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Isolation of a Genomic DNA Fragment Having Negative Vitamin D Response Element

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The vitamin D receptor (VDR) binds to the vitamin D response element (VDRE) in the promoter region of target genes and acts as a ligand-dependent transcriptional regulator. In order to identify novel VDREs and new genes that are regulated by the active form of vitamin D [1,25-$(OH)_2D_3$], rat genomic DNA fragments bound by VDR were isolated. One of these fragments, designated as VBF5, mediated 1,25-$(OH)_2D_3$-dependent negative regulation. Moreover, a genomic DNA region around VBF5 was transcribed and the transcript was down-regulated by 1,25-$(OH)_2D_3$. These data strongly indicate that VBF5 may contain a VDRE regulating negatively an unidentified gene expression.

The active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], exerts various biological effects (1). The cellular actions of 1,25-$(OH)_2D_3$ are thought to be mediated by vitamin D receptor (VDR) which is a member of the large family of ligand-activated transcription factors termed the nuclear receptor family (2,3). A ligand-receptor complex binds to the hormone response element in the regulatory region of the target gene whose transcription rate is influenced by 1,25-$(OH)_2D_3$. Vitamin D response elements (VDREs) have been identified in the regulatory region of vitamin D responsive genes. However, more genes should be identified in order to understand precisely the biological effects of 1,25-$(OH)_2D_3$.

In order to identify any previously unknown genes whose transcription is regulated by specific transcription factors, some recent studies have taken advantage of transcription factors which bind to DNA elements within the genes *in vitro* (4,5). We have contrived a strategy to isolate novel VDREs and identify genes which are regulated by 1,25-$(OH)_2D_3$. We screened rat genomic DNA with a mixture of VDR and retinoid X receptor (RXR) expressed in *Escheirichia coli.* Transient transfection analyses revealed that one of the enriched fragments, named VBF5 (*V*itamin D receptor *B*inding *F*ragment 5), contained a negative VDRE which suppressed transcriptional activity in the presence of 1,25-$(OH)_2D_3$. Moreover, we detected transcripts by Northern blot analysis using a larger genomic DNA fragment around VBF5 as a probe.

MATERIALS AND METHODS

Binding Selection Procedure and Sequence

Enrichment of VDR-binding fragments followed the filter binding method (5). Rat genomic DNA was prepared from spleen, and partially digested with Sau3Al. These fragments (3 μg) were incubated with 10 pmol of VDR and glutathione S-transferase (GST)-RXRβ fusion protein expressed in *E. coli.* (6). The binding mixture was passed through a nitrocellulose filter, and the trapped DNA fragments were subcloned into pBluescript KS. These plasmids (10 μg) were applied to the binding selection procedure again, and this cycle was repeated five times. The VDR-binding fragment was sequenced by the dideoxy-method (7).

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Plasmid Constructions, Cell Culture, Transfection, and DNA-Binding Analysis

The expression vectors for VDR (pHβAPr-VDR) in mammalian culture cells were created by subcloning into pHβAPr-1 (8). For analysis of VBF5, the fragment was inserted into PGV-P (Toyo Ink MFG. Co., Ltd.). The VBF5-DR fragment was produced by PCR and subcloned into PGV-P. The VDRE of mouse osteopontin gene (mSPP-1 VDRE), 5′-gatcACAAGGTTCACGCGGTTCACGTC-3′, was subcloned into PGV-P and used as a positive control for the luciferase assay. CV-1 cells were maintained in Eagle's minimal essential medium (Nissui) supplemented with 10% (vol/vol) fetal bovine serum. Transfection was performed by the calcium-phosphate precipitation method (9) with 1 μg of pHβAPr-VDR, 3 μg of reporter plasmids, and pBluescript KS up to 10 μg of the total amount of DNA. After incubation with 1×10^{-7} M 1,25-$(OH)_2D_3$, the cell extracts were assayed for protein concentration and luciferase activity. The experimental condition for the DNA-binding analysis was described previously (6).

Isolation of the Rat Genomic and cDNA Clones and Northern Blot Analysis

To isolate the genomic clone, a rat liver EMBL3 SP6/T7 -genomic library (CLONTECH) was screened with VBF5. To isolate the cDNA clones, a rat kidney λgt11 cDNA library (CLONTECH) was screened with the genome fragment isolated. GH_3 cells were maintained in Ham's F-10 (ICN Biomedicals Inc.) supplemented with 15% (vol/vol) horse serum and 2.5% (vol/vol) fetal bovine serum. Total RNA isolated with TRIzol reagent (GIBCO BRL) was resolved on 1% denaturing agarose gel, transferred to Hybond-N^+ membrane (Amersham) and hybridized.

RESULTS

Isolation of the VBF5 Regulated by 1.25-$(OH)_2D_3$

We isolated VDR-binding sites in rat genomic DNA according to the filter binding selection method (5). Partially digested rat genomic DNA was incubated with VDR/GST-RXRβ and the DNA fragments bound to proteins were selected on nitrocellulose filters. After the fifth selection cycle, some clones were isolated. These clones were subcloned into reporter plasmid PGV-P having a SV40 early gene promoter. These reporter plasmids were co-transfected with the VDR expression plasmid pHβAPr-VDR into CV-1 cells. The extract of transfected cells was assayed for luciferase activity (Fig. 1A). The reporter plasmid containing mSPP-1 VDRE was used as a positive control, and indeed the activation was observed with 1,25-$(OH)_2D_3$. Interestingly, one of the assayed clones, named VBF5, had vitamin D-dependent negative activity (Fig. 1A). The promoter activity with 1,25-$(OH)_2D_3$ decreased to about 70% of the activity without 1,25$(OH)_2D_3$. The VDR expression plasmid was necessary for this negative activity of VBF5 (data not shown). These results indicate that VBF5 contains negative VDRE.

The entire sequence of VBF5, approximately 1kb, was determined and submitted a search of the DNA to databases. However, no sequence sharing significant similarity to VBF5 was found. It is interesting that there are three 'AGGTCA' motifs arranged as direct repeats (DR) and separated by 4 or 13 bp (DR4 or DR13) between base positions 16 and 93 (designated the DR region) (Fig. 1B). It was known that VDR homodimer and VDR/RXR heterodimer could recognize DR3 type sequence comprising two 'AGGTCA' motifs (6,10,11). Because of this key motif being concentrated here, the DR region was supposed to work as a negative VDRE in VBF5.

Negative VDRE Activity of VBF5-DR

The ability of VDR/RXR complex to bind the DR region was assessed by band shift analysis using VBF5-DR as a probe. VDR alone could not bind to VBF5-DR (Fig. 2A lane 1), while GST-RXRβ could form a weak complex which retarded migration (Fig. 2A lane 2). Compared with these, VDR/GST-RXRβ could bind to VBF5-DR with higher affinity (Fig. 2A lane 3). This major complex was confirmed to contain both VDR and GST-RXRβ by super-shift experiment using the antibodies against VDR and GST-RXRβ (data not shown). In order to investigate negative VDRE activity of the DR region in VBF5, a reporter plasmid containing VBF5-DR was constructed and transfected into CV-1 cells (Fig. 2B). Expectedly, VBF5-DR could mediate the down-regulation by 1,25-$(OH)_2D_3$. The promoter activity with 1,25-$(OH)_2D_3$ decreased to about 60% of the activity without 1,25-$(OH)_2D_3$. This transcriptional response to 1,25-$(OH)_2D_3$ was observed in the presence

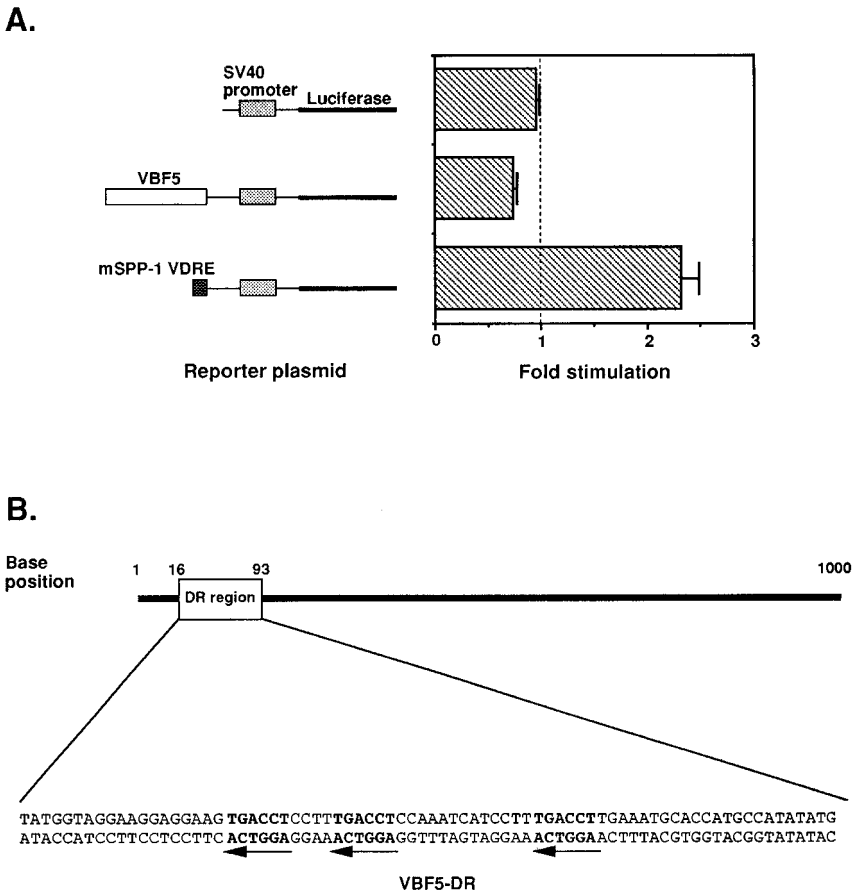

FIG. 1. A: The response of VBF5 to 1,25-$(OH)_{23}$. The reporter plasmids carrying the SV40 early gene promoter and the luciferase gene with VBF5 or mSPP-1 VDRE were transfected into CV-1 cells with the VDR expression plasmid. After incubation in the absence or presence of 1×10^{-7} M 1,25-$(OH)_2D_3$, luciferase activity was determined. The values are represented as a fold compared with the activity without 1,25-$(OH)_2D_3$. Mean values and S.D. ($n = 4$) are shown. The response of the reporter plasmid carrying VBF5 to 1,25-$(OH)_2D_3$ was significantly ($p < 0.05$) different from the control (the reporter plasmid without insert). **B:** Scheme of VBF5 fragment and the nucleotide sequence of direct repeat region in VBF5 (VBF5-DR). Three AGGTCA motifs were shown by arrows.

of the VDR expression plasmid as well as the reporter plasmid containing VBF5 (data not shown). These results indicate that VBF5-DR, rather than the other sequence in VBF5, could function as a negative VDRE. This negative VDRE had a different sequence construction from the DR3 type which was known to be a positive VDRE.

Detection of Transcripts from Genomic DNA Region around VBF5

Since it is expected that VBF5 contributes to gene expression, we screened rat liver genomic DNA library with VBF5 and isolated a phage clone λ1 containing VBF5. The clone λ1 has two SacI sites and divided into three fragments. Each fragment was used for Northern blot analysis to detect the transcript in RNA isolated from kidney. Detectable signals were observed with a 3 kb fragment which aparts about 7 kb from VBF5 (data not shown). Therefore, we screened rat kidney cDNA library by using this fragment as a probe and isolated one clone (λcF3). Furthermore we screened the same cDNA library with the λcF3 cDNA clone to obtain longer cDNA clones. Three positive clones (λc1, λc2, λc3) were isolated and the longest clone (λc3) was further analyzed. To demonstrate that this transcript is actually regulated by 1,25-$(OH)_2D_3$, we treated the GH_3 cells

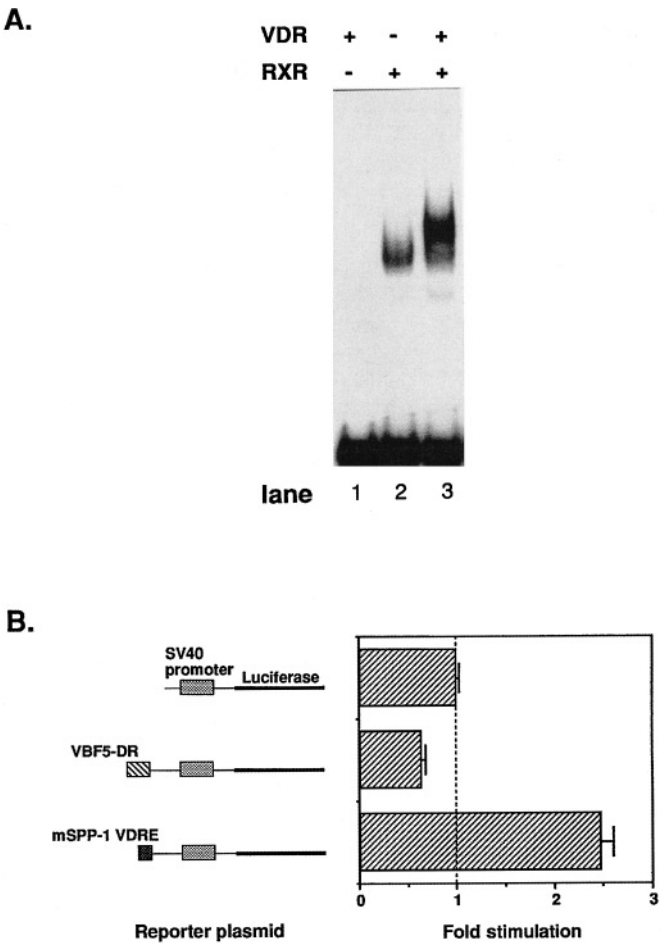

FIG. 2. A: The binding ability of VDR/GST-RXR hetero-complex to VBF5-DR *in vitro.* Gel-shift analysis was performed using radio-labeled VBF5-DR (10 fmol) with VDR (7.5 ng, lane 1), GST-RXR (5 ng, lane 2) or both (lane 3). Each reaction sample was loaded on 4% non-denaturing polyacrylamide gel. **B:** The negative VDRE activity of VBF5-DR. The reporter plasmids carrying the SV40 early gene promoter and the luciferase gene with VBF5-DR or mSPP-1 VDRE were transfected into CV-1 cells with the VDR expression plasmid. Luciferase activity was determined as described in Fig. 1. Mean values and S.D. ($n = 6$) are shown. The response of the reporter plasmid carrying VBF5 to 1,25-$(OH)_2D_3$ was significantly ($p < 0.01$) different from the control (the reporter plasmid without insert).

with 1,25-$(OH)_2D_3$ and performed Northern blot analysis using λc3. Three different length of transcripts were observed, and a signal level of the largest band was down-regulated to about 70% of initial level after 1,25-$(OH)_2D_3$ treatment (Fig. 3).

DISCUSSION

In this report, we isolated VDR/RXR binding fragments from rat genomic DNA. One of these fragments, VBF5, was revealed to contain the negative VDRE in transient transfection assay. We also isolated another fragment which had 1,25-$(OH)_2D_3$ dependent enhancer activity. However, many of the isolated clones could not respond to 1,25-$(OH)_2D_3$. Similar results were reported previously and it was concluded that this phenomenon was due to sequence surrounding the target binding site which had an interfering or silencing activity (5).

The VBF5 was sequenced and the DR region in VBF5 was found to contain DR4 and DR13 with three key motifs ‘AGGTCA’. Each DR was similar but not identical with DR3, which was regarded

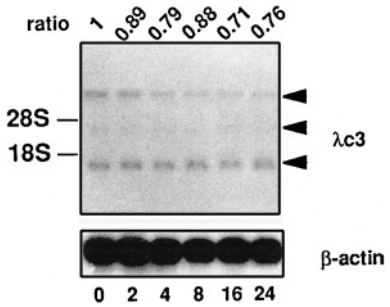

FIG. 3. Northern blot analysis of 1,25-$(OH)_2D_3$-treated GH_3 cells. Total RNA (15 μg) was isolated from GH_3 cells at the indicated hours after 1,25-$(OH)_2D_3$ treatment and analyzed by Northern blot hybridization using λc3 cDNA clone and β-actin as probes. Migration positions of ribosomal RNA markers are shown on the left. Three positive bands were indicated by arrows on the right. The ratios of signal intensity of 10-kb form normalized with β-actin were presented on the top.

as the positive VDRE consensus (10). It is likely that the difference in spacer number and motif number caused the opposite response to 1,25-$(OH)_2D_3$. The efficient binding of VDR/GST-RXRβ complex to VBF5-DR was found *in vitro,* while GST-RXRβ could bind to VBF5-DR less efficiently. VBF5 sequence thus permits binding of the two types of complex. It is possible that alteration of the binding complex might regulate the transcription differently.

Northern blot analysis of RNA isolated from GH_3 cells showed three bands (10 kb, 4 kb, 2 kb). The down-regulation of transcripts by 1,25-$(OH)_2D_3$ has been observed only in the 10-kb form, although we do not characterize this size specific regulation in detail yet. Since this gene would be encoded around VBF5 which had the negative VDRE, the down-regulation might be caused at a transcriptional level. The down-regulation was not dramatically, however, for example, a slight change of heterodimer partner should make great effects *in vivo.* To understand these phenomena, it is important that the complete sequence and the function of this gene product are revealed. The partial sequence of the λc3 cDNA clone was determined and matched up the parts of λ1 genomic DNA clone completely. There is no sequence similarity between the determined sequence and the sequences submitted in the DNA databases. Thus the transcript from genomic regions around VBF5 might be an unidentified gene. Further analysis of this gene product would provide knowledge about the biological function of 1,25-$(OH)_2D_3$.

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