complex marked “L” (for “lower”) is a prominent one and contains one major band and a minor band, which migrates only slightly faster than the major band. The faint faster band is possibly formed due to the lack of one or more small components in forming the DNA-protein complex. We refer to these bands as L complex. The other complex, “H” (for “higher”), is a faint one that migrates slower than L complex. These L and H complexes were competed out by a 100-fold molar excess of unlabeled 57-bp URS probe (Fig. 5A, lane 3), whereas cyclic AMP response element sequence used as a control did not compete them out even at a 200-fold molar excess (Fig. 5A, lanes 9 and 10).

Requirement of the Subregions of 57-bp URS for the Binding to the Nuclear Proteins to Form L Complex—We further examined within the 57-bp URS the presence of possible essential subregions, which are required both for the formation of nuclear protein-DNA complexes and for the suppression by vitamin D treatment. Seven types of mutant oligonucleotides were made to be used for the competition assays (Fig. 5 and Table 1), as well as to construct reporter plasmids for the transcription assays (Fig. 6 and Table 1).

To design these mutant oligonucleotides, we first examined the contribution of Egr-1 site in the context of the 57-bp URS, because it was shown to be involved in the regulation of this promoter by serum (2). We made a mutant oligonucleotide, 57M, by introducing a 4-bp substitution in the core region of Egr-1 site from CGCC to AAAA within the 57 bp (Table 1). Two types of 5′ and 3′ end deletion type mutant oligonucleotides, d5′-37 and d3′-38, retaining intact Egr-1 site were also made to examine the contribution of both the 20-bp 5′-flanking and the 19-bp 3′-flanking regions, respectively. As shown in the Fig. 5A, mutant competitor 57M did not compete against L complex (Fig. 5A, lanes 7 and 8), indicating that the Egr-1 site is required for L complex formation. However, because the other types of competitors, d5′-37 and d3′-38, also failed to compete for L complex (Fig. 5A, lanes 4 and 5), only the presence of the Egr-1 site alone is not sufficient for the L complex formation. Instead, the sequences within the 20-bp 5′ and 19-bp 3′ ends of the 57-bp URS are also required for the L complex formation. Moreover, co-existence of the two oligos 5′d-38 and 3′d-37 was not sufficient to compete out the L complex completely (Fig. 5A, lane 6), indicating that these sequences are simultaneously required on the same oligomer for the L complex formation.

In this competition experiment, we also found that the H complex was competed by 57M. With regard to the deletion mutants, d5′-37 did not compete the H complex out at all, and d3′-38 competed it at intermediate efficiency. These data indicate that the H complex formation does not require the mid-portions of the 57-bp URS corresponding to Egr-1 site, but it requires simultaneously both 20 and 19 bp at the 5′ and 3′ ends of the 57-bp URS.

To further examine the contribution of both ends of the 57-bp URS, we made two additional oligos, 16A16 (replacement with a 25-adenine stretch in the mid-portion) and 8A8 (replacement with a 41-adenine stretch in the mid-portion) (Table 1), containing substitution mutations within the central region of the 57-bp URS. Competition assays using these oligos (Fig. 5B) showed that 8A8 and 16A16 as well as 57M did not compete against the L complex, whereas the 57-bp URS competed the complex formation. As for H complex, 16A16 and 57M competed it out, whereas 8A8 did not compete efficiently, indicating that the simultaneous presence of the 16-bp ends on both 5′ and 3′ sides was again not enough for complex L formation but was required for efficient H complex formation.

FIG. 4. The sequence of the 57-bp URS. The 57-bp URS contains a complete consensus sequence for Spl (dotted underline), WT-1 (dashed underline), and YY-1 binding sites in its mid-portion (bold underline) in its mid-portion. We referred to this 21-bp region as a GC-rich region, indicated by a large shaded box. There are four repeats of a heptamer motif of (A/C)CAGCCC. We refer to these sequences as Hep-1, Hep-2, Hep-3, and Hep-4 (indicated by small boxes). Antisense sequence for Hep1–Hep4 (rHep1–rHep4) are also indicated by arrows. The sequences of Hep1–Hep4 are aligned in the bottom panel of the figure. Mismatched bases are shown in lowercase letters. A putative consensus sequence of the heptamer sequence is indicated on the bottom line. Asterisks indicate the positions selected for the substitution mutations to make mutant oligonucleotides including 57A1, 57A2, 57A3, 57A4, 57A5, and 57A6 as described under "Materials and Methods."
### Table I

Summary of competition and luciferase assays of the mutated URS fragments

| Oligonucleotide | Competition † | Suppression ‡ |
|-----------------|---------------|---------------|
| **L Complex** | **H Complex** | **ND** |
| 57bp URS | + | + | + |
| 57-52 | - | - | ND |
| 57M | + | - | - |
| 16A16 | + | - | - |
| 8A8 | - | - | ND |
| 57A-2 | + | + | + |
| 57-5A | + | + | + |

† The results of the competition assay performed by using the 57-bp URS probe and the nuclear extracts prepared from ROS17/2.8 cells are evaluated based on the intensity of each group of the bands (H and L complexes; +, competed out; –, not competed; +/-, weakly competed).

‡ The results of the luciferase assay with regard to the response to vitamin D treatment in the cells transfected with constructs containing corresponding oligonucleotides ligated immediately upstream of the SV40 promoter of the PGV-P. (+ indicates suppressive response to vitamin D treatment; – indicates no response to vitamin D treatment; ND, not determined.) We found that a sequence has the capacity to respond to vitamin D treatment if the mean values of relative luciferase activities of the construct was significantly different from that of PGV-P construct (p < 0.05), based on the data from more than four independent experiments. The statistical difference was evaluated by Dunnett's test for multiple comparison.

As a control, we additionally made five mutant oligos, 57A-1, 57A-2, 57A-3, 57A-4, and 57A-5, each of which contains a point substitution mutation in one site within the 57-bp URS (at –1138, –1133, –1129, –1106, and –1097, respectively). These five mutant oligos competed for the L and H complex formation (data not shown), indicating that mutation by itself did not affect the binding activity. When all the five sites were mutated simultaneously in another mutant oligo, 57-5A, containing substitutions at –1138 (G to A), –1133 (C to A), –1129 (C to A), –1097 (G to A), and –1106 (C to A) within the 57-bp URS (Table I), the oligo still competed against the L and H complex formation, although the level of competition was slightly less than the intact 57-bp URS as shown in Fig. 5C. Overall, these observations suggest that one large region is necessary for the complex L formation.

The L Complex Is Involved in the Vitamin D Suppression—We then made four types of luciferase constructs (57M-PGVP, 16A16-PGVP, 57A3-PGVP, and 57-5A-PGVP) carrying 57-bp URS with mutations, including 57M (replacement of the middle 4 bases with four adenines), 16A16 (replacement of the middle 25 bases with 25 adenines), 57A-3 (a single point mutation at –1129 from C to A), and 57-5A (five point mutations in one oligomer at –1138, –1133, –1129, –1106, and –1097 as described above), respectively (see Table I and “Materials and Methods”), to examine the responsiveness of these sequences to vitamin D treatment. As shown in Fig. 6, vitamin D suppression was not observed in 16A16-PGVP and 57M-PGVP, which cannot compete against L complex, although they compete against H complex. In the control mutant constructs, 57A3-PGVP and 57-5A-PGVP, vitamin D suppression was observed similar to that of the wild type 57-bp URS (Fig. 6). These data indicate correlation between the L complex formation and vitamin D suppression (Table I) and therefore supported the idea that the L complex is required to mediate vitamin D suppression.

VDR Does Not Bind to 57-bp URS Directly—As stated already, the 57-bp URS did not contain any conserved VDR binding sequences that include VDRE half-site consensus se-
Quence, implying the absence of direct binding of VDR to this 57-bp sequence. To test this point, we examined whether VDR is present in the L or H complex.

We used a monoclonal antibody (10C6) raised against VDR. The radiolabeled 57-bp URS bound to the proteins in the nuclear extracts prepared from pig intestine and formed one major band, which showed similar mobility to the bands L, and clear extracts prepared from pig intestine and formed one complex produced by using pig intestine nuclear extract and 57-bp URS by lanes 7 and 8). However, preincubation with anti-VDR antibody neither affected the intensity of the signals nor the mobility of the retarded band. As a control, OPN-VDRE bound the proteins in the pig nuclear extracts, and the addition of the antibody supershifted the bands (Fig. 7A, lane 2). A competition assay against the binding of the 57-bp URS to the proteins in the crude nuclear extract prepared with ROS17/2.8 cells using eight types of cold VDRE oligonucleotides. EMSA was carried out as described under “Materials and Methods.” A monoclonal antibody (Ab) raised against VDR (10C6) lanes 2 and 8) or nonimmune serum (lane 3, NI) was added to the binding mixture. As competitors, 57-bp URS, 38-bp OPN-VDRE, or 25-bp PTH-VDRE oligonucleotide was added at a 100-fold molar excess in lane 4, 5, or 6, respectively. The closed arrow indicates a major band of the DNA-protein complex formed with 57-bp URS, which was similar to the band L in size. The open arrows indicate binding complexes formed with OPN-VDRE (lane 7), indicating supershift by the addition of the 10C6 antibody as shown in lane 8. B, competition assay against the binding of the 57-bp URS to the proteins in the crude nuclear extract prepared from ROS17/2.8 cells using eight types of cold VDRE oligonucleotides. EMSA was carried out as described under “Materials and Methods.” Incubation was conducted in the presence of 100 molar excess of the competitors including the 57-bp URS (57, lane 2) and the VDREs of mouse OPN (lane 3), rat osteocalcin (OC, lane 4), rat calbindin D28k (489/445) (Cb 9, lane 5), mouse calbindin D28k (200/170) (Cb 28, lane 6), rat 24-hydroxylase (262/238) (OH-u, upstream VDRE, lane 7), (154/134) (OH-d, downstream VDRE, lane 8), human PTH (125/101) (hPTH, lane 9), and rat BSP (31/4) (BSP, lane 10). Arrows indicate the fast migrating (L, bottom) and the slow migrating bands (H, top). As a control, the labeled 57-bp URS probe was loaded without adding competitors (lane 1). Densities of the bands were quantified by densitometer and evaluated by calculating the ratios of the densities of the bands with the total bands in each lane.

![Fig. 6. Mutations in the 57-bp URS alter its responsiveness to vitamin D treatment.](image)

Four constructs carrying mutations in the 57-bp URS (57A3-PGVP, 57A5-PGVP, 57M-PGVP, and 16A16-PGVP) were made as described under “Materials and Methods.” Responsiveness to the treatment with 10^{-8} M 1,25(OH)_{2}D_{3} was examined by transfecting the plasmid into confluent ROS17/2.8 cells as described in the legend to Fig. 1. Relative luciferase activity was calculated as described in the legend to Fig. 2A. Vitamin D suppressed the luciferase activity in the cells transfected with the constructs 57-PGVP, 57A3-PGVP, and 57A5-PGVP but not that of the cells transfected with 57M-PGVP and 16A16-PGVP. Columns represent mean values of relative luciferase activity obtained from more than three independent experiments (the numbers of independent experiments used for calculation are indicated in the parentheses). The error bars indicate standard deviations.

![Fig. 7. The 57-bp URS does not bind VDR.](image)
affect DNA-protein complex formation with regard to the 57-bp URS (data not shown).

**DISCUSSION**

We found that 1,25(OH)$_2$D$_3$ exerted its suppressive effect on Id1 gene transcription via the 57-bp upstream sequence of this gene (−1147/−1090, URS), which was able to confer the repressive response to 1,25(OH)$_2$D$_3$ to a heterologous SV40 promoter. This 57-bp URS shares its upstream boundary with the −1147/−1037 sequence (111 bp), which was reported to be fully active in mediating positive response to serum (2). However, the 57-bp URS is 54 bp shorter than the −1147/−1037 fragment, and serum concentration did not alter the responsiveness of Id1 promoter to 1,25(OH)$_2$D$_3$, suggesting that the responsiveness of the 57-bp URS to 1,25(OH)$_2$D$_3$ is independent from the serum responsiveness, which requires the downstream 53-bp (−1089/−1037) sequence.

The 1,25(OH)$_2$D$_3$ suppression of Id1 promoter activity via the 57-bp URS represents a novel type of repressive vitamin D regulation, because it was completely different from all the six cases of 1,25(OH)$_2$D$_3$-dependent transcriptional repression (16–22), especially in that it does not require direct binding of VDR. The absence of the direct binding of VDR to the 57-bp URS was supported by three different lines of observations on EMSA. First, the L and H complexes, which were formed by the binding of the 57-bp URS to the nuclear extracts prepared from ROS17/2.8 cells, were not competed out by any of the eight known VDREs. Secondly, the bands formed by the 57-bp URS and pig intestine nuclear extracts, which contain VDR and 9-cis retinoic acid receptor proteins, were not competed out by OPN-VDRE or PTH-VDRE. And thirdly, the patterns of these bands did not change in the presence of the two different types of anti-VDR antibodies, 10C6 (Fig. 7A, lane 2) and 5C12 (data not shown). It may be possible that conformational changes of VDR might have occurred to abolish or mask the antibody recognition site by interacting with unknown cofactor proteins. However, the mobility of the DNA-protein complex formed by the 57-bp URS appeared to be faster than those formed by OPN-VDRE (Fig. 7B, lane 1 versus lane 7), suggesting that the components in the DNA-protein complex bound to the 57-bp URS would be different from those bound to OPN-VDRE.

It has been also reported that the promoter DNA fragment of 24-hydroxylase gene does not bind to VDR to participate in vitamin D activation. However, coexistence of VDR binding site (classic type VDRE) in its proximity is necessary to be functional in this case (43). In analogy to this type of regulation by vitamin D, we also tested the possibility that some kind of sequences that bind to VDR might also be involved in the case of transcriptional repression of Id1 gene. We found a VDRE-like consensus sequence (5′-GGTTCAagaaGTCTCA-3′) in a reverse direction at the position −66 to −52 in the Id1 promoter, which bound to VDR as confirmed by anti-VDR-antibody supershift experiments and OPN-VDRE competition assay in EMSA (data not shown). However, neither of the promoter constructs containing this sequence responded to 1,25(OH)$_2$D$_3$ (Fig. 2A; see 5′-del3, 5, 6, 7, and 8), nor did the construct linking the two sequences (57-bp URS and −66/−52) together to the SV40 promoter-luciferase construct (PGV-P) indicate any sign of synergism (data not shown). In this regard, the 57-bp URS should be regarded as a novel type of DNA sequence, which by itself can confer responsiveness to 1,25(OH)$_2$D$_3$ without association with VDRE consensus sequence. The vitamin D effect on Id1 gene could be regarded as a novel type of genomic action that is exerted without requiring new protein synthesis (1) and that does not require binding of VDR to the 57-bp sequence.

The next problem to be solved is how vitamin D represses the Id1 gene expression via this sequence. Several possibilities could be proposed to be the candidate mechanism(s) to mediate vitamin D suppression. The first one is the involvement of additional nongenomic action of vitamin D. It has been reported that 1,25(OH)$_2$D$_3$ and other metabolites of vitamin D exert some part of their effects by binding to yet unidentified receptor(s) on the cell surface and by utilizing the phosphorylation pathway via activating protein kinase C and mitogen-activated protein kinase, which might cause changes in the transcriptional regulation of some genes (45, 46). This type of pathway might be involved in nongenomic action to regulate cell differentiation as reported in the case of monocyte differentiation (9). The 57-bp URS might also be utilized in a similar way to mediate vitamin D suppression of the expression of Id1 gene, although whether this is the case or not is still to be determined.

The second possibility is the involvement of GC-rich region as a binding site for repressor protein(s). The mid-portion of the 57-bp URS, containing a Egr-1 consensus sequence (2), a core region of Sp1 binding consensus sequence (38), and a 12-bp sequence resembling the consensus sequence for WT1 (39), was indicated to be required for vitamin D-mediated repression (57M, Table I). Because Egr-1, YY1, and WT1 are known to be able to act as repressor proteins (23), this region might bind some of these repressor proteins to suppress Id1 gene expression. However, despite the requirement of the Egr-1 binding sequence for forming the L complex in EMSA (Fig. 5A), preliminary experiments using anti-Egr-1, 2, 3, and 4 antibodies failed to show the presence of Egr-1 in the L complex (data not shown). This observation was consistent with the result of EMSA using nuclear extract with or without treatment of 1,25(OH)$_2$D$_3$ (data not shown), which is known to enhance Egr-1 expression (45). Therefore, we assume that other kinds of protein(s), which probably resemble Egr family proteins, might bind to this sequence to suppress the Id1 gene.

The third possibility is the involvement of the heptamer sequences, Hep1–Hep4, located in the 57-bp URS. The sequence, (C/A)CAGCCC, which is assumed to be a consensus sequence for these heptamers, does not correspond to any known consensus sequences for transcription factors reported before. However, because we showed that the full-length 57-bp URS containing all of these heptamers was required for the formation of the L complex and for the vitamin D suppression (Table I), not only the GC-rich region but also the four repeated heptamer sequences, Hep1–Hep4, might be involved in binding of unknown nuclear proteins to exert 1,25(OH)$_2$D$_3$-dependent repression.

Another possibility with regard to the mechanism of vitamin D suppression could be the presence of co-activator(s) or co-repressor(s), which do not bind firmly and thus could not be detected by EMSA. This speculation is based on the fact that no enhancement or shift of the bands was observed in EMSA using nuclear extracts regardless of the treatment of the cells with vitamin D (data not shown).

In analogy to myogenesis (47) and neurogenesis (48), Id1 has been hypothesized to be an inhibitor of a putative master gene, which might regulate expression of multiple target genes important for osteoblastic cell differentiation. However, it is not clear to what extent the vitamin D effect on the induction of cell differentiation is mediated by its suppressive effect on Id1 gene expression. Vitamin D also activates or suppresses quite a variety of genes related to the regulation of osteoblastic differentiation. Vitamin D enhances expression of c-fos, which is also related to osteoblastic differentiation (49). Vitamin D activates genes encoding growth factors, such as bone morphogenetic
protein, insulin-like growth factor-I, basic fibroblast growth factor, and vascular endothelial growth factor (3, 50), which have been reported to increase bone formation. However, these factors alone would not be sufficient to support complete differentiation process of osteoblasts. On the contrary, if the target of Id1, possibly a member of helix-loop-helix type transcription factor family, has a potential to support or activate differentiation of osteoblasts as a master regulatory molecule, the impact of the vitamin D suppression of Id1 would be significant in the differentiation of osteoblasts. Investigation is in progress in our laboratory to identify a repertoire of transcription factors in osteoblastic cells, which could be the targets of Id1 suppression (51).

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REFERENCES
1. Kawaguchi, N., DeLuca, H. F., and Noda, M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4569–4572
2. Tournay, O., and Benezra, R. (1996) Mol. Cell Biol. 16, 2418–2430
3. Minghetti, P. P., and Norman, A. W. (1988) The FASEB Journal 2, 3043–3053
4. Haussler, M. R., Jurutka, P. W., J. C., Thompson, P. D., Selznick, S. H., Faust, M. R., and St-Arnaud, R. (1996) Trends Endocrinol. 7, 387–388
5. Beale, D. D., Pillai, A. S. (1993) Endocrinology 13, 14–19
6. Noda, M., Vogel, R. L., Craig, A. M., Prchal, J., DeLuca, H. F., and Denhardt, D. T. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9995–9999
7. Demay, M. B., Gerardi, J. M., DeLuca, H. F., Kronenberg, H. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 369–372
8. Kerner, S. A., Scott, R. A., Pike, J. W. (1986) Proc. Natl. Acad. Sci. U. S. A. 84, 4455–4459
9. Benezra, R., Yan, W. (1990) Cell 61, 49–59
10. Hanna-Rose, W., and Hansen, U. (1993) Trends Endocrinol. 12, 229–234
11. Johnson, A. D. (1995) Cell 81, 655–658
12. Carr, F. C., and Weng, N. C. W. (1994) J. Biol. Chem. 269, 4175–4179
13. Pedraza, F. J., Ortiz, M. A., and Pfahl, M. (1995) Mol. Endocrinol. 9, 1553–1548
14. Diamond, M. J., Miner, J. N., Yoshinaga, S. K., and Yamamoto, K. R. (1990) Science 249, 1266–1272
15. Droutin, J., and Sunt, Y. L. (1993) EMBO J. 12, 145–156
16. Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Gless, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C. K., and Rosenfeld, M. G. (1995) Nature 377, 397–404
17. Yen, P. M., Liu, Y., Sugawara, A., and Chiu, W. W. (1996) J. Biol. Chem. 271, 10910–10916
18. Liu, S. M., Koszewski, N., Lopez, M., Malluche, H. H., Olivera, A., and Russel, J. (1996) Mol. Endocrinol. 10, 206–215
19. Demay, M. B., Kiernan, M. S., DeLuca, H. F., and Kronenberg, H. M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8097–8101
20. Mackey, S. L., Heymont, J. L., Kronenberg, H. M., and Demay, M. B. (1996) Mol. Endocrinol. 10, 298–305
21. Falzon, M. (1996) Mol. Endocrinol. 10, 672–681
22. Li, J. J., and Sodek, J. (1995) J. Biochem. (Tokyo) 289, 625–629
23. Sakoda, K., Fukuwara, M., Ara, S., Suzuki, A., Nishikawa, J., Imamura, M., and Nishihara, T. (1996) Biochem. Biophys. Res. Commun. 219, 31–35
24. Shima, T., Niwa, H., and Nakamura, Y. (1996) J. Biol. Chem. 271, 9507–9513
25. Boulton, R., Okamura, W. H., and Norman, A. W. (1995) Endocrinology 136, 206–207
26. Friedman, L. P. (1992) Endocrin. Rev. 13, 129–145
27. Beato, M., Herrlich, P., and Shutz, G. (1995) Cell 83, 851–857
28. Bhatia, M., Kirkland, J. B., and Meckling-Gill, K. A. (1995) J. Bone Miner. Res. 10, 15962–15965
29. Saunders, N. A., Bernacki, S. H., Vollberg, T. M., and Jetlen, A. E. (1993) Mol. Endocrinol. 7, 387–388
30. Bixler, D. R., Pillai, A. S. (1993) Endocrinology 13, 14–19
31. Noda, M., Vogel, R. L., Craig, A. M., Prchal, J., DeLuca, H. F., and Denhardt, D. T. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9995–9999
32. Demay, M. B., Gerardi, J. M., DeLuca, H. F., Kronenberg, H. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 369–372
33. Kerner, S. A., Scott, R. A., Pike, J. W. (1986) Proc. Natl. Acad. Sci. U. S. A. 84, 4455–4459
34. Benezra, R., Yan, W. (1990) Cell 61, 49–59
35. Hanna-Rose, W., and Hansen, U. (1993) Trends Endocrinol. 12, 229–234
36. Johnson, A. D. (1995) Cell 81, 655–658
37. Carr, F. C., and Weng, N. C. W. (1994) J. Biol. Chem. 269, 4175–4179
38. Pedraza, F. J., Ortiz, M. A., and Pfahl, M. (1995) Mol. Endocrinol. 9, 1553–1548
39. Diamond, M. J., Miner, J. N., Yoshinaga, S. K., and Yamamoto, K. R. (1990) Science 249, 1266–1272
40. Droutin, J., and Sunt, Y. L. (1993) EMBO J. 12, 145–156
41. Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Gless, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C. K., and Rosenfeld, M. G. (1995) Nature 377, 397–404
42. Yen, P. M., Liu, Y., Sugawara, A., and Chiu, W. W. (1996) J. Biol. Chem. 271, 10910–10916
43. Liu, S. M., Koszewski, N., Lopez, M., Malluche, H. H., Olivera, A., and Russel, J. (1996) Mol. Endocrinol. 10, 206–215
44. Demay, M. B., Kiernan, M. S., DeLuca, H. F., and Kronenberg, H. M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8097–8101
45. Mackey, S. L., Heymont, J. L., Kronenberg, H. M., and Demay, M. B. (1996) Mol. Endocrinol. 10, 298–305
46. Falzon, M. (1996) Mol. Endocrinol. 10, 672–681
47. Li, J. J., and Sodek, J. (1995) J. Biochem. (Tokyo) 289, 625–629
48. Sakoda, K., Fukuwara, M., Ara, S., Suzuki, A., Nishikawa, J., Imamura, M., and Nishihara, T. (1996) Biochem. Biophys. Res. Commun. 219, 31–35
49. Shima, T., Niwa, H., and Nakamura, Y. (1996) J. Biol. Chem. 271, 9507–9513
50. Boulton, R., Okamura, W. H., and Norman, A. W. (1995) Endocrinology 136, 206–207
51. Boulton, R., Okamura, W. H., and Norman, A. W. (1995) Endocrinology 136, 206–207