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Cloning and expression of full-length cDNA encoding human vitamin D receptor

(1α,25-dihydroxyvitamin D_3)

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ABSTRACT **Complementary DNA clones encoding the human vitamin D receptor have been isolated from human intestine and T47D cell cDNA libraries. The nucleotide sequence of the 4605-base pair (bp) cDNA includes a noncoding leader sequence of 115 bp, a 1281-bp open reading frame, and 3209 bp of 3′ noncoding sequence. Two polyadenylylation signals, AATAAA, are present 25 and 70 bp upstream of the poly(A) tail, respectively. RNA blot hybridization indicates a single mRNA species of ≈4600 bp. Transfection of the cloned sequences into COS-1 cells results in the production of a single receptor species indistinguishable from the native receptor. Sequence comparisons demonstrate that the vitamin D receptor belongs to the steroid- receptor gene family and is closest in size and sequence to another member of this family, the thyroid hormone receptor.**

Vitamin D_3 receptors (VDR) mediate the action of their cognate ligand 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] by controlling the expression of hormone-sensitive genes (1). These receptor proteins are intracellular polypeptides of 50–60 kDa that specifically bind 1,25$(OH)_2D_3$ and interact with target-cell nuclei to produce a variety of biologic effects (2, 3). Recently, monoclonal antibodies (mAbs) have been used to recover cDNAs encoding a portion of the chicken VDR mRNA (4). The present study describes the cloning and characterization of cDNAs encoding the human vitamin D receptor (hVDR).¶ Analysis of the deduced amino acid sequence of hVDR demonstrates that this protein belongs to the superfamily of trans-acting transcriptional regulatory factors defined by the steroid and thyroid hormone receptors (5–9). These receptors are characterized by a highly conserved DNA-binding domain rich in cysteine, lysine, and arginine residues and a carboxyl-terminal hydrophobic ligand-binding domain. The primary sequence of the hVDR exhibits this characteristic domain structure and shows that the VDR is more closely related to the thyroid hormone receptor than to the other receptors. Expression of the cloned cDNA in COS-1 cells demonstrates that the single protein produced is indistinguishable from the native receptor in both physical properties and affinity for 1,25$(OH)_2D_3$.

MATERIALS AND METHODS

Identification of hVDR cDNAs. An oligo(dT)-primed λgt10 cDNA library (10) made from 5 μg of human jejunal poly(A)$^+$ mRNA was screened using a 310-base pair (bp) *Eco*RI–*Sau*3A fragment of cVDR (4). The filters were hybridized at 42°C overnight in the following solution: 5× SSC (1× SSC = 150 mM sodium chloride and 15 mM trisodium citrate)/5× Denhardt's solution (11)/0.1% sodium dodecyl sulfate/salmon sperm DNA at 200 μg·ml^{-1}/50% (wt/vol) formamide containing 1 × 10^6 cpm of nick-translated probe per ml (12). After being washed three times at 60°C in 0.5× SSC to remove excess probe, the filters were exposed to X-ray film (Kodak X-Omat S) at −70°C with an intensifying screen. Hybridization-positive phage were isolated, and their inserts were subcloned into the *Eco*RI site of M13mp8. λVDR1 was obtained in this fashion and subsequently used to screen an Okayama–Berg (13) T47D cDNA library (provided by G. Ringold, Stanford University), yielding clone VDR3, and a specifically primed λgt10 T47D library yielding clone λVDR2. The latter was made by substituting the oligonucleotide 5′ ACACACCCCACAGATCCGGGG 3′ for oligo(dT) in the first strand reaction (underlined in Fig. 2).

DNA Sequence Analysis. Three overlapping clones were used to generate the full-length VDR sequence cDNA inserts to be sequenced. These clones were subcloned into the *Eco*RI site of M13mp8 for sequencing by the dideoxynucleotide chain-termination method (14). Primers were either the M13 universal primer or sequence-derived oligonucleotides.

RNA Blot Hybridization. Total RNA was isolated from each of three cell lines (15), and the mRNA fraction was selected by successive passages over oligo(dT)-cellulose (16). The mRNA samples (10 μg) were resolved on a 1% formaldehyde–agarose gel (17) and then transferred electrophoretically to a nylon membrane (Nytran; Schleicher & Schuell). The filter was hybridized to nick-translated hVDR-1 (1 × 10^8 cpm/μg; 1 × 10^6 cpm/ml) using the conditions described above.

Expression of Recombinant hVDR in COS-1 Cells. Two cDNA clones, λVDR1 and VDR3, were joined at a common restriction site (*Stu* I) and cloned into the expression vector P91023B (18) recreating the full-length coding sequence for hVDR. COS-1 cells were transfected with 12 μg of expression plasmid DNA per 100-mm plate using DEAE-dextran (19). After 48 hr, cytosols from 2 × 10^7 cells were prepared by Dounce homogenization in 10 mM Tris·HCl, pH 7.6/0.3 M KCl/5 mM dithiothreitol (designated TK0.3D buffer). Cytosols were diluted to 4 × 10^5 cell equivalents per ml, and 0.1-ml aliquots were incubated with increasing concentrations of 1α,25-dihydroxy[26,27-*methyl*-^{3}H]D_3 {1,25$(OH)_2$[^{3}H]D_3} (176 Ci/mmol, Amersham; 1 Ci = 37 GBq) with or without a 100-fold molar excess of unlabeled 1,25$(OH)_2D_3$. After incu-

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Abbreviations: VDR, vitamin D_3 receptors; 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; hVDR, human VDR; 1,25$(OH)_2$[^{3}H]D_3, 1α,25$(OH)_2$[26,27-*methyl*-^{3}H]D_3; mAb, monoclonal antibody.

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¶The sequence reported in this paper is being deposited in the EMBL/GenBank data base (Intelligenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03258).

bation at 4°C for 20 hr, specific binding was determined by hydroxyapatite binding assay (20). Similarly, other D metabolites (25-hydroxyvitamin D_3, 24,25-dihydroxyvitamin D_3, 1α-hydroxyvitamin D_3, and 1,24,25-trihydroxyvitamin D_3, courtesy of M. Uskokovic, Hoffmann–La Roche) were used to compete for $1,25(OH)_2D_3$ in this assay to determine the binding preference for expressed VDR.

Sucrose Gradient Sedimentation. COS-1 cell cytosols were prepared 48 hr posttransfection, as described above, and then incubated for 4 hr at 4°C with 1 nM $1,25(OH)_2D_3$ with or without a mAb to cVDR (mAb 9A7γ). A parallel incubation was done without the mAb but with a 100-fold molar excess of unlabeled $1,25(OH)_2D_3$. Samples were layered onto 4.6-ml gradients of 10–30% sucrose prepared in TK0.3D buffer and centrifuged at 265,000 × *g* for 18 hr at 4°C. Gradients were fractionated from the top and quantitated in ACS scintillation cocktail (Amersham).

RESULTS

Identification of VDR cDNA Clones. Two mAbs were used to isolate a putative cVDR cDNA clone from a chick intestinal λgt11 cDNA expression library (as reported in ref. 4). The deduced amino acid sequence of this original clone contained a cysteine-, lysine-, and arginine-rich domain. This, together with hybrid-selected translation data and the pattern of mRNA regulation, provided preliminary evidence that this clone encoded the chicken VDR. We have used this 310-bp fragment of cVDR cDNA to isolate the corresponding full-length human cDNA.

An oligo(dT)-primed λgt10 cDNA library was constructed from 5 μg of human intestinal (jejunum) poly(A)$^+$ mRNA. A single hybridizing clone (clone λVDR1) was obtained from this library of 500,000 primary recombinants. After being subcloned into M13mp8, the sequence of the 1100-bp fragment was determined by the dideoxynucleotide chain-termination method of Sanger (14). The 5′ 576 bp of the insert cloned in λVDR1 is highly similar to the corresponding chicken sequence, but thereafter the two sequences completely diverge, and there is no longer an open reading frame in the sequence from clone λVDR1. The presence of a consensus splice sequence at the point of divergence suggests that clone λVDR1 sequence might represent a partially spliced mRNA. Therefore, the λVDR1 sequence was used to screen an Okayama–Berg cDNA library made from T47D cell mRNA (a human breast cancer cell line). Two positive clones, out of 2×10^6 primary recombinants, were obtained and characterized by restriction mapping. The largest of these, clone VDR3, contains an insert of 4.3 kb that overlaps with clone λVDR1 to give a 4.5-kb cDNA sequence, close to the predicted size of hVDR mRNA from RNA analysis (Fig. 1). The encoded open reading frame begins after the *Eco*RI linker sequences and extends for 427 amino acids until a TAG stop codon. To confirm the sequences around the linker and to obtain further 5′ sequence, a specifically primed cDNA library was prepared from T47D mRNA using a 17-mer complementary to a region close to the 5′ end of λVDR1. Four independent clones isolated from this library confirmed and extended the 5′ terminal sequence. A restriction map of the composite cDNA clones is shown in Fig. 1.

Sequence of Human VDR mRNA and Protein. Fig. 2 shows the nucleotide and deduced amino acid sequence of the full-length human vitamin D receptor mRNA. The overlapping cDNA clones λVDR1, VDR3, and λVDR2 comprise 4605 bp and contain a noncoding leader sequence of 115 bp, a 1281-bp open reading frame, and 3209 bp of 3′ noncoding sequence. The functional significance of such a long 3′ untranslated region is unknown, although this is a common feature of all the steroid receptors cloned to date. The sequence AATAAA, thought to be required for polyadenylylation, is represented twice, 25 bp and 70 bp upstream from the poly(A) tract. This

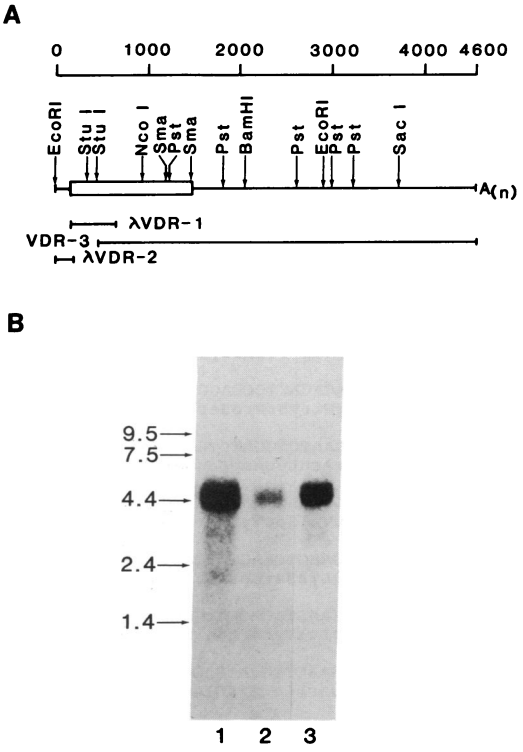

FIG. 1. (*A*) Restriction map of the overlapping hVDR cDNA clones. The boxed region indicates the coding sequence. (*B*) RNA blot hybridization analysis of hVDR transcripts from cell line T47D (lane 1), human intestine ATCC cell line 407 (lane 2), and cell line HL-60 (lane 3) poly(A)$^+$ mRNAs.

is consistent with the RNA data (Fig. 1) in which the predominant mRNA species is ≈4.6 kilobases (kb).

The predicted hVDR translation product is 427 amino acids long with a calculated M_r of 48,295; this agrees well with published data (21, 22) if post-translational modifications are considered. For example, the VDR is known to be phosphorylated (23). Assignment of the initiating methionine was from the general observation that translation of most eukaryotic mRNAs begins at the 5′ proximal AUG (24). Definitive demonstration of this initiation site awaits amino-terminal amino acid sequencing of purified receptor protein.

Identification of the functional domains of hVDR may help establish the mechanisms involved in receptor-mediated transcriptional activation. Comparison of the coding sequence of hVDR with other steroid receptors (5–7, 9) and thyroid hormone receptor (8) shows significant regions of similarity (Fig. 3). The area of strongest identity (C1 in Fig. 3) is an ≈70-amino acid hydrophilic domain that is rich in cysteine, lysine, and arginine residues and lies towards the amino terminus of VDR. Importantly, the position of each of the nine cysteines is conserved among these different proteins, suggesting a common functional domain. This region has been proposed to mediate the DNA binding properties of the steroid receptors (25). Deletion analysis of the glucocorticoid receptor and estrogen receptor has confirmed that this C1 domain is involved in both DNA binding and transcriptional activation (26–28). Preliminary results show that this is also true for the vitamin D receptor (unpublished work). The arrangement of the conserved cysteines led Weinberger *et al.* (25) to suggest that this region can form a Zn^{2+}-dependent "finger" structure analogous to that proposed for the 5S transcription factor TF111A (29). No definitive evidence for this hypothesis exists at present. A second region of lesser conservation, which we have termed C2, lies towards the carboxyl terminus of the VDR protein and is separated from

FIG. 2. Nucleotide and deduced amino acid sequence of full-length hVDR cDNA. Both sequences are numbered with respect to the putative initiating methionine. The oligonucleotide used to generate the specifically primed cDNA library is underlined.

the DNA binding domain by an intervening nonconserved "hinge" of ≈150 amino acids. This domain represents the approximate amino-terminal limits of the ligand-binding domain, based on mutational analysis of glucocorticoid and estrogen receptors (26–28). A third domain, C3, which is further toward the carboxyl terminus of the molecule is quite similar to the equivalent region in the thyroid hormone receptor based on either amino acid- or nucleotide-sequence alignments (Fig. 3 *B* and *C*) but not similar to the other receptors. This suggests a structural and/or evolutionary relationship between these two receptors and between their respective ligands.

Expression of Functional VDR Receptor. To demonstrate that the cloned sequences can direct the synthesis of a functional receptor protein, two of the cDNAs (λVDR1 and VDR3) were joined at a common restriction site and cloned

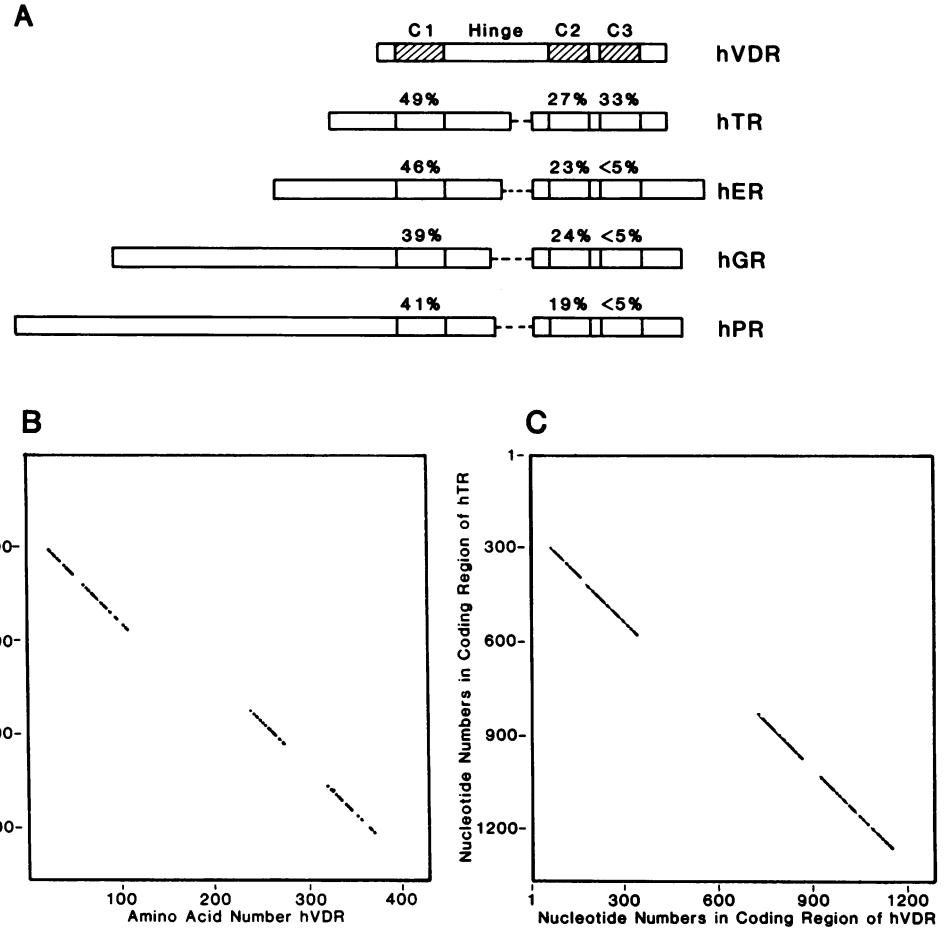

FIG. 3. (*A*) Schematic comparison between the deduced amino acid sequences of hVDR, human glucocorticoid (hGR), human estrogen (hER), human thyroid hormone (hTR), and human progesterone (hPR) receptors (5–9). Sequences were aligned for maximum similarity about their DNA binding (C1) or C2 domains. Percent similarities indicated are with respect to hVDR sequence. (*B* and *C*) Dot matrix plot of the regions of nucleotide- and amino acid-sequence similarity between hVDR and human thyroid hormone receptor.

into the expression vector P91023B in both orientations. This vector uses the adenovirus major late promoter and simian virus 40 origin of replication upstream of the cloned sequences with the mouse dihydrofolate reductase coding region and simian virus 40 polyadenylylation signal downstream (18). The final expression constructs, phVDR1 and phVDR2, contain 2.1 kb of hVDR sequence in either the correct or inverted orientation with respect to the promoter. These two plasmids were used to transfect COS-1 monkey kidney cells, and the resulting transient pools were analyzed for receptor expression.

A competitive binding assay was used to assess the hormone binding properties of the expressed protein. Cytosolic extracts were incubated with $1,25(OH)_2[^3H]D_3$ with or without a 100-fold molar excess of unlabeled steroid, and receptor–hormone complexes were measured using a hydroxylapatite binding assay (20). The phVDR1-transfected cells exhibit substantial $1,25(OH)_2D_3$-binding activity, which is saturable at 1 nM. Scatchard analysis (Fig. 4*A*) indicates the expression of ≈220,000 copies of hVDR per cell with a K_d of 5.2×10^{-11} M. The binding affinity of the steroid for the recombinant hVDR is identical to that reported for vitamin D receptors from a number of sources (3, 30), although the level of receptor expression is some 20-fold higher than that found in receptor-rich cell lines such as 3T6 (30) and T47D (31). The results in Fig. 4*B* illustrate that recombinant hVDR displays selectivity for $1,25(OH)_2D_3$ over such metabolites as 25-hydroxyvitamin D_3, 24(R),25-dihydroxyvitamin D_3, 1α,24(R),25-trihydroxyvitamin D_3, 1α-hydroxyvitamin D_3, and vitamin D_3. The resulting rank order of receptor preference for individual vitamin D metabolites is identical to that of wild-type receptor in all tissues and cells examined to date (31, 32).

Lastly, sedimentation of $1,25(OH)_2[^3H]D_3$-labeled extracts on 10–30% hypertonic sucrose gradients revealed a receptor species sedimenting at 3.2S. Preincubation with a mAb to cVDR shifted this peak to 7S (Fig. 4*C*); both peaks are completely displaceable with excess unlabeled $1,25(OH)_2D_3$. These results are consistent with those found for native hVDR (33).

DISCUSSION

In this paper we have presented the complete nucleotide sequence of the cDNA for hVDR. Expression of hVDR cDNA in COS-1 cells confirms that the protein encoded by the cDNA represents the authentic receptor on the following criteria. (*i*) The protein binds $1,25(OH)_2D_3$ with the same affinity as does the native receptor. (*ii*) The expressed protein exhibits the same rank order binding preferences for other vitamin D metabolites. (*iii*) It sediments on sucrose gradients at 3.2S and displays the characteristic shift to 7S with receptor antibody.

$1,25(OH)_2D_3$ is thought to act via this receptor to effect changes in gene expression, much like the steroid receptors. However, this has been difficult to confirm because the VDR is very low in abundance and few VDR-regulated genes have been identified. The nucleotide sequence data and the deduced amino acid sequence show that considerable similarity exists among VDR, the steroid hormone receptors, and the thyroid hormone receptor. In particular, a cysteine-, lysine-, and arginine-rich domain is ≈40% conserved between VDR, thyroid receptor, and the steroid receptors. This domain seems capable of forming a Zn^{2+}-dependent "finger" and probably harbors both DNA binding and transcriptional activities of the receptors. The presence of this common structural motif in the VDR argues that the mechanism of vitamin D action truly parallels that of the other hormone systems. Furthermore, it has been suggested that VDR and thyroid hormone receptor are closely related biochemically, based

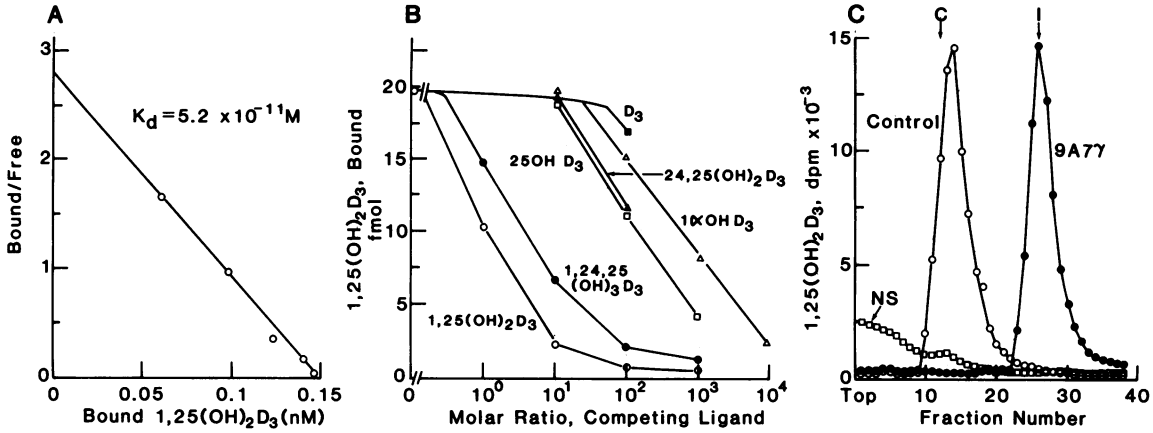

FIG. 4. Expression of hVDR cDNA in transiently transfected COS-1 cells. (*A*) Saturation analysis of recombinant hVDR in phVDR1-transfected COS-1 cells. The Scatchard analysis shows that the expressed receptor has a K_d of 5.2×10^{-11} M and a B_{max} of 2.2×10^5 sites per cell. (*B*) Vitamin D_3 metabolite specificity of hVDR. Transfected COS-1 cell cytosol was incubated with 1,25(OH)$_2$[^{3}H]D$_3$ and the indicated molar concentrations of 25-hydroxy-, 24,25-dihydroxy-, 1α-hydroxy-, 1α,24,25-trihydroxy-vitamin D_3, or vitamin D_3 for 18 hr at 4°C followed by assay using hydroxyapatite (18). (*C*) Sedimentation analysis of recombinant hVDR. Cytosol was prepared 48 hr after transfection and incubated for 4 hr at 4°C with 1,25(OH)$_2$[^{3}H]D$_3$ (1 nM) with (●) or without (○) 9A7 cVDR mAb. A similar incubation was done with 1,25(OH)$_2$[^{3}H]D$_3$, but this was done with a 100-fold molar excess of 1,25(OH)$_2$D$_3$ and without the mAb (□). C, cytosol with labeled D_3; I, cytosol plus mAb with labeled D_3; NS, cytosol with excess unlabeled D_3.

largely on their strong nuclear binding characteristics (34). The overall sequence similarities presented here support a close evolutionary relationship between these two receptors.

Steroid receptors interact with enhancer-like sequences usually found in the 5′-flanking DNA of regulated genes (35). However, little is known about the sequences involved in the presumed genomic action of the VDR. The availability of cloned receptor sequences will expedite studies of vitamin D-responsive regulatory elements. In addition, expression of truncated receptors (unpublished work) and examination of aberrant receptors found in vitamin D-dependent rickets type II (36) will help to define functional domains of the receptor molecule.

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