Difference and similarity of DNA sequence recognized by VDR homodimer and VDR/RXR heterodimer

Jun-ichi Nishikawa, Motoji Kitaura, Mayumi Matsumoto, Masayoshi Imagawa and Tsutomu Nishihara*
Department of Environmental Biochemistry, Faculty of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka 565, Japan

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ABSTRACT
Nuclear receptors for the thyroid hormone and vitamin A and D cooperate with the retinoid X receptor (RXR) in activating the transcription. Although the hormone response elements for these receptors have been proposed in which spacing of the direct repeated motifs determine the specificity (so called 3-4-5 rule), vitamin D response elements (VDREs) in the natural context consist of often imperfect direct repeats. Vitamin D receptor (VDR) alone can bind to the mouse osteopontin (mSPP-1) VDRE, which contains a direct repeat separated by 3 nucleotides, but not to the rat osteocalcin (rOST) VDRE having inexact direct repeat. The presence of RXR not only allows the VDR to bind to the rOST VDRE, but also increases the binding affinity for the mSPP-1 VDRE. The RXR/VDR heterodimer exhibits the similar affinity constants for the mSPP-1 VDRE and the rOST VDRE, in spite of the apparently different affinities for two VDREs of the VDR homodimer. A random oligonucleotide selection procedure revealed that the consensus sequence selected by the RXR homodimer is the direct repeat spaced by one A residue. In contrast, the sequences preferentially selected by the VDR homodimer and the VDR/RXR heterodimer are similar, which are the direct repeats spaced by 3 nucleotides. The difference and similarity of DNA sequence recognition are discussed.

INTRODUCTION
1α,25-Dihydroxyvitamin D3 [1,25-(OH)2D3], the most active metabolite of vitamin D3, has many functions in a multitude of physiological processes including calcium and phosphorus absorption in the intestine, bone remodeling, and differentiation of hematopoietic cells (1). Most of its actions are mediated through its receptor (VDR) that binds to 1,25-(OH)2D3 with high affinity, but not to other metabolites of vitamin D3. This protein belongs to the superfamily of nuclear receptors, which are ligand-binding domains (2,3). The VDR regulates the transcription of their target genes by binding to the cis-acting DNA elements, called vitamin D response elements (VDREs), in the regulatory regions of the genes. These gene products are associated with many kinds of cellular functions. Therefore, information on the sequence that the VDR specifically recognizes is important for understanding the vitamin D3 actions.

The superfamily of nuclear receptors can be classified into two subgroups according to their hormone response elements (HREs). Receptors for steroid hormones such as estrogen, glucocorticoid, progesterone, etc. recognize palindromic DNA sequences as homodimers. On the contrary, the VDR, the retinoic acid receptor (RAR) and the thyroid hormone receptor (TR) bind to direct repeats of 5'-PuG(G/T)TCA motif as heterodimers with the retinoid X receptor (RXR) (4, 5). Based on transient transfection studies using synthetic oligonucleotides as HREs, the transactivation by the VDR-RXR, TR-RXR and RAR-RXR complexes is shown to preferentially occur through direct repeats spaced by 3, 4 and 5 nucleotides, respectively (6, 7). Although VDREs have been identified in human osteocalcin (OST) (8, 9), rat OST (10, 11), mouse osteopontin (mSPP-1) (12), rat calbindin D-9K (13), avian integrin β3 subunit (14) and rat 25-hydroxyvitamin D3 24-hydroxylase gene (15), these VDREs do not have a perfect direct repeat except for the mSPP-1 VDRE. In addition, it has been reported that the VDR DNA binding domain expressed in Escherichia coli can bind to the mSPP-1 VDRE, but not to other VDREs (16,17). However, the presence of RXR allows the VDR to bind various VDREs (5, 13–15, 18). From these facts, we thought that the DNA binding specificity of the VDR could be altered by the heterodimer formation with the RXR.

To define the spectrum of DNA binding sites recognized by the VDR homodimer and the VDR/RXR heterodimer, we have employed the method of polymerase chain reaction (PCR)-mediated random site selection. Contrary to our expectation, the consensus sequence selected by the VDR/RXR heterodimer was essentially the same as the VDR homodimer. However, the RXR/VDR heterodimer had wider spectrum of DNA sequences than the VDR homodimer. We also identified binding sites which
were preferentially selected by the RXR homodimer. The consensus sequences selected by the RXR homodimer, the VDR homodimer and the RXR/VDR heterodimer are 5'-PuGGTGCA-
aAGGTCA, 5'-PuGGTTCAtgtAGTCTAGCAGC-GTCTAGA-3' and 5'-PuGGTGCAtt-
gAGGTCA-3', respectively.

**MATERIALS AND METHODS**

**Expression of recombinant proteins**

The full-length cDNA encoding VDR was cloned into a expression vector pAR2113 carrying T7 promoter φ10 (19). This VDR expression plasmid pAR-VDR was transformed to *E. coli* BL21 (DE3) which carries T7 gene 1 under control of the lac UV5 promoter (19). Wild-type RXRβ was expressed in bacteria as a fusion protein with glutathione S-transferase (GST) using the pGEX-2T expression vector (Pharmacia). The full-length cDNA encoding RXRβ was inserted into pGEX-2T. The recombinant plasmid, pGEX-RXR, was transformed into *E. coli* JM109 cells. All sequences inserted were confirmed by the dyeode method (20).

The transformants, which was harboring pAR-VDR or pGEX-
RXR, were grown overnight at 30°C in LB (Luria-Bertani) medium containing 50 μg/ml ampicillin. The culture was then diluted 100-fold and grown to OD600 0.4–0.6, at that time isopropyl thiolgalactoside (IPTG) was added to a final concentration of 0.3 mM. Then, the cells were allowed to grow for an additional 4 hr, harvested by centrifugation and resuspended in 10% of the original culture volume in TEGDZ200 [50 mM Tris (pH 7.5), 0.5 mM EDTA, 10% glycerol, 2.5 mM dithiothreitol and 200 mM KCl], and broken by sonication. The production of recombinant proteins was examined by staining the gel with coomasie brilliant blue after SDS-PAGE of the cell lysate.

**Protein purification**

The purification was started from the cytosolic fraction of IPTG induced bacteria, which was obtained as supernatant by centrifugation of cell disruption at 10,000×g for 10 min. For purification of VDR, heparin-Sepharose CL-6B (Pharmacia) and DE-52 (Whatman) were used. The sonicated lysate from 1 liter of culture was loaded onto a 1 ml heparin-Sepharose column equilibrated with TEGDZ200, washed with 10 column volume of TEGDZ200 and eluted with TEGDZ400. Fractions containing VDR were pooled, diluted to 50 mM of KCl concentration and loaded onto 3 ml DE-52 equilibrated with TEGDZ400. The purified proteins (6.25 μl) were mixed with the same volume of 20 mM Tris (pH 7.5), 10% glycerol, 2 mM dithiothreitol, 10 mM EDTA, 0.32 mg/ml poly (dl-dC) and radio-labeled probe (10,000 cpm/10 fmol). The binding reaction was performed at 4°C for overnight. Each reaction mixture was loaded on a 4% non-denaturing polyacryl-amide gel, electrophoresed at 150 V for 1 hr, fixed with 10% methanol and 10% acetic acid, and autoradiographed overnight at −80°C.

For Schachard analyses, gel shift assays were performed as a function of the DNA concentration by using a constant amount of protein. Following electrophoresis, gels were fixed, dried and exposed to a imaging plate. Radioactivity associated with free oligonucleotide and with receptor—oligonucleotide complexes was then quantified directly using an image analyzer (Fuji, BAS 2000).

**In vitro binding site selection**

Enrichment for binding sites from a random oligonucleotide pool (5'-AGAGCCACTTCTCTGACGGATCCTGATCTGTC-3') was performed by filter binding method (21). The random sequence oligonucleotide was rendered double stranded by PCR using Tth DNA polymerase (Toyobo Co., Ltd.), primer 1: 5'-AGAGCCACTTCTCTGACGGATCCTGATCTGTC-3' and primer 2: 5'-AGAGCCACTTCTCTGACGGATCCTGATCTGTC-3'. Binding reaction mixture (35 μl) contained purified protein (200 ng of VDR for VDR homodimer, 100 ng of VDR and 200 ng of GST-RXRβ for VDR/RXR heterodimer, or 300 ng of GST-RXRβ for RXR homodimer), 1 μg of double stranded random oligonucleotide, 5.6 μg of poly (dl-dC) and 3.5 μl of 10×binding buffer (100 mM Tris, pH 7.5, 50% glycerol, 10 mM dithiothreitol and 10 mM EDTA). Each mixture was incubated overnight at 4°C. Thereafter, this solution was passed slowly through a pre-soaked nitrocellulose filter (Scheicher & Schuell, BA85). The filter was washed three times with 3 μl of 1×binding buffer before the bound oligonucleotides were eluted with 100 μl of elution buffer containing 20 mM Tris (pH 7.5), 1 mM EDTA, 20 mM NaCl and 0.1% SDS. The eluate was phenolized and then amplified by PCR using primers 1 and 2. Amplified products (1 μg) was put back into a binding reaction and the procedure repeated. After four rounds of enrichment, the selected oligonucleotides were digested with BamHI and XhoI and subcloned into pBluescript SK. Clones were sequenced by the dyeode chain termination method (20).

**RESULTS**

**Difference of DNA binding property between VDR homodimer and VDR/RXR heterodimer**

The ability of recombinant VDR to bind native VDREs was investigated. The interaction of VDR with specific DNA sequences can be assessed through band shift analysis as a protein—DNA complex retarded in migration from the free DNA probe. As shown in Fig. 1, VDR could clearly bind to the mouse osteopontin gene VDRE (mSPP-1 VDRE), but not to the rat osteocalcin gene VDRE (rOST VDRE) even though 100 μg of VDR was added. The mSPP-1 VDRE consisted of a complete direct repeat of the HRE half-sites with a spacing of 3 bp in contrast to the rOST VDRE. It was confirmed by the super-shift experiment using the antibody against the VDR that this band contained the VDR (Fig. 1, lane 6). When the oligonucleotide consisting of a single mSPP-1 half-site was used as probe, the
Figure 1. Gel shift assay using bacterially expressed VDR. The purified VDR (0, 25, 50, 75, 100 ng) was incubated with 32P-labeled oligonucleotide containing mSPP-1 (lane 1–6) or rOST (lane 7–11) VDRE. Anti-VDR polyclonal antibody was used for supershift analysis (lane 6).

Figure 2. Effect of RXRβ on DNA binding of VDR. The 32P-labeled oligonucleotides at the top were used as a probe for in vitro DNA binding analysis with the purified VDR. (A) Each probe was incubated with VDR (50 ng) and increasing amounts of GST-RXRβ. Numbers at the second line indicate the molar ratio of GST-RXRβ to VDR. Arrows indicate VDR homodimer (lower) and GST-RXRβ/VDR heterodimer (upper) complexes. (B) Small amount of VDR (2 ng) was used for gel shift assay. Under this condition, VDR/mSPP-1 VDRE complex was not detected on autoradiogram (lane 1). Labeled probe was incubated with VDR in the absence (lane 1, 4) or presence (lane 3, 6) of GST-RXR0 (3 ng), in vitro DNA binding analysis oligonucleotides at the top were used as a probe for

VDR—DNA complex appeared as one band corresponding to the lower band of Fig. 1 (data not shown). Therefore, the upper band was thought to be a dimeric band, while the lower was a monomer band. It was also reported that recombinant hVDR bound to the similar direct repeat as a homodimer (22).

Although the both VDREs of mSPP-1 and rOST had been shown to be vitamin D inducible by transient transfection assay in mammalian cells (10–12), the VDR binding to two VDREs was apparently different. It was reported that the RXR enhances binding of the VDR, the RAR and the TR to their specific DNA response elements (4, 5). So, we next examined the effect of RXR on the VDR DNA binding. As shown in Fig. 2, the addition of the RXR caused more retardation of the band (A; lane 1 – 5). The lower band corresponds to the VDR homodimer according to the mobility (see also Fig. 1), although the monomer band could not be detected in Fig. 2A, probably due to the weak binding ability. The upper band was super-shifted by adding anti-VDR antibody, which is raised against VDR lacking the DNA binding domain, and disappeared by adding anti-RXR antibody, which is raised against RXR including the DNA binding domain (data not shown). These data strongly indicate that the upper band consists of VDR/RXR heterodimer. Since the RXR was expressed as a fusion protein (RXR; 48 kDa + GST; 26 kDa), the band containing GST-RXR/VDR heterodimer showed further retarded migration than the VDR (VDR; 48 kDa) homodimer. The RXR enhanced the binding of VDR to the mSPP-1 VDRE as well as the rOST VDRE. At the molar ratio of RXR/VDR = 1:1, the most of DNA–protein complex existed as a heterodimer. This result suggested that the RXR/VDR heterodimer had a higher affinity for the mSPP-1 VDRE than the VDR homodimer. Moreover, the RXR/VDR heterodimer could bind to the rOST VDRE as against to the VDR homodimer.

In order to substantiate the difference of binding property seen in the gel shift assay, we also determined the dissociation constants (Kd). Saturation curves and derived Scatchard plots are shown in Fig. 3. These closely parallel the relative binding evidence in Fig. 2. The VDR homodimer bound to the mSPP-1 VDRE with Kd of 1.7 nM, but the affinity for the rOST VDRE was too low to determine Kd value. The heterodimer formation with the RXR increased the affinity of VDR for mSPP-1 VDRE (Kd = 0.68 nM). The addition of RXR remarkably increased the binding affinity of VDR for rOST VDRE (Kd = 0.88 nM) indicating that the affinity constant of the RXR/VDR heterodimer for the mSPP-1 VDRE was nearly in the same level as for the rOST VDRE. These results suggested that the heterodimer formation with the RXR increased the affinity for VDREs and this effect was much larger on the rOST VDRE than the mSPP-1 VDRE.

In vitro selection of DNA sequences preferentially bound by VDR/VDR, VDR/RXR and RXR/RXR

Since the VDR homodimer and the VDR/RXR heterodimer had different binding specificity to native VDREs, the former only bound to the mSPP-1 VDRE and the latter to both of the mSPP-1 and the rOST VDRE, we investigated the details of DNA sequences recognized by both dimers using the method of PCR-mediated random oligonucleotide site selection. The double stranded random oligonucleotides were incubated with either of dimers and the oligonucleotides bound to a dimer were selected by using nitrocellulose filter. After four rounds of binding site selection, the selected DNA fragments were subcloned and sequenced; 81 clones for the RXR homodimer, 90 clones for the VED homodimer and 152 clones for the RXR/VDR heterodimer were sequenced (Table 1).

The RXR recognition sequences were divided into three groups: 75 containing a direct repeat of the AGGTCA motif separated by one residue (DR1); five containing an inverted repeat of the AGGTCA motif with no intervening base pair (iRO); and one containing DR2. An A residue was mainly recovered as the spaced nucleotide (73 out of 75 DR1 clones, Fig. 4A).

The core sequences selected by the VDR homodimer were almost DR3 (89 out of 90 clones), whose half-site motif was PuGTTCA, and one exception was DR4. Important residues, conserved over 90% of total DR3, were as follows: G for the
Figure 3. DNA binding affinities of VDR homodimer and RXR/VDR heterodimer. DNA binding activities of VDR homodimer to mSPP-1 VDRE (A), RXR/VDR heterodimer to mSPP-1 VDRE (B), and RXR/VDR heterodimer to rOST VDRE (C) were analyzed as a function of DNA concentration. Following electrophoresis, gels were fixed and dried. Radioactivity associated with free oligonucleotide and with receptor-oligonucleotide complexes was then quantified directly using a Fuji Imaging Analyzer. In insets, Schachard plots of the same experiments were shown. Affinity constants are shown in (D).

Table 1. Types selected by RXR homodimer, VDR homodimer and RXR/VDR heterodimer

<table>
<thead>
<tr>
<th>Factor</th>
<th>Type</th>
<th>Recovery frequency</th>
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<tr>
<td>RXR/RXR</td>
<td>DR1</td>
<td>75/81</td>
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<tr>
<td></td>
<td>DR2</td>
<td>1/81</td>
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<td></td>
<td>DR0</td>
<td>5/81</td>
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<tr>
<td>VDR/VDR</td>
<td>DR3</td>
<td>89/90</td>
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<tr>
<td></td>
<td>DR4</td>
<td>1/90</td>
</tr>
<tr>
<td>RXR/VDR</td>
<td>DR1</td>
<td>16/152</td>
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<tr>
<td></td>
<td>DR2</td>
<td>1/152</td>
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<td></td>
<td>DR3</td>
<td>132/152</td>
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<td></td>
<td>DR4</td>
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<td>DR5</td>
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These results revealed that RXR homodimer preferentially bound to DR1 and both of the VDR homodimer and the RXR/VDR heterodimer bound to DR3. The distinct difference of sequences selected by the VDR homodimer and the RXR/VDR heterodimer was the third position of the first half-site (T for VDR and G for RXR/VDR). Another difference of sequences selected by the VDR homodimer and the RXR/VDR heterodimer was the number of mismatches from the consensus sequences.

DISCUSSION

In this report, we have described the DNA binding properties of the VDR related complexes. In all experiments, we used the rat recombinant VDR expressed in E.coli. The DNA binding properties of the full-length VDR were identical with the zinc...
finger peptide as previously reported (17). This fact demonstrates that the DNA binding specificity of the VDR is completely dictated by the zinc finger region.

There are many reports that the VDR co-works with other nuclear accessory factor derived from mammalian cells (18, 23, 24). Until now, the most possible candidate is RXRβ (4, 5). Therefore, we tried to elucidate the DNA binding properties of the RXR/VDR heterodimer. Since we wanted to discriminate between the VDR homodimer and the RXR/VDR heterodimer on the gel shift assay, we produced the RXR fused to GST. The results show that the VDR alone could bind to the mSPP-1 VDRE, but not to the rOST VDRE. Both of homo- and heterodimers, however, could bind to either VDREs at the nearly same affinity. The difference of DNA binding specificity between each dimer might possibly produce the diversity of vitamin D action. Because the RXR also forms a heterodimer with the RAR, the TR and the PPAR (4, 5, 25–27), there are possible to exist that the operatable RXR is scarcely present in cells as a result of deprivation by the other nuclear receptors. In such cases, the VDR homodimer may bind to a complete direct repeat such as the mSPP-1 VDRE, but not to other VDREs.

The difference of DNA binding property between the VDR homodimer and the RXR/VDR heterodimer led us to examine the details of sequences recognized by each dimers. At first, we examined the DNA sequences recognized by the RXR alone. The RXR mainly selected the DR1 of the AGGTCA motif. Therefore, the RXR homodimer must preferentially bind to DR1. This result is consistent with previous reports saying that the RXR homodimer can bind to DR1, which is also found in the RXRE of cellular retinol-binding protein type II gene (CPBP II) (28,29).
However, the spaced residue is important because the A residue was the most highly selected at this position (73 out of 75 DR1, Fig. 4A). It is also reported that the RXR can bind to IRO (30). We can see the IRO in the RXR selected group even though it is small population (5 out of 81 clones).

The group selected by the VDR homodimer consists of almost DR3 whose half-sites consensus sequences are AGTTCA (compare to the AGGTCAn motif of the RXR heterodimer). The half-site consensus sequences selected by the RXR/VDR heterodimer are AGGTCAn for the first half-site and AGTTCA for the second half-site. These results support the model proposed by Perlmann et al. (31) that the RXR monomer binds to the 5' portion of the direct repeat and the heterodimeric partner to the 3' portion. The sequence between the half-sites also appears to be important for the DNA binding of the VDR dimers. The TTG sequence at the spacer position was obtained as the consensus for both the VDR homodimer and RXR/VDR heterodimer.

The specificity in DNA binding of unclear receptors is thought to be determined by the spacing and relative orientation of core recognition motifs. As far as the spacing and orientation are concerned, the specificity of the RXR/VDR heterodimer must be determined by the spacing and relative orientation of core recognition motifs. As far as the spacing and orientation are concerned, the specificity of the RXR/VDR heterodimer must be almost determined by the VDR and the RXR can simply increase the DNA binding affinity of the VDR. Because the RXR is the common factor in TR/RAR subfamily, it is reasonable that the RXR partners are responsible for discriminating the cognate HRE. Seeing the example of the mSPP-1 and rOST VDRE, the more a VDRE is distinct from the consensus, the more the RXR is effective.

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REFERENCES