The Vitamin D Response Element-binding Protein

A NOVEL DOMINANT-NEGATIVE REGULATOR OF VITAMIN D-DIRECTED TRANSACTIVATION*

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Vitamin D resistance in certain primate genera is associated with the constitutive overexpression of a non-vitamin D receptor (VDR)-related, vitamin D response element-binding protein (VDRE-BP) and squelching of vitamin D-directed transactivation. We recently cloned and characterized the first member of the hnRNP family capable of modifying steroid hormone-directed transactivation (21, 22), the estrogen response element-binding protein (ERE-BP). The 42-kDa ERE-BP is highly homologous to proteins in the hnRNP subfamily (23). It acts to squelch estrogen receptor (ER)-estrogen response element (ERE)-directed transactivation by competing with the ER homodimer for binding to the ERE. Although discovered because of its overexpression in estrogen-resistant New World primate species, the ERE-BP is also expressed in Old World primate species including man (21). The vitamin D response element-binding protein or VDRE-BP described here is in the second set of non-receptor steroid/hormone response element-binding protein to be discovered. VDRE-BP was also initially identified in New World primates resistant to the vitamin D hormone, 25-hydroxyvitamin D, and vitamin D hormone, 1,25-dihydroxyvitamin D (24). Here we report the purification, cloning, and initial functional characterization of two VDRE-BPs as members of the hnRNP family. One of these VDRE-BPs, VDRE-BP2, is capable of squelching steroid/hormone-mediated transactivation.

EXPERIMENTAL PROCEDURES

Cells—All primate cell lines were obtained from the American Type Culture Collection (Manassas, VA) and cultured as described previously (22). Preparation of nuclear extracts was according to a slight modification (22) of the method of Zerivitz and Akusjarvi (25). Electrophoretic Mobility Shift Assay (EMSA) and Oligonucleotides—Sequences of the various oligonucleotides employed were as follows: consensus vitamin D response element-osteopontin (VDRE-op, consensus recognition sequence underlined), 5'-CTAAGTGCCTGGGGTGTTACCAAGGCTTACGT-3'; vitamin D response element osteocalcin (VDRE-oc, consensus recognition sequence underlined), 5'-TTGGTGACTCACCGGGGTACGGGGCAGCGGGCTGGT-3'; YY1 mutant (mutated sequence underlined), 5'-TTGGTGACTCACCGGGGTACGGGGCAGCGGGCTGGT-3'; YY1 mutant (mutated sequence underlined), 5'-TTGGTGACTCACCGGGGTACGGGGGAGCGGGCTGGT-3'; nuclear factor yin-yang 1 (YY1; consensus recognition sequence underlined), 5'-CGCTCCGCGGCCATCTTGGCG-3'; nuclear factor yin-yang 1 (YY1; consensus recognition sequence underlined), 5'-CGCTCCGCGGCCATCTTGGCG-3'; nuclear factor yin-yang 1 (YY1; consensus recognition sequence underlined), 5'-CGCTCCGCGGCCATCTTGGCG-3'; nuclear factor yin-yang 1 (YY1; consensus recognition sequence underlined), 5'-CGCTCCGCGGCCATCTTGGCG-3'; YY1 mutant (mutated sequence underlined), 5'-CGCTCCGCGGCCATCTTGGCG-3'; YY1 mutant (mutated sequence underlined), 5'-CGCTCCGCGGCCATCTTGGCG-3'

EMSA were performed as described previously by us (22). Nuclear RT-PCR, reverse transcriptase-polymerase chain reaction; ss, single strand; ds, double strand; bp, base pair(s).
extracts from B95-8 or Vero cells or affinity-purified nuclear extracts from B95-8 cells, in the presence or absence of antibodies, 9A7 anti-VDR antibody (Ref. 26; generously provided by J. W. Pike), anti-hnRNP A1, anti-hnRNP A2 (a generous gift from Dr. G. Dreyfuss), and anti-RXRα antibody (27) were incubated with 2 μg of poly(dI-dC), 20 μM oligo(dG) (Promega, Madison, WI), and 10,500 cpm (3H)-labeled VDRE-BP1 or VDRE-BP2, and 10% charcoal-stripped fetal calf serum and allowed to proliferate to 80–90% confluence. Transfections were performed in triplicate with the following DNA suspension: 5.5 μg of VDRE-luciferase reporter plasmid (VDRE-op-LUC; Ref. 26) or an ERE-luciferase reporter plasmid (ERE-LUC; Ref. 23), with or without 0.5 μg of pRSV/β-gal (29) and with or without 0.5 μg of pCMX-T7-Tag1. After 48 h, cells were lysed and luciferase and β-galactosidase activities were measured.

Creation of Cell Lines Overexpressing the VDRE-BPs and ERE-BP—Vitamin D-responsive Old World primate Vero or COS-7 cells were incubated with 5.5 μg of pcR3.1-VDRE-BP1, pcR3.1-VDRE-BP2, and pcR3.1 ERE-BP1 in Lipofectamine solution for 5.5 h followed by the addition of 100-fold excess of radioinert VDRE (Fig. 1A). After an additional 48 h at 37°C, the cells were lysed and luciferase and β-galactosidase activities were measured.

RESULTS

New World Primate Cells Overexpress a Non-VDR-related Protein That Binds to the VDRE—Interaction of the VDR-RXR heterodimer with the VDRE is necessary for transcriptional control of vitamin D-regulated genes, such as the osteopontin gene (33). We initially employed an oligonucleotide containing the consensus sequence for the mouse osteopontin VDRE (VDRE-op) as a probe of protein extracts from New World primate cells, Old World primate cells, or S. cerevisiae cells transformed with the human VDR and RXRα (lane 1), confirming the specific nature of the VDRE-BP-VDRE interaction.

Molecular Cloning of the VDRE-BPs—B95-8 poly(A) + RNA (2.5 μg) was used as template to generate the VDRE-BP cDNA with the Marathon cDNA amplification kit (CLONTECH Laboratories Inc., Palo Alto, CA). Second-strand cDNA synthesis and adapter ligation were performed as instructed in the enclosed manual. The adapter-ligated cDNA was then used as template for annealing adapter- and VDRE-BP-specific primers for the RACE reaction: 5′-CTTGGACCGACGATCCGGTGA-3′ and 5′-TGGTTACTCTGGTACGATGC-3′ with their complementary sequence for the 5′- and 3′-RACE of VDRE-BP1 and VDRE-BP2, respectively. A cDNA for the VDRE-BP1 and VDRE-BP2 was generated by end-to-end amplification using specific 5′ and 3′ primers. The amplified products were then separately subcloned into the PCR 3.1 expression vector (identical as pcR3.1-VDRE-BP1 and pcR3.1-VDRE-BP2) and sequenced.

a) 5′-RACE of VDRE-BP1 and VDRE-BP2, respectively. A cDNA for the VDRE-BP1 and VDRE-BP2 was generated by end-to-end amplification using specific 5′ and 3′ primers. The amplified products were then separately subcloned into the PCR 3.1 expression vector (identical as pcR3.1-VDRE-BP1 and pcR3.1-VDRE-BP2) and sequenced.

b) 5′-RACE of VDRE-BP1 and VDRE-BP2, respectively. A cDNA for the VDRE-BP1 and VDRE-BP2 was generated by end-to-end amplification using specific 5′ and 3′ primers. The amplified products were then separately subcloned into the PCR 3.1 expression vector (identical as pcR3.1-VDRE-BP1 and pcR3.1-VDRE-BP2) and sequenced.

Vitamin D Response Element-binding Proteins

A DNA affinity resin was prepared as described previously by us (23). Extract fractions that were used for annealing were added to the DNA affinity column were desalted and microconcentrated with a Micron-30 filter (30-kDa molecular mass cut-off; Amicon, Beverly, MA). Samples were loaded and electrophoresed through 10% SDS-polyacrylamide gels. Coomassie Blue-staining bands at 34 and 38 kDa were cut out and digested and amino-terminal amino acid sequence determination at the Harvard Microchemistry Facility as described previously (32).

Western Blot Analysis—Nuclear extracts were subjected to electrophoresis on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes as described previously (8). The membranes were blocked with 5% nonfat dry milk for 1 h and then incubated with monoclonal anti-VDR antibody, anti-human hnRNP-A1 antibody, or anti-human hnRNP-A2 antibody for 2 h and with horseradish peroxidase-conjugated secondary antibody for another 1 h prior to detection of antibody-reactive proteins with chemiluminescence reagent (ECL, Amersham Pharmacia Biotech).
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Another; an excess of an oligonucleotide harboring the consensus and a mutated YY1 recognition sequence in the context above suggested that VDRE-BP participated in the vitamin D-resistant state in the vitamin D-resistant Old World primate cells, proof that VDRE-BP is a participant in the vitamin D-resistant multimer in New World primate cells required demonstration that overexpression of the VDRE-BP would convert wild-type hormone-responsive Old World primate cells to a vitamin D-resistant phenotype. As a first step in the recapitulation of the resistant phenotype, we created a double-strand DNA affinity support bearing concatemers of the VDRE-op for purification of VDRE-binding proteins from New World primate cells. Nuclear extracts of vitamin D-resistant New World primate B95-8 cells were loaded onto the affinity column in 0.1 M KC1 and chromatographed over the affinity support through an increasing salt gradient (0.2–1.0 M KC1; Fig. 3A, upper panel). Several Coomassie-stained proteins ranging in size from 34 and 38 kDa were retained on the column and eluted from the support in the 0.2–0.6 M KC1 fractions of the stepwise. Coomassie-stained bands at 34 and 38 kDa were reactive in immunoblots probed with anti-hnRNPA1 (39) and hnRNPA2 antibody, respectively (Fig. 3A, middle panels). The elution positions of these proteins were distinct from the 55-kDa VDR present in the nuclear extracts of B95-8 New World primate cells (Fig. 3A, lower panel). The hnRNPA1-reactive 34-kDa species (henceforth referred to as VDRE-BP1) and the hnRNPA2-reactive 38-kDa species (henceforth referred to as VDRE-BP2) were excised from gels loaded with the 0.4 M KC1 affinity column fractions. The pooled fractions subjected to tryptic digestion and amino-terminal amino acid sequencing.

Three distinct internal tryptic peptides fragments were recovered from the 34-kDa VDRE-BP1 pool and four from the 38-kDa VDRE-BP2 pool (Fig. 3B). All seven tryptic peptides bore sequence similarity with proteins in the hnRNPA family. The sequence of two peptides each in the 34-kDa VDRE-BP1 pool and in 38-kDa VDRE-BP2 pool were closely related; this set of related peptides is found in the highly conserved RNA binding domain of hnRNPs (40). Collectively, the tryptic peptides in the 34-kDa VDRE-BP1 pool possessed a high degree of sequence similarity with proteins of the human hnRNPA subfamily, while peptides from the 38-kDa VDRE-BP2 pool bore the greatest sequence similarity with proteins of the hnRNPA2 subfamily (Fig. 3B).

VDRE-BP mRNA Is Overexpressed in Hormone-resistant New World Primate Cells—Using a panel of degenerate oligonucleotides corresponding to the two tryptic peptide sequences common to both VDRE-BP1 and VDRE-BP2 (see Fig. 3B), we amplified by RT-PCR a 435-bp cDNA that demonstrated 94% sequence identity to the cDNA for human hnRNPA2. This cDNA was used as probe in Northern analysis of RNA extracted from hormone-resistant New World primate B95-8 cells, from OMK New World primate cells with the intermediate, partially hormone-responsive phenotype, and from hormone-responsive Old World primate Vero cells (Fig. 3C).
probe hybridized to a 1.8-kilobase mRNA species that was plentiful in hormone-resistant B95-8 New World primate cells, was less plentiful in New World primate cells with the intermediate phenotype of partial hormone-responsiveness (OMK cells), and was expressed at a much lower level in Old World primate Vero cells with a hormone-responsive phenotype.

**Affinity-purified VDRE-BP Binds Either Double- or Single-strand DNA Bearing the VDRE Recognition Sequence—**hnRNPs are traditionally recognized as single-strand pre-mRNA-binding proteins (40, 41). We theorized that VDRE-BPs would also be capable of binding single-strand DNA. Proof was provided by the experiments shown in Fig. 4. EMSA was performed with the 0.4 M KC1 affinity column fractions containing VDRE-BF1 and VDRE-BF2, with either single-strand VDRE-op or double-strand VDRE-op as probe and with either single-strand or double-strand radioinert response element-containing oligonucleotides as competitors for probe binding. Using the top strand of VDRE-op as probe (Fig. 4A), competition for probe binding was achieved with either the holo double-strand VDRE (lane 3) or the single-strand VDRE (lane 7).

Somewhat less effective competition for VDRE-BP-single-strand VDRE binding was also achieved with an oligonucleotide representing the top strand of the RXRE (5'-AGCTTCAG-GTCAGAGGTCAGAGAGCT-3'; lane 8), indicating that the RXRE harbors a recognition sequence for the VDRE-BP albeit less effective than the VDRE-op upper strand in competing for VDRE-binding. A similar pattern of competition for probe binding was observed when double-strand VDRE-op was used as probe, with double-strand VDRE-op being as effective as single-strand VDRE-op in competition for probe binding (Fig. 4B).

As expected, irrelevant sequences (i.e. CTF/NF, lanes 6 and 9), whether single- or double-strand in nature, had no effect on VDRE-BP binding to the VDRE.

**Molecular Cloning of VDRE-BP—**Confirmation that the VDRE-BPs were members of the hnRNPA family was obtained by cloning cDNAs that encoded the entire open reading frames of VDRE-BP1 and VDRE-BP2. The 435-bp cDNA (see above) was employed to develop 5'- and 3'-nested primers for use in cloning by 5'- and 3'-RACE cDNAs for both VDRE-BPs that extended 5' through the translation start site and 3' through the untranslated region. The 1751-nucleotide VDRE-BP1 cDNA bore 61% sequence identity to the human hnRNPA1 cDNA, and the 1788-nucleotide VDRE-BP2 cDNA possessed 71% sequence identity to the human hnRNPA2 cDNA. Nucleotide sequence identity with human hnRNPA1 and hnRNPA2 was 96% through the open reading frame of the two cDNAs; most sequence divergence was accounted for in the extensive 3'-untranslated region of the two cDNAs. The translated portions of the cDNAs predicted proteins at 34 and 38 kDa, the same size of the anti-hnRNP-reactive proteins observed in immunoblots of eluents from VDRE affinity support (see Fig. 3).

**Overexpression of VDRE-BP—**If the actions of the VDRE-BPs in vitro with the VDR for VDRE binding are operative in vivo, then squelching of VDR-VDRE-directed transactivation should take place in vitamin D-responsive, hormone-resistant New World primate cell line B95-8 either in the absence (panel A) or presence (panel B) of co-transfected VDR (0.5 μg) and VDRE-op-LUC (5.5 μg) in the absence (−) or presence of (+) of a VDR-saturating concentration (10 nM) of 1,25-dihydroxyvitamin D3. Data are the mean ± S.D. of duplicate determinations of luciferase activity in three separate experiments. Asterisks denote a significant decrease in luciferase activity (p < 0.001).
wild-type cells that have been induced to overexpress the protein. In order to investigate this postulate, hormone-responsive, Old World primate Vero cells bearing a fully functional VDR were transiently co-transfected with the pCR3.1-VDRE-BP1, pCR3.1-VDRE-BP2, and VDRE-reporter constructs (Fig. 5). VDRE-reporter activity was unaffected by expression of pCR3.1-VDRE-BP1. On the other hand, basal VDRE-reporter activity was reduced 71% in pCR3.1-VDRE-BP2-transfected Vero cells compared with that observed in the same cells co-transfected with the empty pCR3.1 vector and VDRE-reporter constructs. In fact, basal reporter activity in pCR3.1-VDRE-BP2-bearing cells approached levels observed in hormone-resistant B95-8 cells that was supershifted upon interaction with the VDRE-BP2-probe demonstrating retardation of the probe when B95-8 nuclear extract is added to the gel (middle lane) and supershift of the retarded band upon addition of anti-hnRNPA2 antibody (right lane).

Additional confirmatory evidence that VDRE-BP2 was the naturally occurring dominant VDR-VDRE squelching species in vitamin D-resistant New World primate cells was obtained by Western blot detection of an abundance of the anti-hnRNPA2-reactive VDRE-BP2 only in the B95-8 cell line derived from a vitamin D-resistant New World primate (Fig. 6A) and by demonstration in EMSA of a VDRE-binding protein that was supershifted upon interaction with the VDRE-BP2-cross-reactive anti-hnRNPA2 antibody (Fig. 6B). Recapitulation of the hormone-resistant phenotype in vitro was further demonstrated in VDR-VDRE-directed luciferase activity in wild-type Vero cells stably transfected with VDRE-BP2 (Fig. 7). Six VDRE-BP2-overexpressing cell lines were examined (Fig. 7A); compared with wild-type cells without transfection, the six clones overexpressed VDRE-BP2 6–17-fold. Compared with extracts from wild-type Vero cells, protein extracted from the nuclei of VDRE-BP2-overexpressing cells lines bound to VDRE probe in EMSA (Fig. 7B); VDRE-BP-probe binding in the stably transfected cell lines was similar to that observed in the vitamin D-resistant New World primate cell line from which the VDRE-BPs were initially cloned. VDRE-BP2-overexpressing clones were also assessed for their ability to squelch VDR-VDRE-directed luciferase activity (Fig. 7C). Among the four cell lines examined, luciferase activity was suppressed to 18–27% of wild-type levels with the clone overexpressing VDRE-BP2 to the greatest degree (clone 4) exhibiting the most suppression of reporter activity.

**Specificity of the VDRE-BP for Transactivational Regulation through the VDRE**—We showed that ERE does not compete with the VDRE for binding VDRE-BPs (see Fig. 4). If this is the case, then constitutive overexpression of the VDRE-BPs in cells
should exhibit no squelching of estrogen-ER-ERE-directed transactivation. Panel A of Fig. 8 confirms this postulate. Vero cells transiently transfected with either VDRE-BP1 or VDRE-BP2 did not inhibit luciferase activity driven off a promoter bearing an enhancing ERE, either in the absence or presence of an ER-saturating concentration of estradiol; in fact, VDRE-BP2 induced a modest increase in reporter activity. Specificity of VDRE-BP2 for squelching transactivation from a VDRE was also suggested by experiments performed in wild-type Old World primate COS-7 cells stably overexpressing another dominant-negative acting hnRNP, the estrogen-response element-binding protein or ERE-BP (Ref. 22; Fig. 8B). Compared with wild-type COS-7 cells, COS-7 cells stably overexpressing the ERE-BP and transiently with the VDRE-op-LUC reporter construct demonstrated no change in VDR-VDRE-directed luciferase activity. On the contrary, significant squelching of ERE-directed transactivation was observed in COS-7 cells stably overexpressing ERE-BP1. These data 1) reinforce the concept that the VDRE-BPs do not normally interact with the ERE and 2) imply that modulatory effects of a response element-binding protein are specific for a single hnRNP-related species interacting with a specific response element.

**DISCUSSION**

Our knowledge of the rheostatic control of sterol/steroid hormone action has evolved considerably in the recent past. The earliest concepts of control focused on hormone synthesis, hormone metabolism (or catabolism), and hormone-receptor interaction in the target cell. In the vitamin D hormone system, target cell responsiveness is known to be governed by 1) the cutaneous, nonenzymatic photosynthesis of vitamin D (42); 2) the serial enzymatic modification of vitamin D to its active hormone, 1,25-dihydroxyvitamin D (43); and 3) interaction of the 1,25-dihydroxyvitamin D hormone with its nuclear receptor protein, the VDR (43). For example, without adequate sunlight exposure mammalian species, including *Homo sapiens*, fail to synthesize enough of the prohormone vitamin D to provide a sufficient supply of substrates to the vitamin D-25-hydroxylase and vitamin D-1-hydroxylase enzymes required for 1,25-dihydroxyvitamin D production. The resultant phenotype is failure to properly mineralize the skeleton. The same or similar skeletal phenotype is also seen in humans with inactivating mutations of the vitamin D-1-hydroxylase (44, 45) or the VDR (46). Because they elicit the most dramatic changes in hormone effect and the most definitive phenotype, vitamin D prohormone synthesis, metabolism, and interaction of the hormone with VDR can be considered sites of “macroregulation” of vitamin D action.

However, there are additional factors in the hormone action schema that produce a much less subtle change in the phenotype. These factors serve as “microregulators” to fine tune hormone responses. In the vitamin D system, such microregulators include 1) the circulating vitamin D-binding protein, the shuttle protein in the serum that serves to deliver active vitamin D metabolites to target tissues for metabolism or action and carry away inactive metabolites for catabolism and excretion (47–49); and 2) the dimerization partner of the VDR (i.e., the RXR; Refs. 43 and 50), as well as the receptor-associated co-activators, co-repressors, and co-modulators of transcription (51). Another proposed microregulator in the vitamin D metabolism/action system is the newly discovered family of hsp70-related intracellular vitamin D-binding proteins. These proteins, initially identified in vitamin D-resistant New World primate cells (52, 53), have a high capacity and relatively high affinity (2–20 nM) for 25-hydroxylated vitamin D metabolites (54). Recent work indicates that these proteins have the ability to facilitate hormone action by delivery of 1,25-dihydroxyvitamin D hormone to the nucleus and VDR, which has a higher affinity for hormone than the intracellular vitamin D-binding proteins.

Although the protein-protein interactions that occur among receptor proteins and various co-regulators may provide the most complex means of control of transcription, these interactions are not the most direct mode of transcriptional regulation. For example, even if assembled for maximal transcriptional effectiveness, the transactivating complex must be able to interact with a specific promoter element in order to achieve its transcriptional potential (55). If the VDR-RXR-directed tethering of the transcriptional machinery to promoter elements via the VDRE is not possible because the cis element is occupied by another protein, then transcription will be thwarted although the complex is optimally suited to enhance transcription. The YY1 protein is one such protein with the potential to exert dominant-negative modulatory control over VDR-RXR-directed transcription through the VDRE (38). Guo et al. (38) have shown that YY1 protein can compete with VDR-RXR heterodimer for occupation of the VDRE in the osteocalcin promoter. We found that neither antibodies to the YY1 protein nor exposure to a 100-fold excess of the YY1 recognition sequence altered binding of New World primate cell VDRE-BPs to either the osteocalcin or the osteopontin VDRE (Fig. 1B). These results suggested that the VDRE-BPs in crude extracts of New World primate cells were not related to the YY1 protein family. 

2 S. Wu, unpublished data.
a point later confirmed upon isolation and structural characterization of the VDRE-BPs as members of the hnRNPA family of proteins (see Figs. 3 and 6).

We have not yet determined independent affinity constants of the two known VDRE-BPs for the VDRE, but preliminary results\(^3\) indicate that VDRE-BP2 possesses an avidity for the VDRE that rivals that of the VDR-RXR complex and harbors the same transcriptional squelching potential of YY1 (see Fig. 1B). Hence, when present in relatively small quantities in the nuclear compartment compared with the VDR and RXR, we propose that VDRE-BP2 has the potential to compete with VDR-RXR dimer for binding to the VDRE. When present in relatively large quantities as they are vitamin D-resistant New World primate cells, it is possible that VDRE-BP2 can substantially limit hormone-stimulated gene expression (Fig. 2). Consequently, we theorize that depending on their relative abundance in the target cell the VDRE-BPs have the potential to function as either a microregulator (i.e. when present in relative low abundance compared with VDR-RXR) or as a macro-regulator when the VDRE-BPs(s) overwhelm the VDR-RXR and its access to the VDRE.

hnRNPs are classically recognized as single-strand pre-mRNA-binding proteins (10). Recently proteins in this class have also been shown to possess the ability to bind DNA including double-strand DNA and to alter, principally by silencing, transcription (4–8). The hnRNPA2-related VDRE-BP2 falls into this category. Affinity-purified VDRE-BP2(s) will bind to either double-strand (Figs. 1 and 4) or single-strand (Fig. 4) DNA bearing a VDRE. The effectiveness of a single-strand oligonucleotide bearing the consensus RXRE (consisting of a direct repeat of the AGGTCA motif separated by only a single, not three, nucleotides) in competing for VDRE-BP binding suggests that the specificity for VDRE-BP binding resides in the 6-bp half-site motif (G/A)GGT(G/C)A; variations of this six-nucleotide motif encompass nucleotide sequences present in the VDRE-op (GGGTCA), VDRE-oc (GGGTGA), and RXRE (AGGTCA) used in our DNA binding analyses. Binding specificity was confirmed by failure of the complementary (opposite) strand of the VDRE-op, VDRE-oc, and RXRE to compete for VDRE-BP binding when either single-strand or double-strand VDRE was employed as probe and by the ability of an hnRNPA1 antibody to disrupt VDRE-BP-VDRE binding (data not shown).

We recently described an hnRPC-like protein, which competes with the ER for binding to the ERE (21, 22). This protein, termed the ERE-BP, exerts a profound dominant-negative influence on ER-ERE-directed transactivation in vitro and induces estrogen resistance in vivo when overexpressed (22). The VDRE-BP2 described here appears to act in similar fashion with overexpression of this protein clearly squelching VDR-RXR-directed transactivation (Figs. 2, 5, and 7) via its ability to bind to the VDRE (Figs. 6 and 7). The question remains whether VDRE-BP2 is absolutely specific for the VDRE-BP or whether it might interact with other hormone response elements as well. To address this question, we examined the ability of VDRE-BP2, to alter transactivation directed by a ERE (Fig. 8A). It did not. We also queried whether transient overexpression of the ERE-BP acted to squelch VDRE-directed transactivation. Although a number of separate experiments showed no substantial or consistent effect with transient overexpression of the ERE-BP (data not shown). Stable overexpression of the ERE-BP in wild-type cells had no effect on VDRE-directed transactivation (Figs. 2, 5, 6, and 7) or in cell lines stably overexpressing VDRE-BP2 (Fig. 7) approximated that observed in vitamin D-resistant New World primate cells, which endogenously overexpress VDRE-BP2 and VDRE-BP1, indicates that VDRE-BP2 is the principal dominant-negative agent active at the VDRE.

The fact that the hnRNPA1-related VDRE-BP1 identified here had VDRE binding potential (Fig. 3A) but was without significant squelching potential (Fig. 5) was surprising. It is possible that the affinity of VDRE-BP1 for the VDRE is much lower, hence its ability to remain on the cis element and interfere with VDR-RXR binding is diminished compared with VDRE-BP2. The reduced but still existing affinity of VDRE-BP1 for the VDRE may also be an explanation why VDRE-directed transactivation of a reporter cell lines overexpressing VDRE-BP2 at levels observed in hormone-resistant New World primate cells was not completely squelched (Fig. 7C). If present in sufficient quantity, as VDRE-BP1 appears to be in vitamin D-resistant New World primates cells (Fig. 3A), VDRE-BP1 may compete with VDRE-BP2 for binding to the VDRE. It is also likely that squelching of transcription requires interactions with other proteins in the transcriptional complex. As a consequence it is possible that the reason VDRE-BP1 is ineffective as regulator of transcription (Fig. 5) is because VDRE-BP1 in incapable of interacting with the transcriptional machinery, while VDRE-BP2 is capable of interacting with those proteins.

Are there in vivo equivalents to the microregulatory and macroregulatory phenotypes induced by the VDRE-BP(s) among primate genera? In subhuman primates the answer is yes. The partially hormone-responsive New World primate ge-

\(^3\) H. Chen, unpublished data.
nus *Aotus* and the hormone-resistant New World primate genus *Callithrix* (52) are respective examples of the micro- and macroregulatory phenotype. Because *Aotus* cells express relatively little mRNA (Fig. 3C) and even less protein for the VDRE-BPs, this genus possesses circulating 1,25-dihydroxyvitamin D levels similar to those of Old World primates and a clinical phenotype that is difficult to detect even when challenged with sunlight deprivation (19, 20). On the other hand, *Callithrix*, which expresses high levels of the VDRE-BP mRNA and protein (Figs. 3C and 6A), possesses very high serum levels of 1,25-dihydroxyvitamin D and an extreme propensity to develop rickets when cutaneous vitamin D synthesis is challenged (19, 20, 52). Are these human equivalents to the micro- and macroregulatory phenotypes caused by the VDRE-BPs in subhuman primates? Because the phenotype that would likely result from a relatively low level VDRE-BP expression is likely to be subtle or nonexistent in the basal (unchallenged) state, it is highly unlikely that we have yet identified the human counterpart of the microregulatory phenotype. However, this may not be the case with the macroregulatory phenotype as exemplified by *Callithrix*. There are human families described with the classical phenotype of vitamin D resistance that harbor high serum concentrations of the active vitamin D metabolite 1,25-dihydroxyvitamin D but no recognized mutation in the VDR (56). It is possible that these subjects may suffer from a disturbance resulting in the constitutive overexpression of a protein(s) that interacts with the VDRE.3

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