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Identification of a Novel Vitamin D Response Element from the Rat Genome[1]

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1α,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], the active form of vitamin D_3, has been thought to be a multifunctional agent. In order to discover novel roles of 1,25-$(OH)_2D_3$, we have been looking for new genes that are regulated by 1,25-$(OH)_2D_3$. Because the actions of 1,25-$(OH)_2D_3$ are mediated through the vitamin D receptor (VDR), that is a DNA binding transcription factor, vitamin D regulated genes should have VDR binding sites in their regulatory regions. In this paper, we describe a novel vitamin D response element (VDRE)-containing sequence, clone 3, which was isolated through binding to VDR. DNA sequence analysis of clone 3 did not reveal any significant similarity with sequences reported previously. Clone 3 had two regions consisting of a direct repeated sequence of AGTTCA motifs, both of which bound to VDR independently. Whereas each direct repeat sequence alone could not mediate transcriptional activation efficiently, with their co-existence there was a strong response to 1,25-$(OH)_2D_3$, indicating that these two direct repeated sequences act cooperatively.

Key words: 1,25-dihydroxyvitamin D_3, promoter, rat genomic fragment, vitamin D receptor, vitamin D response element.

Vitamin D_3 is photobiosynthesized from 7-dehydro-cholesterol and metabolized through a series of hydroxylations. 1,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], the most active metabolite of vitamin D_3, exerts various biological effects, including the maintenance of calcium homeostasis, the regulation of bone remodeling, and the modulation of cell growth and differentiation (*1*). The cellular actions of 1,25-$(OH)_2D_3$ are thought to be mediated by the vitamin D receptor (VDR), which is a member of the large family of ligand-activated transcription factors called the nuclear receptor family (*2, 3*). Nuclear receptors primarily act through direct association with specific DNA sequences known as hormone response elements (HREs). These HREs usually consist of two copies of a consensus core sequence. A subset of these nuclear receptors, including VDR, thyroid hormone receptor (TR), retionic acid receptor (RAR), and some orphan receptors, preferentially bind to a direct repeat of the HRE half-site motif, AGGTCA, as either homodimers or heterodimers with the retinoid X receptor (RXR) (*4*). The DNA binding specificity of these receptors is in part determined by the differential spacing between the core recognition motifs (*5*). So far, several vitamin D response elements (VDREs) have been identified in the regulatory regions of vitamin D-inducible genes. Human osteocalcin (*6, 7*), rat osteocalcin (*8, 9*), mouse osteopontin (*10*), rat calbindin D-9K (*11*), the avian integrin β3 subunit (*12*), and rat 25-hydroxyvitamin D_3 24-hydroxylase (*13, 14*) have been reported to be up-regulated by 1,25-$(OH)_2D_3$ through their VDREs. On the other hand, parathyroid hormone (PTH) has been demonstrated to be down-regulated by 1,25-$(OH)_2D_3$ (*15*). Recently, WAF1 was found to be up-regulated by 1,25-$(OH)_2D_3$, which proved to be related to the differentiation of myelomonocytic cells (*16*). However, these genes are not sufficient to explain the various effects of 1,25-$(OH)_2D_3$.

In order to identify novel genes whose transcription is regulated by specific transcription factors, some recent studies have utilized the properties of transcription factors that specifically recognize and bind to DNA elements *in vitro* (*17, 18*). Genomic DNA fragments bound by the transcription factors were selected and amplified. Some of the enriched fragments contained functional *cis*-acting elements which actually regulated the expression of neighboring genes (*19*). In this study, we have contrived a method, reported earlier, for the isolation of 1,25-$(OH)_2D_3$-regulated genes based on identification of VDR binding sites (*20, 21*). While RXR remarkably increases the binding affinity of VDR to VDREs, the recognition of a specific sequence definitely depends on VDR. As a matter of fact, the VDR homodimer showed more restricted DNA binding specificity than the VDR/RXR heterodimer (*20*). Therefore, we decided to use the VDR homodimer for the screening the vitamin D-regulated genes from rat genomic DNA. Here we describe one of the enriched fragments,

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Abbreviations: VDR, vitamin D receptor; RXR, retinoid X receptor; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; DR, direct repeat; VDRE, vitamin D response element; PTH, parathyroid hormone; HRE, hormone response element; GST, glutathione S-transferase.

clone 3, which functions as an active VDRE. Clone 3 has two binding sites for VDR, which act cooperatively in 1,25-$(OH)_2D_3$ dependent transcription. The VDRE isolated in this study seems to function in an unknown fashion.

MATERIALS AND METHODS

Binding Selection Procedure and Sequencing—The enrichment of VDR-binding fragments was carried out by the filter binding method (*18*). Rat genomic DNA was prepared from spleen, and partially digested with *Sau*3AI. The digested DNA fragments were fractionated on a 1% agarose gel, fragments of approximately 1–3 kb being collected. These fragments (3 μg) were incubated with 10 pmol of VDR, which was overexpressed in *Escherichia coli* cells and purified (*20*). The binding reaction was performed in 400 μl of the binding buffer [40 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 0.1 mg/ml BSA, 1×10^{-7} M $1,25(OH)_2D_3$] for 30 min on ice. The binding mixture was filtered through a pre-soaked nitrocellulose filter (Schleicher & Schuell; BA85, 0.45 μm, 25 mm). The filter was washed with 500 μl of TEGDZ (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 10% glycerol, 2.5 mM DTT, and 50 μM $ZnCl_2$) five times. The bound DNA fragments were eluted with 400 μl of the elution buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 20 mM NaCl, and 0.1% SDS), followed by ethanol precipitation. The trapped DNA fragments were subcloned into pBluescript KS and then transformed into JM109 competent cells (Toyobo). Transformants were cultured in LB medium containing 50 μg/ml ampicillin, and plasmids were prepared by the alkaline-SDS method. The plasmids (10 μg) were subjected to the binding selection procedure again. This cycle was repeated five times. The resultant VDR-binding fragment was sequenced by the dideoxy method.

Plasmid Construction—The expression vector of VDR (pHβAPr-VDR) for mammalian cultured cells was constructed by subcloning into pHβAPr-1 (*22*). For the analysis of clone 3, the resultant fragment was inserted into luciferase plasmid PGV-P carrying the SV40 early gene promoter (Toyo Ink Mfg.). A DR region I fragment was synthesized, its sequence being 5′-ctagaCCTCTGAACTC-CTTGAACTGAAAt-3′. A DR region II fragment (374-472) was amplified by PCR. In order to analyze DR regions I and II, each fragment was subcloned into PGV-P. The VDRE of the mouse osteopontin gene (mSPP-1-VDRE) was used as a positive control for the luciferase assay. All mutants were generated by deoxyoligonucleotide-directed mutagenesis, as described by Kunkel *et al.* (*23*). The template DNA for mutagenesis was prepared from PGV-P, which contained the f1 origin. For region I mutants, deoxyoligonucleotides that changed each half site motif (AGTTCA) to TCTAGA were used. The region II mutant was constructed with a deoxyoligonucleotide that deleted 26 nucleotides of region II. All mutants were confirmed by sequencing.

Cell Culture and Transfection—CV-1 cells, an African green monkey kidney cell line, were maintained in Eagle's minimal essential medium (MEM) (Nissui), supplemented with 10% (v/v) fetal bovine serum. The day before transfection, 3×10^5 cells were plated in 60 mm dishes. Transfection was performed by the calcium-phosphate precipitation method (*24*) with 1 μg of pHβAPr-VDR, 3 μg of reporter plasmids, and pBluescript KS, up to 10 μg of the total amount of DNA. Fresh medium with or without 1×10^{-7} M $1,25(OH)_2D_3$ was added 20 h after transfection. After incubation with $1,25(OH)_2D_3$ for 24 h, the cell extracts were assayed for the protein concentration and luciferase activity.

Southern Blot Analysis—Rat genomic DNA (10 μg) from spleen was digested with *Bam*HI, *Bgl*II, and *Xba*I. The digested DNA was resolved on a 1% agarose gel, transferred to a Hybond-N^+ membrane (Amersham), and then hybridized to the clone 3 fragment as a probe.

DNA-Binding Analysis—The DR region I and II fragments described under "Plasmid construction" were radiolabeled using [α-^{32}P]dCTP (Amersham) and the Klenow fragment (Toyobo). The purified proteins (6.25 μl) were mixed with the same volume of 20 mM Tris-HCl, pH 7.5, 10% glycerol, 2 mM DTT, 10 mM EDTA, 0.32 mg/ml poly (dI-dC), and radio-labeled probe (10 fmol). The binding reaction was performed at 4°C overnight. Each reaction mixture was loaded on a 4% non-denaturing polyacrylamide gel, electrophoresed at 150 V for 1 h, fixed with 10% methanol and 10% acetic acid, and then autoradiographed overnight at −80°C.

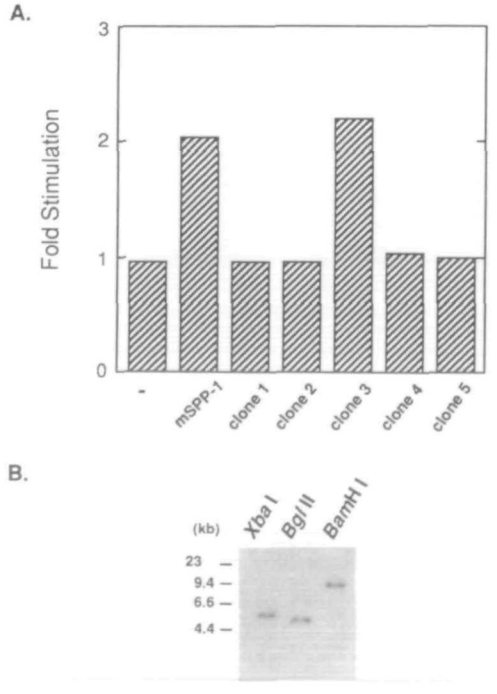


Fig. 1. **(A) The responsiveness of VDR-binding genomic fragments to $1,25\text{-}(OH)_2D_3$.** The reporter plasmids carrying the SV40 early gene promoter and the luciferase gene with the isolated genomic fragment were transfected into CV-1 cells with the VDR expression plasmid. After incubation in the absence or presence of 1×10^{-7} M $1,25\text{-}(OH)_2D_3$, luciferase activity was determined. The values are represented as Fold Stimulation compared with the activity without $1,25\text{-}(OH)_2D_3$. The mSPP-1 VDRE was used as a positive control, and clones 1-5 were rat genomic fragments selected as to the VDR binding property. The response of the reporter plasmid carrying clone 3 to $1,25\text{-}(OH)_2D_3$ was significantly ($p<0.001$) different from the control (the reporter plasmid without the insert, represented as -). **(B) Southern blot analysis of clone 3.** Rat genomic DNA (10 μg) was digested with *Bam*HI, *Bgl*II, or *Xba*I. The digested DNA samples were resolved on a 1% agarose gel, transferred to a membrane, and then hybridized with clone 3.

RESULTS

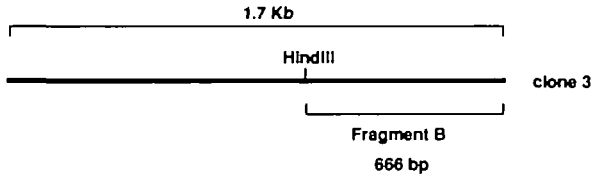
Isolation of the Rat Genomic DNA Fragment Having a VDRE—We had already screened VDR-binding sites in rat genomic DNA according to the filter binding selection method (*18*). Partially digested rat genomic DNA was incubated with VDR and the DNA fragments that bound to the protein were trapped on a nitrocellulose filter. After the fifth selection cycle, five independent clones were isolated. These clones were subcloned into reporter plasmid PGV-P containing the SV40 early gene promoter linked to the luciferase gene. Each reporter plasmid was co-transfected together with the VDR expression plasmid into CV-1 cells. An extract of transfected cells was assayed for luciferase activity (Fig. 1A). The reporter plasmid with the VDRE of the mouse osteopontin gene (mSPP-1 VDRE) was used as a positive control. As shown in Fig. 1A, only clone 3 showed a response to $1,25(OH)_2D_3$.

To exclude the possibility of a cloning artifact or contamination during the amplification, Southern blot analysis was performed using clone 3 as a probe (Fig. 1B). Rat genomic DNA was digested with three different restriction enzymes (*Bam*HI, *Bgl*II, and *Xba*I), and then resolved on a 1% agarose gel. For each restriction enzyme, there was a single band positive for clone 3, indicating that clone 3 was not derived from repetitive sequences but present as a single copy in the rat genome.

Sequence Analysis of a Vitamin D-Responsive Fragment—The entire sequences of all five clones selected with VDR were determined. It has been reported that the spacing of core binding motifs is important in dictating the selective transcriptional effects of nuclear receptors (*5*). Spacers of 4 (DR4) and 5 bp (DR5) lead to relatively specific transcriptional responses to 3,5,3′-triiodothyronine and retinoic acid, respectively, while a spacer of 3 bp (DR3) leads to a transcriptional response to 1α,25-dihydroxyvitamin D_3 (*5*). Furthermore, VDR prefers the AGTTCA sequence as a half-site motif to AGGTCA (*20*). So, we checked sequences by taking notice of the direct repeated AGTTCA motif. As a result, we found only one DR3 in clone 3, but none in the other four clones, with instead have a widely spaced direct repeat of the AGTTCA motif. Next, we examined the VDRE activity of clone 3 digested with some restriction enzymes. One of the resultant fragments, fragment B (Fig. 2A), exhibited vitamin D responsiveness as well as clone 3. Looking closely at the sequence of this fragment, we found another AGTTCA-rich region in addition to a typical DR3. This region consists of four consecutive direct repeats of the half-site motif, which was partially overlapped (Fig. 2, B and C).

In Vitro Binding of VDR or the VDR/RXR Complex to DR Regions I and II—Since clone 3 was selected according to its VDR binding ability, VDR should bind to DR region I or II. The ability of VDR to bind to DR regions I and II was assessed by band-shift analysis. When we used a relatively high amount of VDR, VDR alone could bind to DR regions I and II (Fig. 3A). Whereas it was reasonable that VDR could bind to DR region I consisting of DR3, it was surprising that DR region II was also recognized by VDR with an affinity similar to that for region I. DR region II contained four AGTTCA motifs, of which one pair consisted of DR4. Because VDR binds to the DR4 type sequence with lower affinity than to DR3 (*25*), the binding of VDR to DR region II might be due to the feature of four AGTTCA motifs rather than the DR4 type sequence. It is well-known that the VDR/RXR heterodimer can bind to VDREs with higher affinity than the VDR homodimer, and mainly functions on

A.



B.

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AAGCTTGAGCCTTGGACTTGGTCTGTGTCTATCTATCTACCTCAGGCTGGGAACTTCTAG   60
CTCGTAAACAATCTTCTGCAAGTCCAGTCCTTAAGGCTTCAGCTTCGAGCTTCTGTCTGC  120
TAACATAGGCCTATTGGTGTTTCTGCATCTGAGTCTGACTGCTGAATAAATTCACCCTTT  180
GTAGCTCTTTCTGAACTCTGGCTGGCTGACAGAACTCACCTATATGTCTCGTCTCAAACT  240
AGTTTCCAAACTGACTGATTCAAATTGGCTTCTCTGAGCTTTGACTGAATGTCTACTAGA  300
GTGACAGCCTCTGAACTCCTTGAACTGAAACTGAAATGAAACTGCCTTGAACTCAACTGT  360
TCTGCTCTGAAATGAGTTTAACTGCACTCAACTGAACTGAACCAAACTGAACTCTCCCTC  420
ACTGCTCTTAAATAGCTTTCTATTCTTGTCTGTTCTCATGAGGTTTGGGTGTACCTTAAC  480
TCTGATTCATTCTGTCAAGTCATTTTTCTGATTCTCCACTTTGTCTGACCCTCAACTAGA  540
TGTCAATGTGTTATAGTTTGGGAATAAAGGTATGTCCTAAAGTTGTGTGTGTATTTCAGC  600
CAAGGGATTAAATGTGTGTAGCAAGGGCATATCTGTATTCCATCCAGTCATATCTTTGAT  660
ATGATC                                                         666 bp
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C.

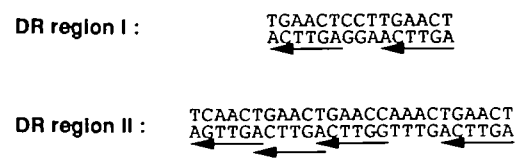

Fig. 2. **(A) Scheme of clone 3 and fragment B. (B) Nucleotide sequence of fragment B.** The DR regions are indicated by boxes. **(C) Organization of the direct repeat sequences in the DR regions.** The "AGTTCA" motifs are indicated by arrows.

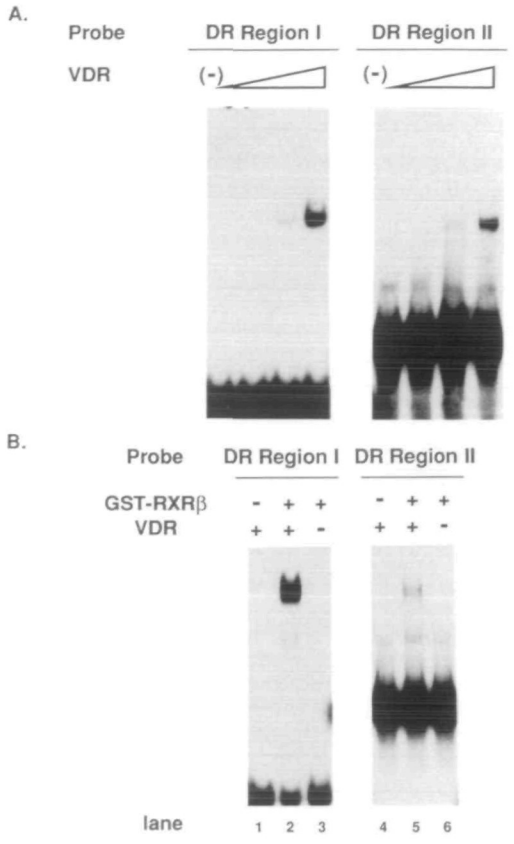

Fig. 3. **(A) The binding ability of VDR as to DR region I or DR region II *in vitro*.** Gel shift analysis was performed using DR region I and DR region II as radio-labeled probes (10 fmol) with VDR (0, 13, 26, or 39 ng). **(B) The binding ability of the VDR/GST-RXR hetero-complex as to DR region I or DR region II *in vitro*.** Gel shift analysis was performed using DR region I and DR region II as radio-labeled probes (10 fmol) with VDR (6.5 ng, lanes 1 and 4), GST-RXR (10 ng, lanes 3 and 6), or both (lanes 2 and 5). Each reaction sample was loaded on a 4% non-denaturing polyacrylamide gel.

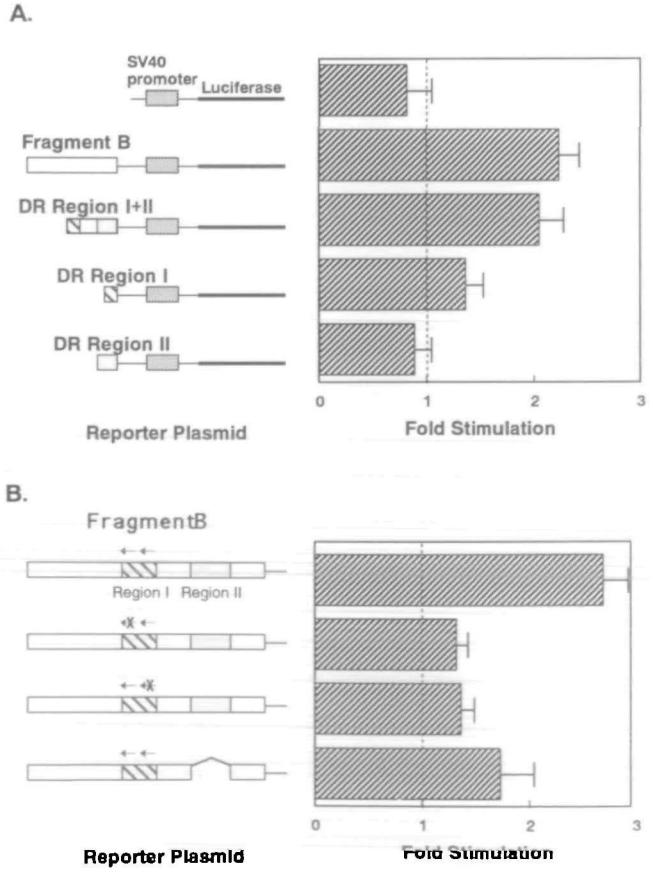

Fig. 4. **(A) Luciferase assay with DR region I and DR region II.** The reporter plasmids carrying the SV40 early gene promoter and the luciferase gene with fragment B, DR region I+II, DR region I, and DR region II, respectively, were transfected into CV-1 cells with the VDR expression plasmid. After incubation in the absence or presence of 1×10^{-7} M 1,25-$(OH)_2D_3$, luciferase activity was determined. The values are represented as Fold Stimulation compared with the activity without 1,25-$(OH)_2D_3$. Mean values and SD ($n=3$) are shown. The response of the reporter plasmid carrying DR region I to 1,25-$(OH)_2D_3$ was significantly ($p<0.05$) different from the control (the reporter plasmid without the insert) and the reporter plasmid with DR region I+II. **(B) Luciferase assay of mutant fragment B.** Mutations were introduced in each half-site of region I. Region II was deleted completely. All mutants were inserted into the reporter plasmid and transfected into CV-1 cells with the VDR expression plasmid. After incubation in the absence or presence of 1×10^{-7} M 1,25-$(OH)_2D_3$, luciferase activity was determined. The values are represented as Fold Stimulation compared with the activity without 1,25-$(OH)_2D_3$. Mean values and SD ($n=5$) are shown.

VDREs *in vivo*. Therefore, the ability of the VDR/RXR complex to bind to DR regions I and II was assessed (Fig. 3B). When we used a small amount of VDR, the VDR-DNA complex was not detectable (Fig. 3B, lanes 1 and 4). GST-RXRβ also did not give retarded migration band with each probe (Fig. 3B, lanes 3 and 6). On the contrary, the VDR/RXR heterodimer could bind to both probes (Fig. 3B, lanes 2 and 5). While VDR showed similar levels of binding to DR regions I and II, VDR/RXR gave a much more retarded band with DR region I than DR region II.

VDRE Activity of DR Regions I and II—In order to determine the VDRE activity of each DR region in fragment B, a reporter plasmid containing DR region I or II or both (DR region I+II) was constructed and transfected into CV-1 cells (Fig. 4A). DR region I+II could mediate 1,25-$(OH)_2D_3$-activated transcription. We observed a similar level of activation by DR region I+II to that of the entire fragment B. On the other hand, DR region I alone could mediate only weak 1,25-$(OH)_2D_3$ dependent activation and DR region II could mediate no 1,25-$(OH)_2D_3$ dependent activation, despite that each region was bound to the VDR/RXR complex strongly. In order to confirm the importance of regions I and II, we constructed mutants of fragment B by site-directed mutagenesis (Fig. 4B). The destruction of either half-site of DR3 in region I significantly reduced the 1,25-$(OH)_2D_3$ dependent activation. On deletion of region II, the activity of fragment B also decreased, albeit weak activity remained. These results indicate that the sequences of regions I and II seem to act cooperatively to mediate 1,25-$(OH)_2D_3$-dependent activation. It is likely that this VDRE has a novel feature in VDR binding and transcriptional activation.

DISCUSSION

In this paper, we described the isolation of VDR binding fragments from rat genomic DNA. After five rounds of selection, we finally obtained five independent clones. On transient transfection analysis using the luciferase gene as a reporter, one of them, clone 3, showed strong VDRE activity, but the others did not.

The entire sequence of clone 3 was determined and two putative VDR binding regions (DR regions I and II) were found. DR region I consisted of a direct repeat of the AGTTCA motif separated by three residues (DR3), which is the well-known VDRE (*20, 25*). On the other hand, DR region II did not have the DR3 sequence, instead having four AGTTCA motifs, of which one pair consisted of DR4. The VDR homodimer could bind to this DR region II with approximately same affinity as to DR region I. DR region II is a novel sequence which is recognized by VDR alone.

DR region I in clone 3 contained a typical VDRE sequence, and both the VDR homodimer and VDR/RXR heterodimer could bind to DR region I. However, DR region I showed only weak 1,25-$(OH)_2D_3$-dependent activation compared to the mSPP-1 derived VDRE, which is another typical DR3. It remains unclear why the same type of response elements (DR3) act differently in natural contexts. The sequence around AGTTCA motifs might play some role in transcriptional activation.

It has been reported that the VDRE derived from the rat 24-hydroxylase gene has two DR3 type sequences, which function cooperatively (*26*). Clone 3 showed a similar type of action. DR region I exhibited weak VDRE activity. By the addition of DR region II to DR region I, it turned to exhibit strong responsiveness, even though DR region II itself did not exhibit any VDRE activity.

On the selection of genomic fragments using the recombinant VDR, we mainly recovered widely spaced direct repeats of the AGTTCA motif. This means that VDR can bind to this type of sequence, although it does not show VDRE activity. Using the random oligonucleotide selection method, we previously showed that the sequence preferentially selected by VDR is DR3 (*20*). However, this method is restricted by the lengths of oligonucleotides. So, we have to point out here that VDR specifically binds to DR3 as long as the distance between the two AGTTCA motifs does not exceed 5 bp, despite that VDR promiscuously recognizes widely spaced direct repeated AGTTCA motifs. The binding of VDR to DNA without transcriptional activation possibly plays some role in the vitamin D_3 action. We are now looking for a gene of which transcription is regulated by clone 3 by means of gene walking.

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REFERENCES

1. DeLuca, H.F. and Schnoes, H.K. (1983) Vitamin D: Recent advances. *Annu. Rev. Biochem.* **52**, 411-439
2. Evans, R.M. (1988) The steroid and thyroid hormone receptor superfamily. *Science* **240**, 889-895
3. O'Malley, B.W. (1990) The steroid receptor superfamily: More excitement predicted for the future. *Mol. Endocrinol.* **4**, 363-369
4. Yu, V.C., Delsert, C., Anderson, B., Holloway, J.M., Devary, O.V., Näär, A.M., Kim, S.Y., Boutin, J.-M., Glass, C.K., and Rosenfeld, M.G. (1991) RXRβ: A coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. *Cell* **67**, 1251-1266
5. Umesono, K., Murakami, K.K., Thompson, C.C., and Evans, R.M. (1991) Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D_3 receptors. *Cell* **65**, 1255-1266
6. Kerner, S.A., Scott, R.A., and Pike, J.W. (1989) Sequence elements in the human osteocalcin gene confer basal activation and inducible response to hormonal vitamin D_3. *Proc. Natl. Acad. Sci. USA* **86**, 4455-4459
7. Ozono, K., Liao, J., Kerner, S.A., Scott, R.A., and Pike, J.W. (1990) The vitamin D-responsive element in the human osteocalcin gene. *J. Biol. Chem.* **265**, 21881-21888
8. Demay, M.B., Gerarde, J.M., DeLuca, H.F., and Kronenberg, H.M. (1990) DNA sequences in the rat osteocalcin gene that bind the 1,25-dihydroxyvitamin D_3 receptor and confer responsiveness to 1,25-dihydroxyvitamin D_3. *Proc. Natl. Acad. Sci. USA* **87**, 369-373
9. Markose, E.R., Stein, J.L., Stein, G.S., and Lian, J.B. (1990) Vitamin D-mediated modifications in protein-DNA interactions at two promoter elements of the osteocalcin gene. *Proc. Natl. Acad. Sci. USA* **87**, 1701-1705
10. Noda, M., Vogel, R.L., Craig, A.M., Prahl, J.P., DeLuca, H.F., and Denhardt, D.T. (1990) 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. *Proc. Natl. Acad. Sci. USA* **87**, 9995-9999
11. Darwish, H.M. and DeLuca, H.F. (1992) Identification of a 1,25-dihydroxyvitamin D_3-response element in the 5′-flanking region of the rat calbindin D-9k gene. *Proc. Natl. Acad. Sci. USA* **89**, 603-607

12. Cao, X., Ross, F.P., Zhang, L., MacDonald, P.N., Chappel, J., and Teitelbaum, S.L. (1993) Cloning of the promoter for the avian integrin $\beta 3$ subunit gene and its regulation by 1,25-dihydroxyvitamin D_3. *J. Biol. Chem.* **268**, 27371-27380
13. Zierold, C., Darwish, H.M., and DeLuca, H.F. (1994) Identification of a vitamin D-response element in the rat calcidiol (25-hydroxyvitamin D_3) 24-hydroxylase gene. *Proc. Natl. Acad. Sci. USA* **91**, 900-902
14. Ohyama, Y., Ozono, K., Uchida, M., Shinki, T., Kato, S., Suda, T., Yamamoto, O., Noshiro, M., and Kato, Y. (1994) Identification of a vitamin D-responsive element in the 5'-flanking region of the rat 25-hydroxyvitamin D_3 24-hydroxylase gene. *J. Biol. Chem.* **269**, 10545-10550
15. Demay, M.B., Kiernan, M.S., DeLuca, H.F., and Kronenberg, H.M. (1992) Sequences in the human parathyroid hormone gene that bind the 1,25-dihydroxyvitamin D_3 receptor and mediate transcriptional repression in response to 1,25-dihydroxyvitamin D_3. *Proc. Natl. Acad. Sci. USA* **89**, 8097-8101
16. Liu, M., Lee, M.-H., Cohen, M., Bommakanti, M., and Freedman, L.P. (1996) Transcriptional activation of the Cdk inhibitor p21 by vitamin D_3 leads to the induced differentiation of the myelomonocytic cell line U937. *Genes Dev.* **10**, 142-153
17. Kinzler, K.W. and Vogelstein, B. (1989) Whole genome PCR: Application to the identification of sequences bound by gene regulatory proteins. *Nucleic Acids Res.* **17**, 3645-3653
18. Inoue, S., Kondo, S., Hashimoto, M., Kondo, T., and Muramatsu, M. (1991) Isolation of estrogen receptor-binding sites in human genomic DNA. *Nucleic Acids Res.* **19**, 4091-4096
19. Inoue, S., Orimo, A., Hosoi, T., Kondo, T., Ikegami, A., Orimo, H., and Muramatsu, M. (1993) Genomic binding-site cloning reveals an estrogen-responsive gene that encodes a RING finger protein. *Proc. Natl. Acad. Sci. USA* **90**, 11117-11121
20. Nishikawa, J., Kitaura, M., Matsumoto, M., Imagawa, M., and Nishihara, T. (1994) Difference and similarity of DNA sequence recognized by VDR homodimer and VDR/RXR heterodimer. *Nucleic Acids Res.* **22**, 2902-2907
21. Sakoda, K., Fujiwara, M., Arai, S., Suzuki, A., Nishikawa, J., Imagawa, M., and Nishihara, T. (1996) Isolation of a genomic DNA fragment having negative vitamin D response element. *Biochem. Biophys. Res. Commun.* **219**, 31-35
22. Gunning, P., Leavitt, J., Muscat, G., Ng, S.-Y., and Kedes, L. (1987) A human β-actin expression vector system directs high-level accumulation of antisense transcripts. *Proc. Natl. Acad. Sci. USA* **84**, 4831-4835
23. Kunkel, T.A., Roberts, J.D., and Zakour, R.A. (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**, 367-382
24. Chen, C. and Okayama, H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**, 2745-2752
25. Nishikawa, J., Matsumoto, M., Sakoda, K., Kitaura, M., Imagawa, M., and Nishihara, T. (1993) Vitamin D receptor zinc finger region binds to a direct repeat as a dimer and discriminates the spacing number between each half-site. *J. Biol. Chem.* **268**, 19739-19743
26. Zierold, C., Darwish, H.M., and DeLuca, H.F. (1995) Two vitamin D response elements function in the rat 1,25-dihydroxyvitamin D 24-hydroxylase promoter. *J. Biol. Chem.* **270**, 1675-1678