Biochemical and Biophysical Research Communications **283,** 188–194 (2001)
doi:10.1006/bbrc.2001.4734, available online at http://www.idealibrary.com on IDEAL®

Isolation of Genomic DNA Sequences That Bind Vitamin D Receptor Complexes

Nicholas J. Koszewski,[1] Stefan Kiessling, and Hartmut H. Malluche
Division of Nephrology, Bone and Mineral Metabolism, University of Kentucky Medical Center, 800 Rose Street, Lexington, Kentucky 40536-0298

Received March 6, 2001

Vitamin D signaling is believed to be transduced by a heterodimeric receptor complex that binds to specific sequences of DNA termed vitamin D response elements (VDREs) in the promoter regions of target genes. However, recent studies have suggested that considerable flexibility exists in the types of binding sites the vitamin D receptor (VDR) is capable of recognizing, including some that bind VDR homodimers. In this report, a screening method involving immunoselection and PCR amplification was utilized to examine genomic binding sites for the receptor. Four individual fragments ranging in size from ca. 250–320 bp were nominally isolated from the amplified pool of captured fragments for further analysis. Each of the four sequences was capable of forming specific, unique VDR complexes using recombinant human VDR (rhVDR) alone or rhVDR heteromers formed in conjunction with the addition of recombinant human retinoid X receptor α (rhRXRα). Two of these fragments exhibited significant hormone-dependent repression of luciferase activity when linked to a thymidine kinase driven reporter vector. DNaseI footprinting revealed specific binding over DR+3 or related half-site sequences found within both of these DNA fragments. The results from this study demonstrate that specific, functional binding sites for the VDR can be successfully isolated from genomic DNA and should aid in the discovery of genes regulated by the steroid hormone.

Key Words: **vitamin D; gene regulation; hormone response element; genome.**

The VDR is a member of the nuclear receptor superfamily (NRI11) (1), and responds to the binding of the hormone, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), by altering transcriptional events within the target cell (for review see (2)). Numerous target genes have been identified that are directly affected by binding of the VDR to DNA response elements. These VDREs largely follow a direct repeat motif with a three base-pair spacing between the hexanucleotide half-sites (DR+3). The VDR also requires an accessory nuclear factor for high-affinity DNA–binding interactions, and members of the RXR family have been identified that can act as heterodimer partners with the VDR to promote such interactions (3, 4). However, several studies have reported VDREs that do not adhere to the DR+3 motif, and include inverted palindromes, direct repeats with greater than three base-pair spacing and even single half-sites (5–9). Thus, the heterogeneity that exists in functional DNA-binding sites may reflect the diversity of responses inherent in different cell types in terms of the expression and interaction with other transcription factors or comodulators. This also implies that identifying VDREs in the human genome by computational methods that focus on a DR+3 motif may reveal only a fraction of the potential binding sites used by the receptor to modulate target gene expression.

At least one DR+3 VDRE, isolated from the murine osteopontin gene, can bind VDR dimeric complexes in the absence of an added accessory factor (10, 11). Binding site selection analysis using short degenerate oligonucleotides also indicated that homodimers of VDR could specifically recognize an analogous DR+3 type of sequence (12, 13). Addition of VDR and RXR proteins to binding reactions with these DNA sequences results in heterodimeric complexes that are distinct from the homodimeric entities. Takeshita *et al.* demonstrated that VDR homodimers were stabilized by interactions with coactivator proteins and observed increased transcriptional responses in their presence (14). However, other studies have shown that VDR alone failed to produce significant amounts of reporter activities from mOP VDRE-driven reporters, while the combination of VDR and RXR proteins yielded strong, positive hormone-induced transcriptional responses (15, 16).

In addition to the mOP VDRE, various other DNA elements have been described that appear to bind the

[1] To whom correspondence should be addressed. Fax: 859-323-0232. E-mail: njhosz0@pop.uky.edu.

0006-291X/01 $35.00

VDR in the absence of an accessory factor (7, 9, 17–19). Most of these sites were identified in the course of defining the hormone's effects on a particular target gene. Sakoda *et al.* used recombinant VDR expressed in *Escherichia coli* together with a filter binding selection process to identify a rat genomic DNA sequence that bound VDR alone and could positively respond to hormones by increasing transcription of a reporter gene (20). The present study was undertaken to determine the feasibility of screening genomic DNA for functional VDR binding sites using the recombinant human protein and an anti-VDR C-terminal antiserum that specifically recognizes liganded VDR homodimers bound to DNA (21). Because of the readily apparent heterogeneity in VDR binding sites, and the potential to discover response elements that function with the VDR alone, the screen utilized only rhVDR in the selection process. Results are presented from four sequences that were evaluated in detail following their isolation from the pool of recovered rat genomic DNA fragments.

MATERIALS AND METHODS

General. Restriction and DNA-labeling enzymes were purchased from New England Biolabs (Beverly, MA). γ-^{32}P-ATP (6000 Ci/mmol) and α^{32}P-dATP (3000 Ci/mmol) were purchased from Dupont NEN Research Products (Wilmington, DE). Radioactive probes for EMSA were generated by fill-in labeling reactions using Klenow fragment and α-^{32}P-dATP. Radioactive probes for DNaseI footprinting were generated by end-labeling reactions using T4 polynucleotide kinase and γ-^{32}P-ATP. Rat genomic DNA, Advantage Genomic Polymerase Mix and pT-Adv cloning vector were purchased from Clontech (Palo Alto, CA). The Tf1 polymerase and DNaseI were purchased from Promega (Madison, WI). Protein A/G affinity matrix was purchased from Pierce (Rockford, IL). Spreadex-800 gels were purchased from Amresco (Solon, OH). PCR reactions were performed in an Ericomp PowerBlock I thermal cycler in 0.2 ml tubes.

Isolation of binding sites. A binding reaction containing 150 mM KCl, 20 mM Tris (pH 7.5), 1.5 mM EDTA, 2 mM DTT, 5% glycerol, 0.5% Chaps, 10 mM NaF, 100 μM Na_3VO_4, 12 μg dIdC, 0.5 mM leupeptin, and 250 nM $1,25(OH)_2D_3$ in a 240 μl volume was assembled. Ten microliters of rhVDR extract were added and incubated at 4°C for 30 min. After this time, rat genomic DNA (8 μg), digested with the combination of *Ava*II, *Eco*RI, *Hin*dIII, and *Xho*I restriction enzymes, was added to the mixture and incubation continued at 4°C for 30 min. Subsequently, 5 μl of Ab195, an antiserum raised against the C-terminal portion of the rat VDR (21), was added to the sample and the incubation continued at 4°C for 1 h. Protein A/G slurry was then added and mixed intermittently at 4°C for 75 min. The slurry was pelleted at 3000*g* for 3 min and the resulting pellet washed five times with 150 mM buffered KCl solution. The DNA was recovered by resuspending the pellet in TE/0.2% SDS and warming to 55°C for 15 min. The material was pelleted at 3000*g* for 3 min and the supernatant was transferred to a clean tube, treated with proteinase K at 50°C for 20 min, and extracted one time with phenol/chloroform (1:1). The DNA was precipitated, washed, dried, and treated with Tf1 polymerase at 72°C for 20 min. Following recovery of precipitated DNA, the sample was reconstituted in ligation buffer containing the pT-Adv cloning vector. Following transformation of the ligated products, the *E. coli* cells were batch grown overnight in liquid culture and plasmid DNA recovered. The plasmid DNA population was digested with *Hin*dIII/*Xho*I to liberate the insert sequences together with flanking vector "arms" possessing primer sites for PCR amplification. The oligonucleotides utilized for PCR amplification of the genomic inserts were 5′ TAGTAACGGCCGCCAGTGTGCT and 5′ CGGCCGCCAGTGTGATGGATAT. The digested plasmid DNA was then used in a DNA-binding reaction with rhVDR as outlined above. Following proteinase K treatment and phenol extraction, the pelleted DNA was resuspended in 10 μl of water and 5 μl was used for PCR amplification (94°C/4 s, 68°C/3 min, 32 cycles). Following amplification, the rhVDR immunoselection/PCR procedure was repeated an additional two times using the newly amplified material in each successive round.

To isolate individual PCR fragments for further analysis, an aliquot of the amplified DNA was separated through a Spreadex-800 gel at 200 V for 3 h in TAE buffer. Individual gel plugs were obtained and PCR-amplified directly using the primers and conditions indicated above.

Electrophoretic mobility shift assays. Briefly, the standard-binding buffer consisted of 120 mM KCl, 20 mM Tris (pH 7.5), 1.5 mM EDTA, 2 mM DTT, 5% glycerol, 0.5% Chaps, 10 mM NaF, 100 μM Na_3VO_4, 1 μg dIdC, 0.5 mM leupeptin, and 250 nM $1.25(OH)_2D_3$. All samples were incubated at 4°C. Cytosols of recombinant protein extracts were diluted 1:50 in KTEDG buffer (400 mM KCl, 20 mM Tris (pH 7.5), 1 mM EDTA, 2 mM DTT, and 10% glycerol) prior to use. For rhVDR binding, 2 μl of diluted extract were used in a 20 μl final volume. In the case of combined rhVDR/rhRXRα binding, the amount of diluted rhVDR cytosol was reduced to 1 μl and supplemented with the addition of 1 μl of similarly diluted rhRXRα cytosol. Following 30 min in this buffer to allow for hormone binding, the radiolabeled probe was added and the incubation continued for an additional 30 min. The samples were then applied to cooled, prerun 4% polyacrylamide gels (29:1) in 0.5 X TBE buffer and electrophoresis initiated at 14 V/cm for 3.5 h. Gels were transferred, dried, and autoradiography performed.

The procedure for evaluation of the antisera with complexes of the rhVDR was described in detail previously (21). After allowing the extracts to incubate in the buffer with ligand at 4°C for 30 min, the antiserum or control serum (1 μl each) was added and incubation continued for another 30 min period. After this time the radiolabeled probe was added and the incubation continued for an additional 30 min prior to loading onto the gel. Cold competition binding reactions were carried out using the chicken vitellogenin II estrogen response element (cVitII ERE) (5′ GATCCCTGGTCAGCGTGACCGGAG) or murine osteopontin (mOP) VDRE (5′ CTAGACAAGGTTCACGAGGTTCACGTG) double-stranded oligonucleotides.

DNaseI footprinting. Binding reactions for the mobility shift assays were assembled as described above. Either buffer or increasing amounts (2, 4, or 6 μl) of diluted rhVDR/rhRXRα mixture were added to the binding reactions and allowed to incubate at 4°C for 30 min. The single end-labeled DNA fragments (25,000 cpm) were mixed with the binding mixtures and incubation continued at 4°C for 30 min. Samples were then brought to 22°C and a $Mg/CaCl_2$ mixture added to final concentrations of 10 mM/5 mM, respectively. Diluted DNaseI was added and incubation continued at 22°C for 2 min. Samples were immediately extracted with phenol/chloroform (1:1) and the radiolabeled DNA precipitated from the aqueous phase by addition of sodium acetate and ethanol. Following centrifugation and washing of the DNA pellets with 75% ethanol, the samples were dried in a rotary evaporator and resuspended in a loading buffer. Samples were separated through 6% sequencing gels, dried, and autoradiography performed. Positions of footprint regions were determined by comparison to guanine chemical sequencing of the radiolabeled probes.

Transient transfection analysis. Opossum kidney (OK) cells were obtained from American Type Culture Collection (CRL-1840). Cells were maintained in Dulbecco's modified Eagle media/F-12 (1:1) with 10% charcoal-stripped fetal bovine serum containing penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C. Cells were plated

in triplicate in 24-well plates, transfected with the appropriate 195-N.2/tk-Luc construct (100 ng), CMV-β-galactosidase expression vector (50 ng) and carrier plasmid DNA (250 ng) using lipofectamine. Cells were treated with or without 100 nM 1,25-$(OH)_2D_3$ for 48 h, harvested and lysates prepared. Luciferase activity was determined and normalized with respect to the values for β-galactosidase enzymatic activity.

RESULTS

The strategy employed to isolate VDR binding sites from genomic DNA was as follows. Briefly, rat genomic DNA was cleaved by restriction enzyme digestion to produce a distribution of DNA fragments with an average size of ca. 1000 bp (data not shown). This material was then used in a DNA binding reaction with protein extracts of rhVDR in the presence of hormone. Following this binding reaction, an antiserum directed against the C-terminal region of the VDR was added to the samples and the immunoglobulin–receptor–DNA complexes isolated by affinity chromatography. The recovered DNA was then subcloned into a plasmid vector and, following transformation of *E. coli* and recovery of the plasmids, the DNA fragments were released with restriction enzymes utilized previously to initially digest the genomic material. Thus, these released fragments contained short sequences of DNA, or "arms," from the cloning vector that permitted PCR primers to be used for amplification of recovered DNA. The DNA was then subjected to three additional rounds of selection, recovery and PCR amplification, by which time there was no discernible change in the pattern of recovered DNA fragments by agarose gel electrophoresis following the second and third rounds of selection (data not shown).

The recovered DNA fragments from the third round of selection were separated by gel electrophoresis and a number of amplified products were observed (Fig. 1A). Subsequent experiments focused on the four fragments indicated in Fig. 1A solely because their smaller size lended them to the rapid generation of radiolabeled probes for use in examining specific binding interactions in EMSA, footprinting, and DNA sequence analysis. Gel plugs were obtained and these samples reamplified by PCR, which indicated that single bands of the appropriate size were recovered (data not shown).

In an initial attempt to ascertain if these sequences represented specific binding sites, each of the amplified products was used as a cold competitor in EMSA experiments featuring the rhVDR homodimer complex. The genomic screen was based on rhVDR binding to DNA sequences without the addition of an accessory factor, so the initial evaluation focused on homodimer binding to the mOP VDRE, an *in vitro* interaction extensively characterized in this laboratory as well as by others (10, 11, 14, 15). As seen in Fig. 1B, binding by the homodimer complex was completely eliminated by a 400-fold molar excess of unlabeled mOP VDRE oligonucleotide (lane 1), but was not disrupted by a 400-fold excess of an oligonucleotide competitor comprised of the cVitII ERE (lane 2). Because of limitations on the amounts of recovered, PCR-amplified material, and accounting for the relative increased size of these fragments, it was only possible to achieve 40-fold molar excess amounts of each of the genomic sequences (lanes 3–6). In all cases, these amounts of the amplified fragments strongly diminished the observable binding by the rhVDR homodimer complex, equivalent to the amount of competition observed with a 40-fold excess of the mOP oligonucleotide (lane 7). These results indicated that these genomic fragments possessed sequence elements that could specifically inhibit binding by the rhVDR homodimer complex.

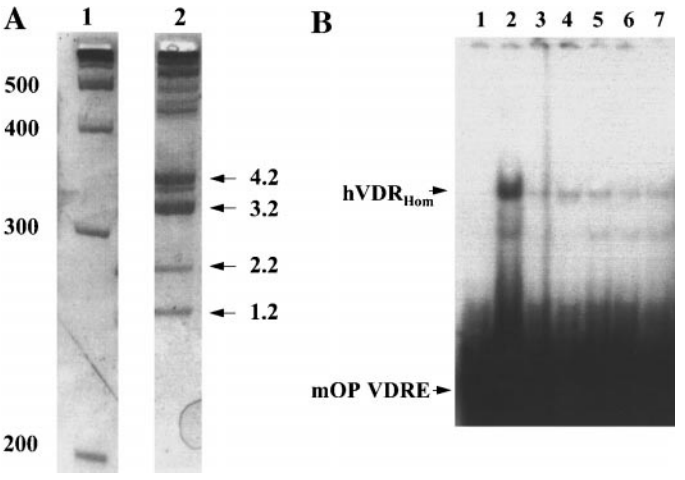

FIG. 1. Gel analysis of amplified rat genomic DNA fragments. (A) Following three rounds of immunoselection/amplification the products were separated on Spreadex-800 gels. Lane 1, mol. wt. markers; lane 2, PCR-amplified products. The four samples that underwent further analysis are indicated. (B) Cold competition in mobility shift assay for rhVDR homodimer binding to the radiolabeled mOP VDRE. Specific complex is indicated by arrow. Lane 1, 400-fold excess of unlabeled mOP oligonucleotide; lane 2, 400-fold excess of cVitII ERE; lane 3, 40-fold excess PCR-amplified 195-1.2 competitor; lane 4, 40-fold excess 195-2.2 competitor; lane 5, 40-fold excess 195-3.2 competitor; lane 6, 40-fold excess 195-4.2 competitor; lane 7, 40-fold excess mOP competitor.

The recovered fragments were then individually radiolabeled and tested for the ability of each to form specific rhVDR complexes. Shown in Fig. 2 are the EMSA profiles obtained from binding of the recombinant protein extracts to 195-2.2 (A) and 195-4.2 (B) radiolabeled probes. In both cases, no binding was observed when Sf9 control extracts from uninfected cells were used in the binding reaction (lanes 1). Inclusion of Sf9 cell-derived rhVDR in the binding buffer produced a single complex with 195-2.2, while two bands were observed with the 195-4.2 probe (lanes 2). These complexes were not observed with the inclusion of a 400-fold molar excess of mOP VDRE oligonucleotide, while a 400-fold excess of the cVitII ERE was unable to

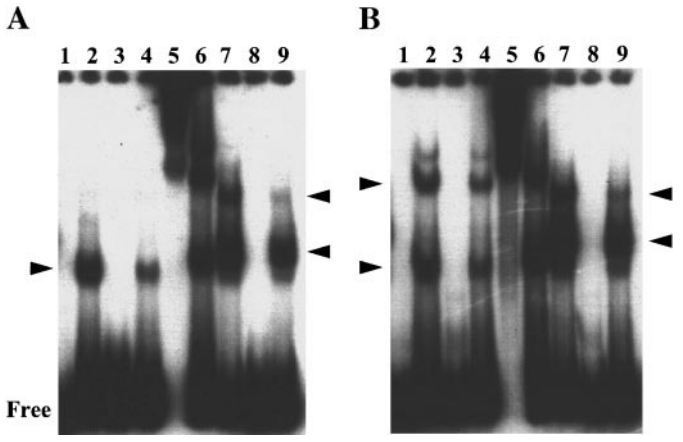

FIG. 2. EMSA to define specificity of rhVDR homomeric– and rhVDR/rhRXRα heteromeric–binding complexes to radiolabeled 195-2.2 and 195-4.2. (A) Complexes formed with radiolabeled 195-2.2 fragment: lane 1, Sf9 cell control extract; lane 2, rhVDR extract from baculovirus-infected Sf9 cells; lane 3, rhVDR with 400-fold excess cold mOP VDRE; lane 4, rhVDR with 400-fold excess cold cVitII ERE; lane 5, rhVDR with Ab195 anti-VDR antiserum; lane 6, rhVDR with control rabbit serum; lane 7, combination of rhVDR/rhRXRα extracts; lane 8, rhVDR/rhRXRα with 400-fold cold mOP VDRE; lane 9, rhVDR/rhRXRα with 400-fold cold cVitII ERE. (B) Radiolabeled 195-4.2 fragment was used with lane designations as described above. Positions of predominant homomeric (left side arrowheads) and heteromeric (right side arrowheads) in (A) and (B) are indicated.

effectively compete for binding complexes in either case (lanes 3 and 4). Complete supershifts were observed when the anti-VDR antiserum, Ab195, was included in the binding reactions (lane 5), while normal rabbit serum had no effect (lane 6). These experiments indicated that rhVDR was capable of forming specific binding complexes with both of these genomic fragments. Analogous results were obtained when the other two fragments, 195-1.2 and 195-3.2, were radiolabeled and used in binding reactions with rhVDR-containing extracts (data not shown).

Because of the propensity of the VDR to form high affinity heterodimeric DNA–binding complexes (4, 10, 15), the added presence of rhRXRα in the binding buffer was evaluated for any effect on complexes that formed with these DNA fragments (Figs. 2A and 2B, lanes 7–9). New, more retarded complexes were readily evident when both receptors were present relative to the complexes observed with rhVDR alone in the binding sample (indicated by arrowheads). In addition, even more slowly migrating complexes of lesser intensity were also evident with both radiolabeled probes. Inclusion of a 400-fold molar excess of the mOP VDRE prevented formation of all complexes with either radiolabeled genomic fragment (lane 8), while the excess cVitII ERE was ineffective in competing for binding to these complexes (lane 9). Experiments that included the anti-VDR antisera confirmed the presence of the VDR in these complexes, and analogous results were obtained with the other two genomic fragments (data not shown). Therefore, while the earlier results indicated that rhVDR-containing extracts alone could produce complexes with these sequences, the added presence of rhRXRα in the binding buffer generated new complexes of slower mobility that also exhibited specific DNA–binding interactions.

Sequence analysis of the four fragments using the Blast 2.0 search program (22) indicated that 195-1.2 corresponded to rat satellite I DNA (GenBank V01570, E Value 8e-52), but also matched well with an EST sequence identified from rat PC12 cells (GenBank H32702, E Value 6e-41). The sequences of the other three fragments failed to yield a significant match with any of the queried databases. Based on this information the transcriptional potential of each of the remaining three unknown fragments was then evaluated in transient transfection assays. The genomic fragments were subcloned into a luciferase reporter vector driven by the thymidine kinase promoter (tk-Luc), transfected into OK cells, and treated in the absence or presence of vitamin D. As seen in Fig. 3, fragment 195-3.2 produced a 41% decline in luciferase activity in response to hormone. The clone containing 195-4.2 exhibited higher basal levels of expression, but also produced a repressive response (29% decline) in luciferase activity following the addition of hormone. The construct containing 195-2.2 exhibited the weakest basal activity and failed to yield a significant response to hormone in these cells.

Based on the results from the transfection experiments, DNaseI footprint analysis was utilized to further localize the potential binding sites on the 195-3.2 and 195-4.2 fragments that could be attributed to the observed repressive response. The rhVDR/rhRXRα

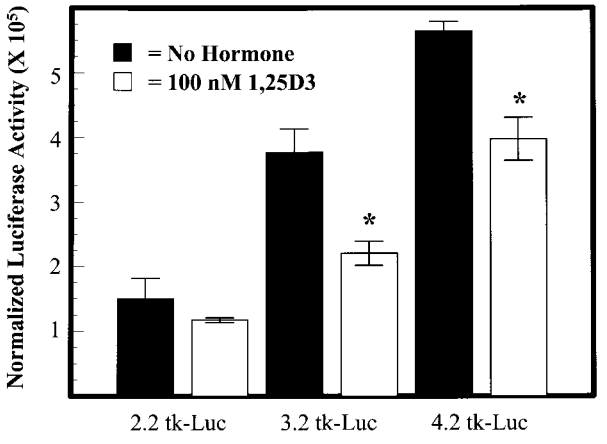

FIG. 3. Transient transfection studies in OK cells. OK cells were transfected with 195-N.2 tk-luciferase reporter constructs and an expression vector for the β-galactosidase gene. Following transfection, cells were treated with either no hormone or 100 nM $1,25(OH)_2D_3$ for 48 h and analyzed for luciferase and β-galactosidase enzyme activities. Average values are shown from treatments performed in triplicate wells and results are representative of three independent experiments. The asterisk indicates $P < 0.05$.

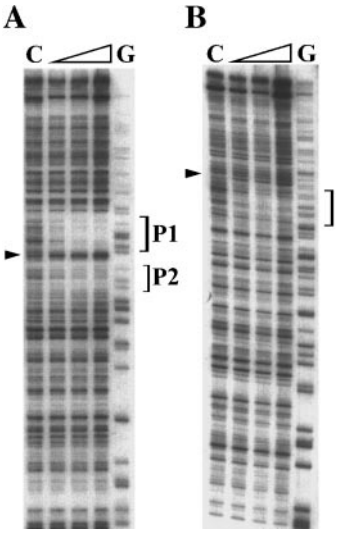

FIG. 4. DNaseI footprints generated from rhVDR/rhRXRα complexes. End-labeled 195-3.2 (A) or 195-4.2 (B) fragments were incubated with buffer control (C) or increasing amounts of rhVDR/rhRXRα extracts (triangle) and then treated with DNaseI for 2 min. Samples were analyzed on 6% sequencing gels and relative positions of footprints determined by a guanine sequence ladder (G). Bracketed areas indicate protected regions, including the two (P1 and P2) footprint areas observed for 195-3.2. Arrowheads denote hypersensitive cleavage sites observed upon addition of protein extracts to the binding reaction.

complex was utilized in this analysis because of the much stronger binding complexes observed in the mobility shift analysis (Fig. 3), and the likelihood that heterodimers constitute the active transcriptional complex (16). Using increasing concentrations of a mixture of rhVDR and rhRXRα-containing extracts resulted in clear protected regions for both fragments (Fig. 4). The 195-3.2 fragment exhibited two footprint regions: a strong region that covered approximately 30 base-pairs (bracket, P1) in conjunction with a smaller, weaker footprint region that was ca. 15 base-pairs in length (bracket, P2). Fragment 195-4.2 revealed a single footprint region covering some 20–25 base-pairs in length (bracketed region, Fig. 4B).

DISCUSSION

Vitamin D has been shown to regulate the transcription of a variety of gene products through direct interactions of the liganded VDR with DNA response elements (2). Typically, this process involves treatment of cells with a hormone, a differential screening method to identify gene products whose transcripts are altered in response to this treatment and then working back into the promoter or upstream DNA regions to identify the putative VDRE responsible for transducing the hormone's signal. The approach utilized in the present study was to exploit the availability of both rhVDR and anti-VDR antisera to screen genomic DNA for receptor-binding sites. Following their isolation, a subset of these sequences was then analyzed in detail to determine the specificity of rhVDR binding and functional activity in transfection assays. All four sequences could specifically bind rhVDR homomer or heteromer complexes in EMSA. In transfection experiments both the 195-3.2 and 195-4.2 genomic fragments resulted in strong, hormone-dependent repression of reporter gene activity. Thus, using rat genomic DNA as a model, the experimental strategy was successful in identifying specific DNA-binding sites which exhibited hormone-dependent transcriptional responses.

Comparison of the 195-3.2 binding site identified by the footprint analysis with other VDREs associated with transrepression of target genes by the vitamin revealed some striking similarities. The footprint analysis of this fragment revealed two protected regions (Fig. 5A). The stronger of these two footprint regions encompassed a region that potentially could be attributed to complex VDREs in two different orientations. The first possibility involves a DR+3 sequence in association with an additional half-site located 5′ from the DR+3 to produce an overlapping DR+2/DR+3 element, 5′ GGTTCA TG GGGTAA CTC AGTTTA. The DR+3 portion of the sequence is highly reminiscent of the proximal negative VDRE identified from the rat parathyroid

A

```
TTAGCTTTTC ACCTCCGTAC TTTTCTCCAT
TAATGCTTCC AGCGTTTACC TACGGCATGG
TAGGATAATG CAAGTTCACA GATCTTTCCT
CGTAGGTTCA TGGGGTAACT CAGTTTAAAA
CTGGCAGGTT TCTTCTCAAA ATGGCACCAG
ACATCCCCTC GAAGCGTCAT AAAATTCAGA
CCTTCGGACC CATGTCCAGA GGAATTACTT
TCTCACTCAA ACCTGAAGCT AGGTTTGAGC
CTGTGAAGAA
```

B

```
TTACCAGCAA TTAGCTTGTC TTAAAAAAAA
AAAAAGAGGA AGTTTTAAAA CCTGTTTAAG
GATCGTCTTT CTTAAAAAGA GACTTATAGT
CAGAATGGTC TTCAGATCCT CAGGAAACCA
CTGAAGCAAA AAGCCTGCGA TTCCCGGGCC
AGATTTCTCT GAAGAATGGA GATCCCGTAG
TTCAGGCAAA CCTTGCTGCA CTCAGCAGAC
ATTGCCTAGC GGCTAAATCA TTTGATTCCA
AGCTGATATT CCTATAACCG GCAGGATCCA
A
```

FIG. 5. Sequences of 195-3.2 (A) and 195-4.2 (B). The regions protected in the DNaseI footprint analysis are boxed. The dashed box in (A) indicates the second weaker footprint region found in the analysis of 195-3.2.

hormone-related peptide gene that bears the sequence, 5′ AGGTTA CTC AGTGAA (23). Alternatively, a contiguous DR+3/DR+2 sequence can also be proposed, GGTTCA TGG GGTAAC TC AGTTTA. In this latter case the 5′ DR+3 is strikingly reminiscent of the more distal negative VDRE associated with the rat parathyroid hormone gene (24). Thus, the strong similarities of either interpretation of this complex site with other known transrepressing VDREs suggest that these motifs may be more widely used in the rat genome to confer vitamin D-dependent repression of target gene activity.

In addition, a second, weaker footprint region was also observed in the 195-3.2 fragment. A sole half-site sequence, 5′ AGTTCA, was centered in this footprint region. It raises the possibilities that either this half-site functions independently to bind a single receptor subunit, or that it is linked to the aforementioned 5′ distal half-site in the other footprint region to create a more widely spaced (+16) direct repeat format (25) in conjunction with the DR+3.

The 195-4.2 footprint region contained a half-site, 5′ AGTTCA, that did not appear to be part of a DR+3 element (Fig. 5B). In a similar fashion, repression of the human parathyroid hormone gene by vitamin D appears to be mediated by a similar single half-site element (8, 17). However, closer inspection of the opposite strand of 195-4.2 within the footprint region revealed another half-site element, 5′ AGGTTTG, thus creating potential imperfect palindromes spaced by 2 or 3 base pairs.

Collectively, the footprint data indicated that the nature of these DNA elements is complex. A DR+3 motif was identified in 195-3.2 that was within the context of an extended footprint covering other half-site sequences, and no DR+3 was evident in the footprint covered in fragment 195-4.2. Sequence analysis also indicated the presence of DR+3 and isolated half-site motifs within the 195-1.2 and 195-2.2 fragments. The DNaseI footprint analyses also revealed hypersensitive cleavage sites resulting from receptor binding either between the two footprint regions (195-3.2, Fig. 4A) or immediately adjacent to the site (195-4.2, Fig. 4B). Further studies will be required to pinpoint the exact nucleotides contacted within the footprint regions of the these fragments as well as determine the relative affinities of these two sequences for VDR binding as compared to other known positive and negative VDREs. It should also be possible to design primers from these two different respective sites in order to "walk" the rat genome and identify nearby gene products that may indeed be regulated by these putative response elements.

The analysis involving just these four sequences failed to yield a DNA fragment unique for VDR homomeric binding. Thus, eventhough the selection strategy utilized only rhVDR in the binding reaction, all of the studied sequences were capable of binding both VDR homomers and heteromers, which is analogous to the situation for the mOP VDRE (10, 11). There are numerous, larger fragments (Fig. 1A, lane 2) from the amplified pool of DNA that await further analysis and may yet reveal a sequence that is capable of binding only VDR homomeric complexes.

The strategy outlined in this report should provide another means of identifying response elements that are regulated by vitamin D. In particular, because it is based solely on avidity for DNA-binding, it is, as demonstrated in these results, capable of identifying sequences that result in the repression of target gene activity, an area that receives less attention than its counterpart. This technique should also be amenable to higher throughput screening to quickly investigate the transcriptional potential of captured DNA fragments, and localize potential VDREs within those sequences. With the vast amounts of DNA sequence information currently being deposited into the human genome database it should also be possible to quickly assess the likelihood that a particular sequence captured by this method using human genomic material is indeed linked to nearby genes. Their specific regulation as targets for action by the vitamin could then be investigated more thoroughly using the appropriate *in vitro* or *in vivo* models.

ACKNOWLEDGMENTS

The authors thank Ms. Holli Gravatte and Ms. Anne Rowan for their excellent technical assistance. This work was supported by NIH Grants DK-47883 and DK-54276 (N.J.K.) and Dialysis Clinic Incorporated (N.J.K., S.K.).

REFERENCES

1. Nuclear Receptor Nomenclature Committee (1999) *Cell* **97,** 161–163.
2. Haussler, M. R., Whitfield, G. K., Haussler, C. A., Hsieh, J. C., Thompson, P. D., Selznick, S. H., Dominguez, C. E., and Jurutka, P. W. (1998) *J. Bone Miner. Res.* **13,** 325–349.
3. Kliewer, S. A., Umesono, K., Mangelsdorf, D. J., and Evans, R. M. (1992) *Nature* **355,** 446–449.
4. Thompson, P. D., Jurutka, P. W., Haussler, C. A., Whitfield, G. K., and Haussler, M. R. (1998) *J. Biol. Chem.* **273,** 8483–8491.
5. Schrader, M., Nayeri, S., Kahlen, J. P., Muller, K. M., and Carlberg, C. (1995) *Mol. Cell Biol.* **15,** 1154–1161.
6. Gill, R. K., and Christakos, S. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 2984–2988.
7. Polly, P., Carlberg, C., Eisman, J. A., and Morrison, N. A. (1996) *J. Cell Biochem.* **60,** 322–333.
8. Demay, M. B., Kiernan, M. S., DeLuca, H. F., and Kronenberg, H. M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 8097–8101.
9. Towers, T. L., and Freedman, L. P. (1998) *J. Biol. Chem.* **273,** 10338–10348.
10. Cheskis, B., and Freedman, L. P. (1994) *Mol. Cell Biol.* **14,** 3329–3338.

11. Koszewski, N. J., Reinhardt, T. A., and Horst, R. L. (1996) *J. Steroid Biochem. Mol. Biol.* **59,** 377–388.
12. Colnot, S., Lambert, M., Blin, C., Thomasset, M., and Perret, C. (1995) *Mol. Cell Endocrinol.* **113,** 89–98.
13. Nishikawa, J., Kitaura, M., Matsumoto, M., Imagawa, M., and Nishihara, T. (1994) *Nucleic Acids Res.* **22,** 2902–2907.
14. Takeshita, A., Ozawa, Y., and Chin, W. W. (2000) *Endocrinology* **141,** 1281–1284.
15. Lemon, B. D., and Freedman, L. P. (1996) *Mol. Cell Biol.* **16,** 1006–1016.
16. Lemon, B. D., Fondell, J. D., and Freedman, L. P. (1997) *Mol. Cell Biol.* **17,** 1923–1937.
17. Mackey, S. L., Heymont, J. L., Kronenberg, H. M., and Demay, M. B. (1996) *Mol. Endocrinol.* **10,** 298–305.
18. Kahlen, J. P., and Carlberg, C. (1994) *Biochem. Biophys. Res. Commun.* **202,** 1366–1372.
19. Freedman, L. P., Arce, V., and Perez Fernandez, R. (1994) *Mol. Endocrinol.* **8,** 265–273.
20. Sakoda, K., Suzuki, A., Arai, S., Imagawa, M., Nishikawa, J., and Nishihara, T. (1997) *J. Biochem. (Tokyo)* **121,** 89–94.
21. Koszewski, N. J., Reinhardt, T. A., Langub, M. C., Malluche, H. H., and Horst, R. L. (1998) *Arch. Biochem. Biophys.* **349,** 388–396.
22. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410.
23. Kremer, R., Sebag, M., Champigny, C., Meerovitch, K., Hendy, G. N., White, J., and Goltzman, D. (1996) *J. Biol. Chem.* **271,** 16310–16316.
24. Russell, J., Ashok, S., and Koszewski, N. J. (1999) *J. Bone Miner. Res.* **14,** 1828–1837.
25. Kato, S., Sasaki, H., Suzawa, M., Masushige, S., Tora, L., Chambon, P., and Gronemeyer, H. (1995) *Mol. Cell Biol.* **15,** 5858–5867.