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Intron Retention Generates a Novel Isoform of the Murine Vitamin D Receptor That Acts in a Dominant Negative Way on the Vitamin D Signaling Pathway

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We identified and characterized a novel rat vitamin D receptor isoform (rVDR1), which retains intron 8 of the canonical VDR (rVDR0) during alternative splicing. In this isoform protein directed by the stop codon in this newly identified exon, a part of the ligand binding domain (86 amino acids) is truncated at the C-terminal end but contains 19 extra amino acids. The rVDR1 transcript was expressed at a level 1/15 to 1/20 of that of rVDR0 in the kidney and intestine in adult rats but not in embryos. The recombinant rVDR1 protein showed no ligand binding activity. Homo- and heterodimers of the recombinant rVDR0 and rVDR1 proteins bound to a consensus vitamin D response element (VDRE) but not to consensus response elements for thyroid hormone and retinoic acid. However, unlike rVDR0, rVDR1 did not form a heterodimeric complex with RXR on the VDRE. A transient expression assay showed that this isoform acted as a dominant negative receptor against rVDR0 transactivation. Interestingly, the dominant negative activities of rVDR1 differed among VDREs. Thus, the present study indicates that this new VDR isoform negatively modulates the vitamin D signaling pathway, through a particular set of target genes.

Most of the biological actions of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], such as regulating calcium homeostasis and cytodifferentiation, are exerted through gene expression mediated by the nuclear vitamin D receptor (VDR) (for a review, see reference 8). VDR is a member of the nuclear receptor superfamily, which functions as a ligand-inducible transcription factor (for reviews, see references 2, 10, 13, and 39). This family includes nuclear receptors for steroid hormones, thyroid hormone, retinoic acid, and unknown ligands (orphan receptors). Together with the retinoid receptors (RARα, -β, and -γ and RXRα, -β, and -γ) and thyroid hormone receptors (TR α and β), VDR forms a subfamily, based on structural and functional similarities, in the nuclear receptor superfamily. Like RARs and TRs, VDR heterodimerizes with RXR. These heterodimers bind distinct but similar target enhancer elements composed of two directly repeated core AGGTCA (or related hexamer core) motifs. The spacer between the two core motifs (3 bp [DR3] for RXR/VDR, 4 bp [DR4] for RXR/TR, and 2 [DR2] and [DR5] 5 bp for RXR/RAR) discriminates among these nuclear receptors for recognizing target enhancer elements (for reviews, see references 12, 19, 22, 41, and 50). In addition to the heterodimeric form of VDR with RXR, VDR forms a homodimer with some vitamin D response elements (VDRE), suggesting the presence of two signaling pathways for vitamin D (5, 48). Irrespective of the functional and structural similarities of these receptors, only one type of VDR protein has been found in sharp contrast to multiple subtypes and isoforms of RAR, RXR, and TR (17, 24, 53). The RAR and TR isoforms are generated by alternative splicing and/or by direction of differential promoters. Functional analysis by a transient-expression assay showed that the transactivational activity differs among the receptor isoforms (34). Moreover, genetic investigations using several lines of mice lacking the receptor isoforms and subtypes of RAR and RXR clarified the tissue- and developmental-stage-specific roles of each subtype and isoform in the retinoid signaling pathway (16, 25, 46). Thus, the subtype and isoform of the nuclear receptor seem to differentially modulate the biological actions of the ligand.

The biological significance of VDR in the vitamin D signaling pathway has been demonstrated with in vitro functional analysis of VDR (30, 35) and the genetic mutations in hereditary 1,25$(OH)_2D_3$ resistance rickets (HVDRR) patients (15, 21, 27). More recently, it was reported that allelic variations mapped to intron 8 of the human VDR gene (hVDR) are significantly related to circulating osteocalcin and bone density (32, 33). On the basis of these observations, they suggest that these allelic variations in the hVDR gene may be used to predict bone density, which determines the risk of bone fracture in adulthood. Though they raised a possibility that the differential sequences of the 3′ untranslated regions (3′UTRs) derived from the allelic difference in the hVDR gene may vary the half-life of the transcript (32), the molecular basis of how the allelic variance affects the vitamin D signaling pathway remains unclear.

Thus, because of the wide spectrum of vitamin D effects and the range of metabolic 1,25$(OH)_2D_3$ derivatives that modulate these actions (see reference 51), we speculated that there are other types (subtypes or isoforms) of VDR protein that also affect the actions of vitamin D. By screening various cDNA libraries with fragments of cDNAs encoding rat VDR, we identified one rat VDR isoform generated by retention of intron 8 of the canonical VDR (rVDR0) during alternative splicing. This VDR isoform (rVDR1) directed by the stop codon in the newly identified exon (intron 8 for rVDR0) lacks

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86 amino acids of the ligand binding domain at the C-terminal end of the canonical VDR (rVDR0) but has another 19 amino acids. Recombinant rVDR1 showed no ligand binding activity, bound as a homodimer to VDRE, and formed a heterodimeric complex with rVDR0 but not with RXR. A transient-expression assay showed that rVDR1 acted as a dominant negative receptor against the ligand-induced transactivation of rVDR0, but this dominant negative activity on VDREs was sequence specific. Thus, our results provided the first evidence that a functionally distinct isoform is generated by retention of the intron for the canonical receptor of the nuclear receptor superfamily. Furthermore, the present study implies that this novel isoform negatively modulates the vitamin D signaling pathway, through a particular set of target genes.

MATERIALS AND METHODS

cDNA and genomic DNA cloning. The *Xho*I-*Pst*I restriction fragment including the ligand binding domain of rVDR0 (43) was radiolabelled and used as probe to screen a rat kidney cDNA library (Clontech). The hybridization mixture contained 50% formamide, 1× Denhardt's solution, 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]), 100 μg of denatured salmon sperm DNA per ml, and 10^6 cpm of ^{32}P-labelled probe DNA. Duplicate nitrocellulose membranes were hybridized for 16 h at 42°C and washed twice at 55°C for 30 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS). The membranes were exposed for 16 h at −80°C by using an intensifying screen. From 10^6 plaques, 10 positive clones were analyzed by PCR. Two clones encoding rVDR1 were further analyzed.

A *Sma*I-*Kpn*I fragment of rVDR1 cDNA was used as a probe to clone a genomic fragment of about 20 kb with the *Bam*HI sites from a rat genomic library (Clontech). The 3.5-kb DNA fragment with *Pst*I sites containing intron 8 was subcloned and completely sequenced.

Plasmid construction. The chloramphenicol acetyltransferase (CAT) reporter genes were constructed by inserting synthetic oligonucleotides with *Hin*dIII and *Xba*I sites into the corresponding sites of pGCAT (18, 19). The oligonucleotide sequences used for both the CAT reporter gene and the gel shift assays as probes (5, 11, 18, 19, 43) were as follows: DR3G, 5′-AGCTTCAGGTCAGGAAGGT CAGT-3′; DR3T, 5′-AGCTTCAGTTCGGAAGTTCAGT-3′; DR4, 5′-AGCT TAGGTCACGGAAGGTCAGT-3′; DR5, 5′-AGCTTCAGGTCACCGGAAG GTCAGT-3′; human osteocalcin (OC) VDRE, 5′-AGCTTGCTCGGGTAGG GGTGACTCACCGGGTGAACGGGGGCATCTCGACTCGT-3′; mouse osteopontin (OPN) VDRE, 5′-AGCTTGCTCGGGTAGGGTTCACGAGGTTC ACTCGACTCGT-3′. The rVDR1 expression vector was constructed by replacing the *Sac*I-*Bam*HI rVDR0 fragment with the PCR-amplified rVDR1 fragment (from 911 to 1071 bp in Fig. 1).

RNA isolation and Northern blots. Total RNA was extracted from rat tissues and total embryos by using acid guanidinium thiocyanate-phenol-chloroform. Poly(A)$^+$ RNA (3 μg) was electrophoresed through 1% agarose–1.1 M formamide gels and transferred to nitrocellulose filters (Schleicher and Schuell BAS85) (28, 43). The DNA fragment specific for intron 8 of the rat VDR gene was labelled with [^{32}P]dCTP by random priming. Hybridization was carried out at 42 to 45°C for 12 h in 5× SSPE (1× SSPE is 0.15 M NaCl–10 mM NaH_2PO_4–1 mM EDTA [pH 7.0])–50% formamide–0.2 mg of denatured salmon sperm DNA per ml–1× Denhardt's reagent. The most stringent wash was at 65°C in 0.1× SSPE containing 1.0% SDS and 0.03% sodium PP_i. The membranes were prehybridized by a 30-min incubation in 0.1× SSPE–0.1% SDS at 90°C, followed by hybridization with the rat β-actin probe to estimate the relative amount of rat RNA in each tube (28). Autoradiography was usually performed over two different periods of exposure at −80°C with an intensifying screen. The amounts of transcript were quantified by densitometric analysis of the autoradiograms using the β-actin transcript as the internal control. The relative abundance of transcripts was calculated for three different rats.

Reverse transcription PCR. The procedure of semiquantitative reverse transcription PCR was the same as that reported elsewhere (28, 37). The 633-bp PCR product specific for rVDR1 cDNA (see also Fig. 1) was generated by using a pair of oligonucleotides designated a and b (nucleotides 1483 to 1502 and 2097 to 2116 in the rVDR1 transcript) by 30 cycles of PCR (one cycle was at 93°C for 1 min, 53°C for 1 min, and 72°C for 3 min). The 458-bp PCR product specific for rVDR0 and 1,590-bp PCR product specific for rVDR1 were generated by using a pair of PCR primers designated 6 and 9 (nucleotides 605 to 624 in exon 6 and 1044 to 1063 in exon 9 of the VDR0 open reading frame by 30 cycles of PCR (one cycle was at 93°C for 1 min, 53°C for 1 min, and 72°C for 3 min). The 267-bp PCR product common for the rVDR0 and rVDR1 transcripts was generated by using a pair of primers designated 2 and 3 (nucleotides 1 to 20 in exon 2 and 248 to 267 in exon 3 of the VDR open reading frame by 30 cycles of PCR. The specific PCR products were confirmed by Southern blot analysis with the specific cDNAs.

Expression and purification of recombinant VDR proteins. The cDNAs encoding rVDR0 and rVDR1 were amplified by PCR with *Bam*HI and *Eco*RI sites and inserted into the corresponding sites of pGEX-2T (Pharmacia). *Escherichia coli* cells (DH5α) were transformed and induced with isopropyl-β-D-thiogalactopyranoside IPTG (0.1 mM). The glutathione *S*-transferase (GST) fusion proteins were purified by glutathione Sepharose 4B according to the manufacturer's specifications. Five hundred micrograms of each GST-fused protein was digested with thrombin (5U) and then sequentially passed through the glutathione Sepharose to remove thrombin and GST. The flowthrough fraction was further applied on a Sephadex G-200 column equilibrated with a 50 mM Tris-HCl buffer (pH 8.0) containing 1 M NaCl, 1 mM dithiothreitol, and 10% glycerol) to purify the VDR proteins. The purity of these proteins was over 95% in SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Cell transfection and CAT assay. HeLa cells were maintained in Dulbecco's modified Eagle's medium without phenol red, supplemented with 5% dextran-coated charcoal-stripped fetal calf serum. The cells were transfected at 40 to 50% confluence in 9-cm petri dishes with a total of 20 μg of DNA by using calcium phosphate. Two micrograms of a CAT reporter plasmid (DR3G, DR3T, DR4, DR5, OC-VDRE, or OPN-VDRE) was transfected with 500 ng each of receptor expression vectors (unless stated otherwise). All assays were performed in the presence of 3 μg of pCH110 (Pharmacia), a β-galactosidase expression vector used as internal control to normalize for variations in transfection efficiency. Bluescribe M13+ (Stratagene) was used as a carrier to adjust the total amount of DNA. Cognate ligands (all-*trans* retinoic acid at 1 μM and thyroid hormone and vitamin D at 0.1 μM) were added to the medium 1 h after transfection and at each exchange of medium. After a 20-h incubation with the calcium phosphate-precipitated DNA, the cells were washed with fresh medium and incubated for an additional 20 to 24 h. Cell extracts were prepared by freeze-thawing and assayed for CAT after normalization for β-galactosidase activity as described elsewhere (18, 19, 43).

Gel retardation. Electrophoretic mobility shift assays and antibody supershifts were performed as described elsewhere (18, 19, 43). Rat VDR0 and rVDR1 were expressed in *E. coli* as GST fusion proteins and purified by digestion with thrombin followed by a chromatography affinity column. Digested samples were applied to Sephadex G-100 to further purify the rVDR0 and rVDR1 proteins. The following purified receptors (6) were also used for this assay: RAR, a partially purified mouse RARα lacking the AB region (mRARαΔAB) synthesized in *E. coli*; RXR, a partially purified mouse RXRα lacking the region AB (mRXRαΔAB) synthesized in *E. coli*; and TR, a partially purified chicken TRα synthesized in *E. coli*. The monoclonal antibody 4RX (for RXR) was used for antibody supershifts. The binding reaction mixtures containing the receptors, the ^{32}P-5′-end-labelled synthetic oligonucleotide (DR3T, DR3G, DR4, or DR5), and poly(dI-dC) (Pharmacia; 2 μg) were incubated at 25°C for 15 min in binding buffer (10 mM Tris-HCl [pH 7.5], 1 mM dithiothreitol, 1 mM EDTA, 100 mM KCl, 10% glycerol). The antibody was added when the incubation was started. The complexes were resolved on 5% polyacrylamide gels as described elsewhere (18, 19, 43). The reduced DNA binding of rVDR0/RXR by rVDR1 was expressed as an average for three independent experiments (see Fig. 6E).

Assay of ligand binding to rVDRs. All procedures were performed essentially as described elsewhere (43). Increasing amounts of unlabelled 1,25$(OH)_2D_3$ with [^{3}H]1,25$(OH)_2D_3$ (13 Ci/mmol) (Amersham) dissolved in ethanol were dried under N_2 gas in the bottoms of tubes; then, 4 μl of the VDR solution and 21 μl of TENM buffer (20 mM Tris-HCl [pH 7.3], 1 mM EDTA, 50 mM NaCl, 2 mM 2-mercaptoethanol) were added. After a 16-h incubation at 4°C, 50 μl of stop solution (0.5% Norit A, 0.05% dextran T-70, 10 mM Tris-HCl [pH 7.5]) was added; then, the reaction mixture was centrifuged for 5 min at 10,000 × *g* at 4°C. Specific binding was calculated from the radioactivity in the supernatant by using a liquid scintillation counter.

RESULTS

Retention of intron 8 for the canonical VDR (rVDR0) generates a novel VDR isoform (rVDR1). VDR forms a subfamily including all-*trans* retinoic acid receptors (RARs), 9-*cis* retinoic acid receptors (RXRs), and TRs. As RAR, RXR, and TR have multiple subtypes and isoforms, we investigated whether there are isoforms and/or subtypes of VDR. We screened cDNA libraries from various murine tissues, using DNA fragments encoding various regions of the canonical rat VDR (designated rVDR0) and oligonucleotides of the P box in the DNA binding domain (for reviews, see references 2, 10, 13, 39, 49). Using the ligand binding domain of rVDR0 (4, 43), we isolated 10 positive clones closely related to rVDR0 cDNA. These clones had heterogeneous 5′ and 3′ UTRs (unpublished results), indicating the presence of unknown exons located in upstream and downstream regions of the VDR gene. We analyzed these clones by means of PCR with pairs of primers corresponding to the exons encoding rVDR0 protein (4) and found two clones encoding an identical isoform (designated

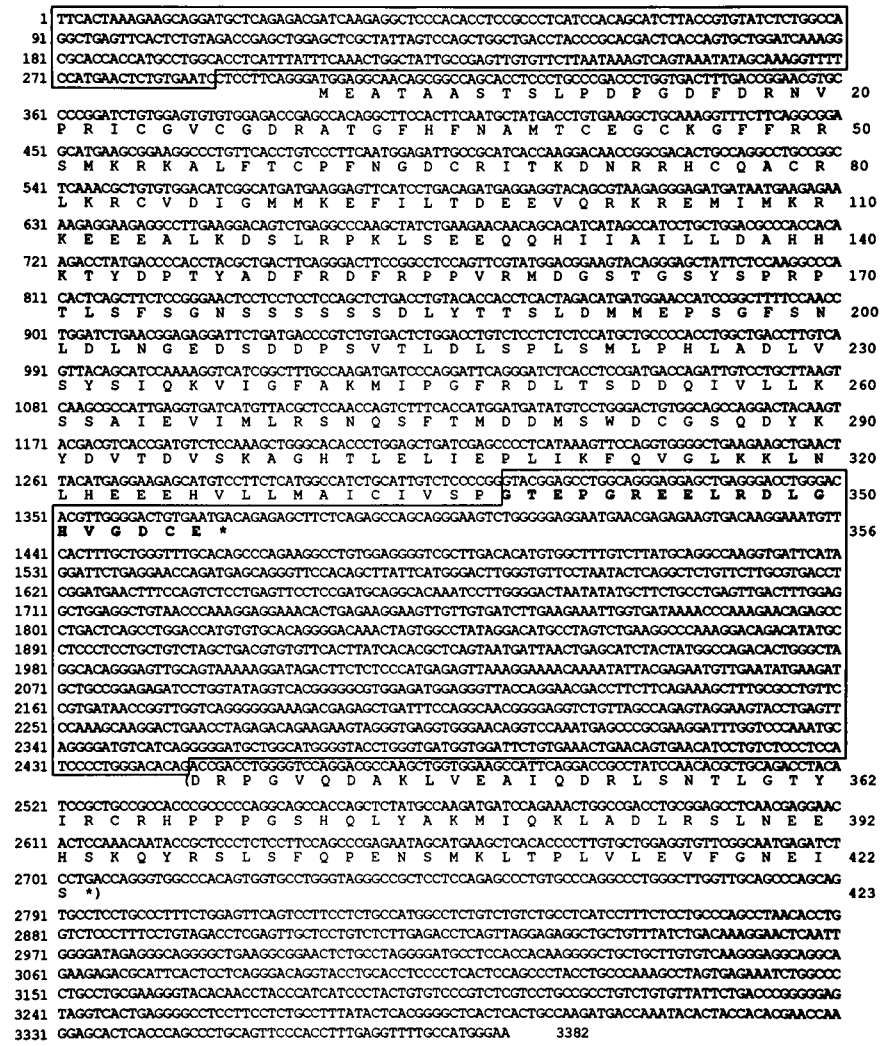

FIG. 1. Nucleotide and deduced amino acid sequences of cDNAs of rVDR0 and rVDR1 isolated from a rat kidney cDNA library. The 5′UTR and inserted sequences specific for rVDR1 are boxed, and the newly identified amino acid sequence of rVDR1 is boldfaced. The 11-bp sequence of the 5′UTR is common for rVDR0 and rVDR1.

rVDR1). The sequences of the 83-nucleotide 5′UTR specific for rVDR0 and 285 nucleotides for rVDR1 were identified (Fig. 1). A 1,134-bp fragment is inserted into the ligand binding domain of rVDR0, though the other sequence of rVDR1 is identical on the open reading frame of rVDR0 (Fig. 1).

Analysis of the genomic structure and sequence of the rVDR gene demonstrated that this inserted sequence is derived from the entire intron between exons 8 and 9 (Fig. 2) and that the sequences of the exon-intron junction match the general rule, suggesting that this isoform is generated from the primary transcript by alternative splicing. The retention of intron 8 as an exon for rVDR1 in the gene locus generates a relatively large exon located at the most downstream region of the gene locus. This gene organization of rat VDR rather resembles that of the other members of this receptor superfamily, which often harbor a large exon (see reference 14).

We used this exon of rVDR1 as a specific probe in Northern blots to detect the rVDR1 transcript. We found this transcript in the kidney and intestine, where rVDR0 is expressed (Fig. 3A), though no specific transcript was detected by a nonspecific probe consisting of 1,042 bp of the other intron between exons 6 and 7 (data not shown). The amounts of the rVDR1 transcript seemed to be 1/15 to 1/20 of that of rVDR0 according to densitometric analysis of the specific bands. Reverse transcription was required for PCR with poly(A)$^+$ mRNA from various tissues (37) to detect the rVDR1 transcript in the cytosolic mRNA fraction in the kidney (Fig. 3B), confirming the presence of the rVDR1 transcript in adult rats. It is notable that an rVDR protein with a lower molecular weight has been found in the rat kidney (20). This may represent the rVDR1 protein. These findings suggest that the rVDR1 transcript is localized in the cytosol as mature mRNA for translation. However, in 11.5- and 15.5-day embryos, expression of rVDR1 was not detected by reverse transcription PCR when the sets of the PCR primers corresponding to intron 8 (a-b in Fig. 3B) and exons 6 and 9 (6-9 in Fig. 3B) were used. Under this condition, the amplified cDNA fragments for rVDR1 were detected in the rat kidney and intestine (Fig. 3B). Thus, the expression of the rVDR1 transcript seems developmental stage specific.

rVDR1 acts as a dominant negative isoform. The putative amino acid sequence of rVDR1 lacks 86 amino acids at the C-terminal end but contains additional 19 amino acids dictated

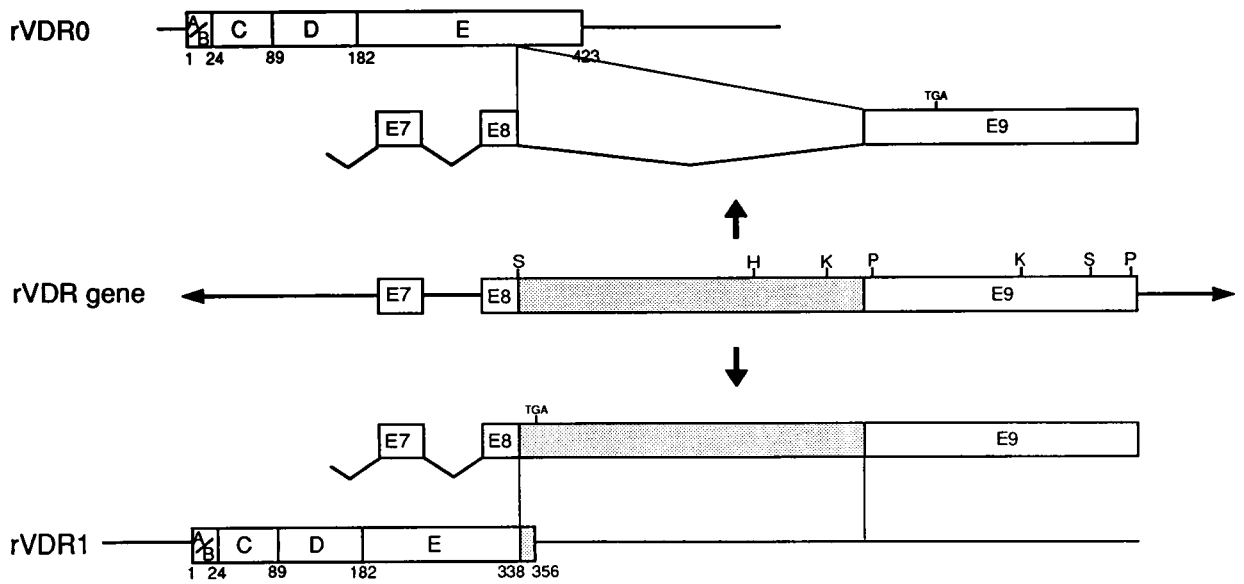

FIG. 2. A map of the rat VDR genomic region of interest and two rVDR isoforms generated by alternative splicing. The rat VDR genomic region around exons 7 to 9 (rVDR gene) and the putative protein structures of rVDR0 and rVDR1 are shown. The rVDR A to E regions are schematically represented, with the numbers of amino acid residues indicated. The sites for restriction enzymes in the rat VDR genomic region are abbreviated as follows: S, *Sma*I; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I.

by a stop codon located in the 1,134-bp sequence (Fig. 1 and 2). The recent report that the precise location of the ligand binding domain at the C-terminal end of hVDR was mapped from 403 to 427 amino acid residues (399 to 423 in rVDR0) (1, 35) suggests that rVDR1 has an impaired ligand binding activity. Although one (heptad 9, 399 to 407 amino acids in rVDR0) of the two domains required for heterodimerization with RXR is lacking, another region (heptad 4, 321 to 328 amino acids in rVDR0) responsible for specific DNA binding is present in rVDR1. We assessed the transactivation function of rVDR1 by means of a transient-expression assay (CAT assay) (18, 19). We used vectors expressing rVDR0, rVDR1, and rat RXRα and the CAT reporter plasmid containing a consensus VDRE consisting of two directly repeated AGTTCA motifs with a 3-bp spacer (DR3T). This is reportedly a stronger binding motif for VDR than AGGTCA (11, 48). We found that rVDR1 itself had no transactivation activity but rather inhibited the ligand-induced transactivation by rVDR0 in all cells tested (representative results in HeLa cells are shown) when the expression vectors for rVDR0 (0.5 μg) and rVDR1 (2 μg)

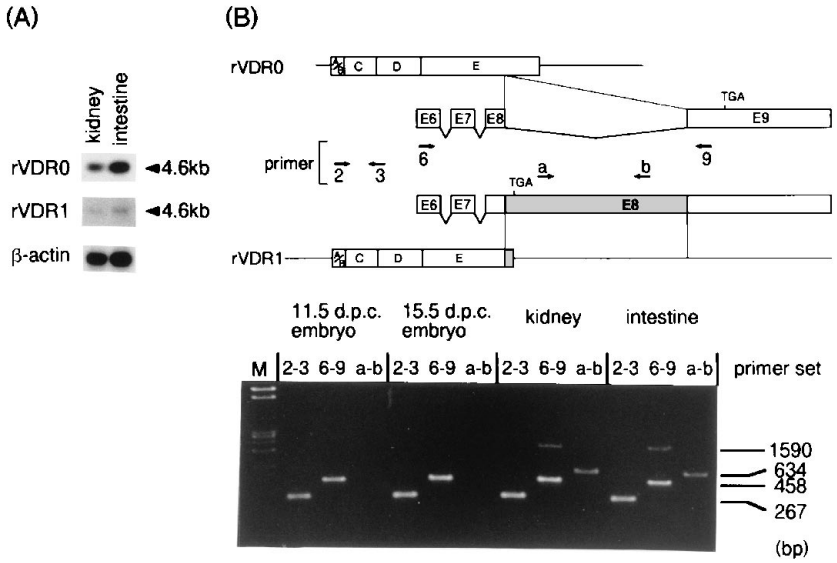

FIG. 3. Expression of the rVDR1 transcript in the intestine and kidney but not in embryos. (A) The expression of rVDR0 and rVDR1 transcripts was analyzed by Northern blotting with 3 μg of poly(A)$^+$ mRNA from the rat intestine and kidney. Representative results of Northern blots are shown. The relative abundance of rVDR transcripts was calculated from more than three samples by densitometric scanning of specific bands. (B) The rVDR1 transcript was not detected in 11.5- and 15.5-day postcoitum embryos. Expression of rVDR0 and rVDR1 transcripts was analyzed by reverse transcription PCR with 3 μg of poly(A)$^+$ mRNA from the rat adult tissues and embryos as described in Materials and Methods. The structures of the rVDR0 and rVDR1 transcripts and the PCR primers are shown at the top. Representative results are shown below.

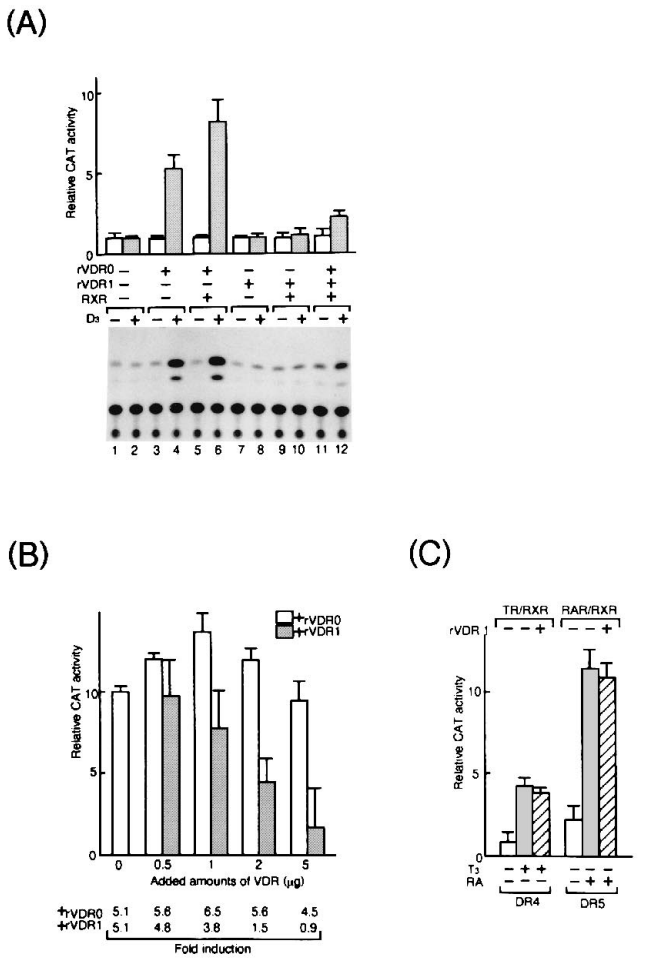

FIG. 4. rVDR1 acts as a dominant negative isoform in the vitamin D signaling pathway. (A) Dominant negative activity of rVDR1 against rVDR0. HeLa cells were transfected with a CAT reporter plasmid containing two directly repeated 5′-AGTTCA motifs separated by 3 bp (DR3T) and vectors expressing mouse RXRα (0.5 μg), rVDR0 (0.5 μg), and rVDR1 (2 μg). The transfected cells were maintained for 44 h in the absence (−) or presence (+) of $1,25(OH)_2D_3$ (10 nM), and CAT activities were normalized relative to the β-galactosidase activities expressed by the pCH110 internal control vector and are reported as means + standard deviations, calculated from the values of at least three independent experiments. Representative results of a CAT assay are shown below. (B) Dose-dependent activity of the dominant negative rVDR1. The cells were transfected with 0.5 μg of the rVDR0 expression vector and the VDR expression vector (either rVDR0 or rVDR1) at the indicated amounts in the presence of $1,25(OH)_2D_3$ (10 nM). CAT activity was calculated as described in above. (C) The thyroid hormone and retinoic acid signaling pathways were not inhibited by rVDR1. The cells were transfected with the CAT reporter plasmids containing two 5′-AGGTCA direct-repeat motifs of 4 bp (DR4 is a consensus thyroid hormone response element) or 5 bp (DR5 is a consensus retinoic acid response element) and vectors (0.5 μg each) expressing mouse RXRα with chicken TRα (for DR4) or RXRα with mouse RARα (for DR5) in the absence (−) or presence (+) of the cognate ligands (10 nM thyroid hormone [T_3] and 1 μM all-*trans* retinoic acid). The effect of rVDR1 was tested with cotransfection of the rVDR1 expression vector (2 μg). CAT activities were calculated as described above.

were cotransfected (Fig. 4A). The inhibitory activity of rVDR1 was more pronounced when increased amounts of the rVDR1 expression vector were added in the presence of rVDR0 (0.5 μg of the expression vector) (Fig. 4B). This inhibition was not simply due to squelching between the two isoforms for limiting nuclear factors (31, 47), since even when 5 μg of the rVDR0 expression vector was added, ligand-induced transactivation of rVDR0 was not suppressed (Fig. 4B). There were no apparent differences in these studies among the three RXR subtypes and no significant effect of 9-*cis* retinoic acid on the dominant negative activity of rVDR1 (data not shown).

The dominant negative activity of rVDR1 is sequence specific. The degree of rVDR1 inhibition against rVDR0 was sequence specific, and it was more prominent upon the mouse osteopontin than the human osteocalcin VDRE, possibly because of the binding preference of the VDR homodimers to target VDREs (7, 11, 48) (see Fig. 6B). The notion that the AGTTCA motif is a better binding core site for VDR than AGGTCA is further supported by the fact that DR3T was more active as a VDRE than DR3G (Fig. 5). On the other hand, rVDR1 did not suppress ligand-induced transactivation on the consensus response elements for retinoic acids (DR5) and thyroid hormone (DR4) in the presence of the cognate receptors (Fig. 4C). There was no apparent effect of rVDR1 upon the other retinoic acid response elements (DR1 and DR2) and estrogen response elements (consensus ERE) in the presence of the cognate receptors (data not shown). Thus, these results suggest that this rVDR1 isoform acts as a dominant negative receptor against rVDR0.

rVDR1 binds as a homodimer to VDRE and forms a heterodimeric complex with rVDR0 but not with RXR. To explore the molecular mechanism of the dominant negative activity of rVDR1, we examined its binding to the consensus VDRE (DR3T) and compared it with that of rVDR0. We bacterially produced rVDR0 and rVDR1 proteins. The open reading frames of rVDR0 and rVDR1 cDNAs predict proteins of 48 and 40 kDa, respectively (Fig. 1). The purified recombinant rVDR0 and rVDR1 proteins migrated with the predicted molecular weights on an SDS-polyacrylamide gel (Fig. 6A). As expected, rVDR1 showed no ligand binding activity (Fig. 7). The purified recombinant rVDR0 protein bound DR3T as a homodimer without RXR as described elsewhere (7, 11, 48) (Fig. 6B). The rVDR1 homodimer also bound DR3T to a similar extent. Adding mouse RXRα to rVDR0 caused a drastic increase in DNA binding by heterodimerization, and a specific monoclonal antibody for RXRα shifted up this binding complex (6, 23, 43), confirming the presence of RXR in this complex. However, rVDR1 could not form a heterodimer with RXRα, probably because it lacks the domain at the C-terminal end responsible for heterodimerization, like the C-terminally truncated mutant of hVDR (35). There was no specific binding

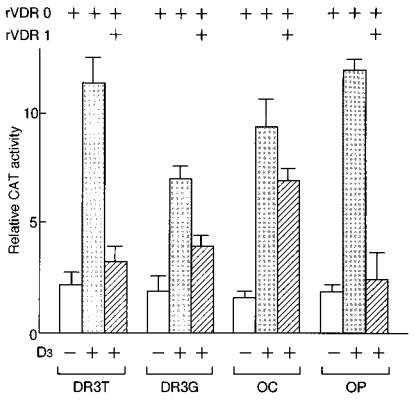

FIG. 5. The dominant negative activity of rVDR1 is sequence specific. HeLa cells were transfected with the CAT reporter plasmids (2 μg) containing DR3G, DR3T, human osteocalcin VDRE (OC), or mouse osteopontin VDRE (OP) together with the expression vectors (0.5 μg each) for RXR and VDRs in the absence (−) or presence (+) of $1,25(OH)_2D_3$ (10 nM). CAT activities were calculated as described for Fig. 4A.

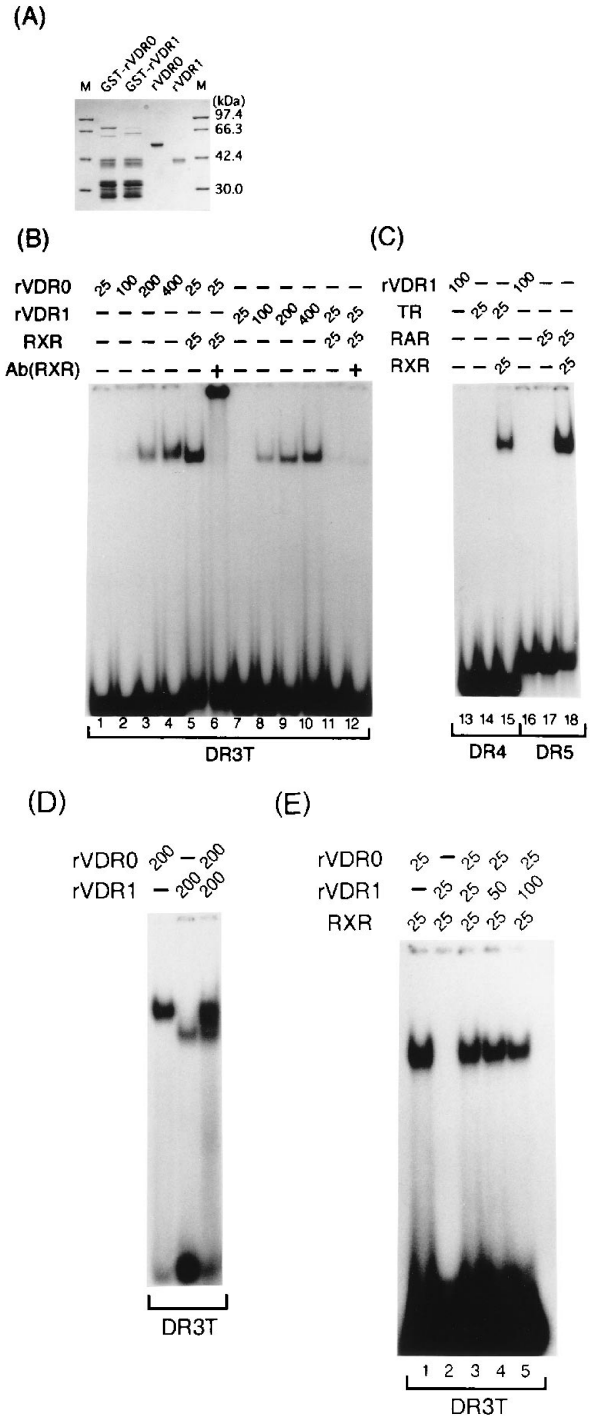

FIG. 6. DNA binding of homodimeric rVDR1, but not its heterodimer with RXR, to VDRE. (A) Expression and purification of rVDR0 and rVDR1 produced in *E. coli* as GST fusion proteins. The GST-fused VDR proteins expressed in *E. coli* were purified by glutathione Sepharose 4B following enzyme digestion. The GST-fused rVDRs (GST-rVDR0 and GST-VDR1) and purified rVDR proteins (rVDR0 and rVDR1) were analyzed by SDS–10% PAGE. Lanes M, molecular weight markers. (B) The rVDR1 homodimer binds to a consensus VDRE (DR3T). The gel shift assay was performed with DR3T as a probe and the indicated amounts of purified mouse RXRα (RXR) and purified rVDR0 and rVDR1 as shown in panel A. The nature of the DR3T-RXR-VDR complexes was verified by supershift studies using a monoclonal antibody (Ab) (4RX for mouse RXRα). (C) Lack of binding of the rVDR1 homodimer to consensus thyroid hormone or retinoic acid response elements (DR4 and DR5). The gel shift assay was performed with DR4 and DR5 as probes and the indicated amounts of purified rVDR1, chicken TRα (TR), and mouse RARα (RAR). (D) Heterodimeric formation of rVDR1 with rVDR0. The gel shift assay was performed with longer electrophoresis with DR3T as a probe and the purified rVDR0 and rVDR1 proteins. (E) DNA binding of rVDR0/RXR is suppressed in the presence of rVDR1. The gel shift assay was performed with DR3T as a probe and the purified rVDRs and RXR.

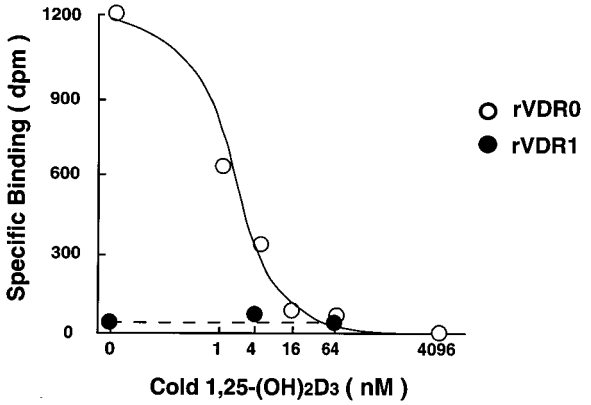

FIG. 7. Lack of $1,25(OH)_2D_3$ binding activity in rVDR0. The purified rVDR proteins were incubated overnight with 1 nM [^{3}H]$1,25(OH)_2D_3$ in the absence or presence of various concentrations of unlabelled $1,25(OH)_2D_3$ at 4°C. Unbound vitamin D_3 was removed by centrifugation, and the receptor-bound radioactivity was measured with a liquid scintillation counter. Each point represents an average of duplicate samples in the absence of unlabelled vitamin D_3. Only one representative result is shown for three independent experiments.

of rVDR1 to the consensus thyroid and retinoic acid response elements (DR4 and DR5, respectively) (Fig. 6C), in agreement with the previous observations that dimerization interfaces formed between the DNA binding domains specify binding recognition by the receptor dimers of the cognate response element in VDR as well as RAR, RXR, and TR (26, 40, 41, 48, 52). Moreover, no binding to such DR elements was observed in the presence of both rVDR0 and rVDR1 (data not shown). Taken together, these results suggest that the specificity of rVDR1 to target enhancer elements is identical with that of rVDR0. To test this possibility, we examined heterodimeric formation between rVDR0 and rVDR1. At the high concentrations of rVDR0 and rVDR1 required for DNA binding as homodimer, a heterodimeric formation of rVDR0 and rVDR1 was observed (Fig. 6D). Moreover, the effect of rVDR1 on the DNA binding of the rVDR0/RXR heterodimer was examined to explore the dominant negative activity of rVDR1. As shown in Fig. 6E, the DNA binding of RXR/rVDR0 was suppressed in the presence of rVDR1 (a reduction of about 15% at 50 ng of rVDR1 and about 30% at 100 ng). Note that this suppression is not simply due to competitive DNA binding between the rVDR1 homodimer and the rVDR0/RXR heterodimer, since at this condition, clear DNA binding was not found in the rVDR1 homodimer. Considering the fact that rVDR1 does not form a heterodimer with RXR, it seems that the heterodimeric formation of rVDR1 with rVDR0 prevents rVDR0 from heterodimerization with RXR to some extent.

Thus, taken together with the results of transactivation assay, these findings indicate that rVDR1 acts as a dominant negative receptor in the vitamin D signaling pathway, by competitively binding as a homodimer to the target VDRE.

DISCUSSION

Here we identified a novel isoform of rat VDR (rVDR1). Like the transcript of canonical VDR (rVDR0), that of rVDR1

seems to be generated from the primary rVDR transcript(s) by alternative splicing, but it harbors an additional exon (the retained intron 8 of rVDR0) (Fig. 1 and 2). The stop codon in this new exon (intron 8 of rVDR0) truncates a part of the ligand domain (86 amino acids) at the C-terminal end but adds 19 amino acids (Fig. 1). From the structural and functional similarities, VDR forms a subfamily with RAR, RXR, and TR within a nuclear receptor superfamily. However, VDR seems unique in this subfamily, since the isoform proteins of RAR, RXR, and TR are composed of diverse exons combined by alternative splicing and/or differential promoter usage (16, 24, 52). Though it has been already found in some genes that the retention of the intron as an exon generates a functionally distinct isoform protein (36), this is the first example of a receptor isoform in the nuclear receptor gene superfamily. For this reason, unlike TR, RAR, and RXR, the VDR isoform might have been missed for several years after its cDNA cloning (1, 4). Furthermore, the present study raises the notion that by intron retention a functionally distinct isoform protein of VDR, as well as of other nuclear receptors, may be differentially produced in a spatiotemporal manner. In fact, expression of the rVDR1 transcript was observed only in adult tissues and not in embryos (Fig. 3).

Transient-expression assay showed that rVDR1 acts as a dominant negative isoform against rVDR0, but interestingly, this activity of rVDR1 differed among VDREs (Fig. 4 and 5). However, this dominant negative activity was specific for VDR, since the ligand-induced transactivation of RXR-TR and RXR/RAR was not suppressed in the presence of rVDR1 (Fig. 4C). A gel shift assay demonstrated that rVDR1 binds as homodimer to VDRE and forms a heterodimeric complex with rVDR0 but not with RXR (Fig. 6). In agreement with the results of ligand-induced-transactivation assay (Fig. 4C), no binding complex with rVDR1 was observed in the consensus elements for thyroid hormone and retinoic acid (DR4 and DR5) (Fig. 6C) or any other tested response elements for retinoic acid and estrogen (data not shown). Taken together with the fact that the binding specificity of the nuclear receptor for its cognate response element is dictated by the DNA binding domain in VDR as well as RAR, RXR, and TR (26, 40, 41, 48, 52), it is likely that the binding specificities of rVDR0 and rVDR1 for VDRE are identical. VDR has a binding preference for the two AGTTCA motifs with a 3-bp spacer (DR3T) rather than for the two AGGTCA motifs (DR3G) (11, 48), though the latter seems to be the most potent binding sites for RAR, RXR, and TR, as well as many other nuclear receptors. We therefore speculate that the VDRE composed of an AGT TCA motif is more preferable for rVDR1 binding than that composed of AGGTCA. In fact, the transactivation function of rVDR0 and the dominant negative activity of rVDR1 were more prominent on DR3T than DR3G (Fig. 5). Thus, the difference in the dominant negative activity of rVDR1 to the rVDR0/RXR heterodimer between two naturally occurring VDREs (Fig. 5) may be due to the binding preference of the rVDR1 homodimer to the VDREs. In addition, the rVDR0/rVDR1 heterodimer may prevent rVDR0 from forming the heterodimer with RXR (Fig. 6D and E). These observations imply that the dominant negative isoform effectively suppresses a particular set of vitamin D target genes.

As only rVDR0, and not rVDR1, forms a strong heterodimeric complex with RXR (12), the RXR content in the cells may modulate the dominant negative activity of rVDR1, especially on the VDRE for which the RXR/VDR heterodimer has a higher binding preference than the VDR homodimer (Fig. 6B). Though no functional difference among RXRα, -β, and -γ was evident by in vitro analysis, tissue-specific roles of each RXR subtype have been suggested from analyses of gene-targeted mice deficient in RXR (16, 25, 46). Moreover, the three RXR genes are differentially expressed (28). Thus, tissue-specific actions of vitamin D mediated through VDR isoforms may be positively and negatively modulated by the presence as well as the level of RXRs.

Hereditary $1,25(OH)_2D_3$-resistant rickets (HVDRR) is a rare autosomal recessive disease characterized by target organ resistance to the action of $1,25(OH)_2D_3$. The molecular basis of HVDRR is genetic mutations in hVDR (15, 21, 27). In agreement with the results of the functional analysis of VDR in vitro (35), heterogeneity of the defect derived from the mutations in the functional domains has been found in HVDDR. Mutations in the DNA binding domain of hVDR had normal ligand binding, but no ligand-induced transactivation, because of the inability of the mutated receptor to bind VDREs (15). Truncation of more than half of the C-terminal end of VDR (the ligand binding domain) directed by ochre nonsense mutations also causes HVDRR (27), demonstrating that the C-terminally truncated VDR impairs the function of ligand-induced transactivation in vivo. These observations support our findings on the dominant negative activity of rVDR1, which also lacks 86 amino acid residues of the C-terminal end of rVDR0. In contrast to the genetic mutations that exchange amino acid residues, it is known that these nonsense mutations produce a reduced-transcript phenotype and decreased levels of the encoded gene product (38, 44). These facts may explain in part the low level of the rVDR1 transcript in adult tissues.

Morrison et al. (32, 33) showed that allelic variations in the hVDR gene are significantly related to circulating osteocalcin and bone density. Bone density is assumed to determine the risk of osteoporotic fracture in adulthood (3, 29, 33, 45). To our surprise, they reported that this allelic variation of the hVDR gene predicting bone density maps to intron 8 (32), as we had found that rVDR1 is generated by retention of intron 8 of the rat VDR gene. Differential sequences of the 3′UTR reflecting the gene allelic variations have indicated that the half-life of the hVDR transcript is modulated by the 3′UTRs, since 3′UTR is well known to regulate the half-life of the mRNA (42). When intron 8 of the hVDR gene is retained as an exon during alternative splicing like the rVDR1 transcript, the hVDR gene also generates a C-terminally truncated isoform, which is presumed to act in a dominant negative manner, as a stop codon in the open reading frame is present in intron 8 (1). It is therefore of interest, for understanding the molecular basis of how the allelic variations in the hVDR gene are related to bone density, to know whether a dominant negative isoform like rVDR1 is generated by intron retention in humans.

Here we identified and characterized a dominant negative isoform of rat VDR. The altered levels of this isoform, the expression of which may be not only controlled by genetic variance but also induced through a pathophysiological process such as osteoporosis (9), may negatively modulate the vitamin D signaling pathway through a particular set of target genes.

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