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Isolation and expression of rat 1,25-dihydroxyvitamin D_3 receptor cDNA

(vitamin D function/calcium metabolism/bone/intestine/steroid receptors)

JAMES K. BURMESTER*, NOBUYO MAEDA†, AND HECTOR F. DELUCA*

Departments of *Biochemistry and †Genetics, University of Wisconsin–Madison, Madison, WI 53706

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ABSTRACT **The cDNA for the 1,25-dihydroxyvitamin D_3 receptor has been isolated by screening a rat kidney λgt11 expression library with a mixture of three previously identified anti-receptor monoclonal antibodies. Protein produced from this cDNA reacts with each of the three monoclonal antibodies but does not react with an antibody specific for the porcine receptor or an irrelevant antibody. In addition, the expressed protein specifically binds 1,25-dihydroxyvitamin D_3 but does not bind estradiol, progesterone, testosterone, cortisol, or aldosterone. The nucleotide sequence for this cDNA has been determined and used to deduce the amino acid sequence of the protein. This amino acid sequence contains a region that matches perfectly the partial amino acid sequence determined for the receptor isolated from porcine intestine. This amino acid sequence also closely matches the reported amino acid sequence of the putative DNA-binding finger of the avian 1,25-dihydroxyvitamin D_3 receptor.**

1,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], the most active metabolite of vitamin D, is required for maintaining calcium homeostasis (1). In the intestine, 1,25-$(OH)_2D_3$ stimulates calcium absorption. Working with parathyroid hormone, 1,25-$(OH)_2D_3$ causes calcium reabsorption in the kidney and stimulates the mobilization of calcium from bone.

The actions of 1,25-$(OH)_2D_3$ are mediated through its receptor. This protein binds 1,25-$(OH)_2D_3$ with high affinity and low capacity (2). Following this interaction, the activated receptor is believed to bind DNA, altering gene expression. The absence of a functional receptor results in the disease vitamin D-dependent rickets type II (3, 4).

Studies of the 1,25-$(OH)_2D_3$ receptor have involved the tracking of a trace protein in a crude mixture. Initial analysis of the receptor involved radiolabeled hormone–receptor binding techniques (2). More recently, monoclonal antibodies against the chicken receptor (5, 6) and the porcine receptor (7) have been isolated. These have been used to study regulation of the intestinal receptor during rat development (8), to immunoprecipitate the receptor from among *in vitro* translation products (9), and to identify the receptor via immunoblot analysis during its purification to homogeneity (10). In addition, the avian receptor has been cloned, providing a limited amino acid sequence (11). Yet, the large quantities of receptor necessary for detailed physical and biochemical studies are not available.

We have isolated and sequenced a cDNA that clearly encodes a major portion of the 1,25-$(OH)_2D_3$ receptor. This cDNA when expressed in *Escherichia coli* produces a protein that specifically binds 1,25-$(OH)_2D_3$. This paper reports this and the deduced partial amino acid sequence of the receptor protein.‡

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MATERIALS AND METHODS

Chemicals. 1,25-$(OH)_2$[26,27-^{3}H]D_3 was prepared as described (12). Nonradioactive 1,25-$(OH)_2D_3$ was a gift from Hoffmann–La Roche. Progesterone, testosterone, estradiol, hydrocortisone, aldosterone, and isopropyl β-D-thiogalactopyranoside (IPTG) were purchased from Sigma. Goat anti-mouse IgG alkaline phosphatase-conjugated antibody and the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color development substrates were obtained from Promega Biotec (Madison, WI).

Buffers and Solutions. Tris-buffered saline/Tween 20 (TBST) for screening the expression library was composed of 10 mM Tris·HCl, pH 8.0/150 mM NaCl/0.05% Tween 20. For color development, alkaline phosphatase buffer contained 100 mM Tris·HCl (pH 9.5), 100 mM NaCl, and 5 mM $MgCl_2$. The color development reaction was stopped by rinsing several times with water. Phage dilution buffer consisted of 10 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$. TED was 50 mM Tris·HCl, pH 7.4/1.5 mM EDTA/5 mM dithiothreitol. $TEDK_{300}$ was TED plus 300 mM KCl. TED/Triton was TED plus 0.5% (vol/vol) Triton X-100.

Screening the Expression Library. The rat kidney λgt11 cDNA expression library was purchased from Clontech (Palo Alto, CA) and was screened by the procedure of Young and Davis (13). Briefly, phage were plated at 1×10^5 plaque-forming units per plate (150 × 15 mm). After a 3.5-hr incubation at 42°C, the plate was overlaid with a nitrocellulose filter saturated with IPTG and incubated at 37°C for 3.5 hr. After this incubation, the nitrocellulose filter was removed, rinsed once with TBST, and then blocked for 30 min at room temperature with TBST/5% nonfat dry milk. The blocked filter was incubated with anti-receptor antibody for 30 min at room temperature, rinsed three times with TBST, and then incubated for 30 min at room temperature with alkaline phosphatase-conjugated goat anti-mouse IgG. After washing, color was developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate diluted in alkaline phosphatase buffer. Positive plaques were further purified by the same procedure.

Isolation of *E. coli* Lysogens. *E. coli* lysogens carrying the receptor cDNA insert and the chicken ovalbumin cDNA insert (from Clontech) were isolated for use in determining the steroid-binding specificity of recombinant receptor. *E. coli* strain Y1089 hfl was infected with the appropriate phage at a ratio of ≈5 phage per *E. coli* cell. Individual *E. coli* colonies were picked and replica-plated at 32°C and 42°C. Colonies that grew at 32°C but not at 42°C carried a lysogenic phage.

Abbreviations: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; IPTG, isopropyl β-D-thiogalactopyranoside.

‡The sequence reported in this paper is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03630).

Preparation of Fusion Protein Cell Extracts. The putative receptor lysogen and the ovalbumin lysogen were used to inoculate their respective cultures. When the cultures had grown to an OD_{600} of 0.5, IPTG was added to 10 mM final concentration. After 60 min further incubation, the cells were harvested by centrifugation at 5000 × *g* for 5 min. They were then resuspended in $TEDK_{300}$ buffer and immediately frozen on dry ice. Before use, the cells were thawed, sonicated, and the cellular debris was removed by centrifugation. The resulting extract was used for demonstrating steroid-binding and DNA-binding activity.

Hydroxylapatite Binding Assay. The extract (16.1 mg/ml) produced from the receptor lysogen was diluted 1:4 with TED buffer, and 2 nM 1,25-$(OH)_2$[26,27-^{3}H]D_3 or 2 nM 1,25-$(OH)_2$[26,27-^{3}H]D_3 and 200 nM nonradioactive competitor hormone was incubated with the diluted extract for 3 hr at 4°C. After this incubation, hydroxylapatite was incubated with the labeled extract for 15 min with Vortex mixing every 5 min. The hydroxylapatite was centrifuged and the pellet was washed two times with TED/Triton. The pellets were transferred with ethanol to scintillation vials and the radioactivity was counted. The ovalbumin cell extract was treated identically.

DNA Cellulose-Binding Assay. The extract produced from the receptor lysogen was diluted 1:4 with TED buffer. This extract was then incubated with either 2 nM 1,25-$(OH)_2$[26,27-^{3}H]D_3 or 2 nM 1,25-$(OH)_2$[26,27-^{3}H]D_3 and 200 nM 1,25-$(OH)_2D_3$ for 2 hr at 4°C. DNA cellulose, prepared according to the method of Litman (14), was added to the labeled extracts and a 1-hr incubation at 4°C was done. After this incubation, the mixture was centrifuged and the precipitate was washed twice with TED/Triton. The pellets were transferred with ethanol to scintillation vials and the radioactivity was counted. Receptor isolated from porcine intestine according to the procedure of Dame *et al.* (7) was tested by using the same procedure as a positive control. Additional samples were assayed for hormone binding by the hydroxylapatite procedure.

Sequencing of the cDNA Inserts. Phage were plated on host *E. coli* cells and grown at 37°C until the plate was almost confluent. Phage dilution buffer was then added to the plate and the plate was incubated at 4°C overnight. The phage dilution buffer was collected, and phage that had diffused into the medium were precipitated by the addition of PEG 6000 and sodium chloride to 10% and 1 M final respective concentrations. The precipitation was done at 4°C for 1 hr. Phage were then centrifuged at 8000 × *g* for 20 min and the supernatant was discarded. The pellet was resuspended in phage dilution buffer and extracted once with an equal volume of chloroform to remove PEG 6000. The supernatant was saved and $NaDodSO_4$ and EDTA were added to 0.5% and 50 mM respective final concentrations. After heating to 68°C for 20 min, proteins were removed by phenol/chloroform extraction and the phage DNA was precipitated.

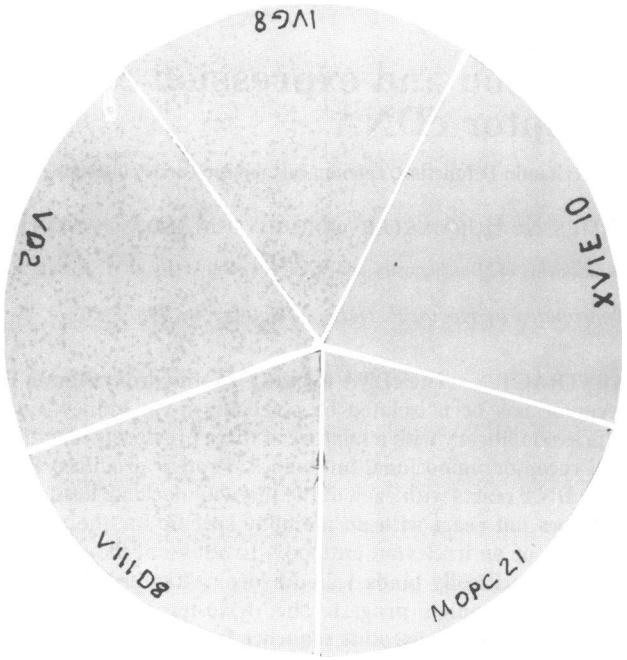

FIG. 1. Expression of 1,25-$(OH)_2D_3$ receptor epitopes by the λgt11 clone. *E. coli* cell line Y1090 was infected by the recombinant phage, plated on an agar plate, and grown for 3.5 hr at 42°C. The plate was then overlaid with a nitrocellulose filter saturated with IPTG and incubated an additional 3.5 hr at 37°C. This filter was removed, blocked with nonspecific protein, and then slices were incubated with their respective monoclonal antibody or the control antibody MOPC21. Positive plaques were detected by a goat anti-mouse alkaline phosphatase-conjugated second antibody. Antibodies IVG8, VD2, and VIIID8 recognize distinct epitopes of the receptor. Antibody XVIE10 reacts only with 1,25-$(OH)_2D_3$ receptor from pig. MOPC21 is the control antibody.

Table 1. Specific binding of 1,25-$(OH)_2$[26,27-^{3}H]D_3 by recombinant fusion protein

Ligand	dpm ± SD	Specific binding, dpm	% specific binding
	Induced receptor lysogen		
1,25-[26,27-^{3}H]	57,804 ± 2133	50,601*	100
1,25-[26,27-^{3}H] + 1,25-$(OH)_2D_3$	7,203 ± 540	—	—
1,25-[26,27-^{3}H] + progesterone	54,235 ± 3834	47,032	93
1,25-[26,27-^{3}H] + estradiol	54,706 ± 3010	47,503	94
1,25-[26,27-^{3}H] + testosterone	51,999 ± 2374	44,796	89
1,25-[26,27-^{3}H] + cortisol	56,884 ± 2352	49,681	98
1,25-[26,27-^{3}H] + aldosterone	54,301 ± 2579	47,098	93
	Induced ovalbumin control		
1,25-[26,27-^{3}H]	4,256 ± 382	0	0
1,25-[26,27-^{3}H] + 1,25-$(OH)_2D_3$	4,414 ± 931		

Steroid binding of the recombinant receptor–β-galactosidase fusion protein. An *E. coli* lysogen carrying the receptor was isolated by picking colonies that grow at 32°C but not at 42°C. After receptor–β-galactosidase fusion protein induction by IPTG for 1 hr, the cells were collected and resuspended in $TEDK_{300}$. An extract was prepared by sonicating the cells and centrifuging out the debris. Steroid binding was assessed by labeling with 2 nM 1,25-$(OH)_2$[26,27-^{3}H]D_3 (1,25-[26,27-^{3}H]) or 2 nM 1,25-$(OH)_2$[26,27-^{3}H]D_3 and 200 nM nonlabeled competitor. Hydroxylapatite was used to recover the labeled receptor.

*Approximately 173 fmol per mg of protein.

Table 2. Precipitation of 1,25-$(OH)_2$[26,27-^{3}H]D_3 bound to porcine receptor or recombinant protein by DNA cellulose

Precipitant	1,25-$(OH)_2D_3$	PNE*	Recombinant protein†
Hydroxylapatite	−	60,625 ± 2346	58,247 ± 1903
	+	5,257 ± 165	5,526 ± 469
DNA-cellulose	−	33,174 ± 585	451 ± 53
	+	1,550 ± 132	827 ± 11

An extract was prepared from the *E. coli*/recombinant phage lysogen that was induced with IPTG. This extract was labeled with 2 nM 1,25-$(OH)_2$[26,27-^{3}H]D_3 or 2 nM 1,25-$(OH)_2$[26,27-^{3}H]D_3 and nonradioactive 1,25-$(OH)_2D_3$ for 2 hr. DNA-cellulose was then incubated with the appropriate mixtures for 1 hr at 4°C. After washing, the radioactivity in the pellets was determined. Parallel samples of pig nuclear extract were tested as a positive control. Samples were assayed for hormone binding using hydroxylapatite.
*Pig nuclear extract, 1 mg of protein per ml.
†Recombinant receptor, 3 mg of protein per ml.

Phage DNA was isolated and then digested with *Eco*RI to release the cDNA inserts. The digested DNA was then mixed with dephosphorylated *Eco*RI-cut pUC-18 vector DNA. The mixed DNA was ligated together and then used to transform competent JM109 *E. coli* cells. Sequencing of the DNA was done by the method of Maxam and Gilbert (15). All sequences were determined from at least two different restriction enzyme sites and were read from both DNA strands.

RESULTS

Isolation of cDNA Clones. Monoclonal antibodies recognizing four distinct epitopes of the porcine intestinal receptor have been generated (7). Of these antibodies, IVG8, VD2, and VIIID8 crossreact with receptor from other mammalian or avian species, whereas antibody XVIE10 shows no reactivity with receptor from any species other than pig. Therefore, a mixture of the antibodies IVG8, VD2, and VIIID8 was used to isolate the rat receptor cDNA.

The screening of a rat kidney cDNA λgt11 expression library with this mixture of antibodies produced five positive clones in the first round. Approximately 1.2×10^6 independent recombinants were screened. When each of these phage was purified and tested for which antibody recognized the protein produced, four of the five phage were positive with only VIIID8. The other phage produced protein containing the IVG8, VD2, and the VIIID8 epitopes but not the pig-only epitope recognized by the antibody XVIE10 (Fig. 1). This protein was not reactive with the irrelevant antibody MOPC21.

Steroid Binding and DNA Binding by the Fusion Protein. The receptor was first identified because of its high affinity and low capacity for binding 1,25-$(OH)_2D_3$ (2). Specific binding of hormone to the recombinant protein would provide undisputable proof that the receptor cDNA had been isolated.

To test for hormone binding by the recombinant protein, an *E. coli* lysogen carrying the receptor cDNA was isolated. After induction of the β-galactosidase fusion protein by IPTG, an *E. coli* cell extract was prepared. This extract was tested for specific binding of 1,25-$(OH)_2D_3$. A control extract prepared from an induced lysogen carrying the chicken ovalbumin cDNA insert was also tested for hormone binding.

The recombinant fusion protein specifically binds 1,25-$(OH)_2$[26,27-^{3}H]D_3 (Table 1). This binding can be readily displaced by an excess of nonradioactive 1,25-$(OH)_2D_3$ but is not significantly displaced by an excess of either proges-

ArgPheThrCysProPheAsnGlyAspCysArgIleThrLysAspAsnArgArgHisCys (I)
GlnAlaCysArgLeuLysArgCysValAspIleGlyMetMetLysGluPheIleLeuThr
AspGluGluValGlnArgLysArgGluMetIleMetLysArgLysGluGluGluAlaLeu
LysAspSerLeuArgProLysLeuSerGluGluGlnGlnHisIleIleAlaIleLeuLeu
AspAlaHisHisLysThrTyrAspProThrTyrAlaAspPheArgAspPheArgProPro
ValArgMetAspGlySerThrGlySerTyrSerProArgProThrLeuSerPheSerGly
AsnSerSerSerSerSerSerAspLeuTyrThrThrSerLeuAspMetMetGluProSer
GlyPheSerAsnLeuAspLeuAsnGlyGluAspSerAspAspProSerValThrLeuAsp
LeuSerProLeuSerMetLeuProHisLeuAlaAspLeuValSerTyrSerIleGlnLys
ValIleGlyPheAlaLysMetIleProGlyPheArgAspLeuThrSerAspAspGlnIle
ValLeuLeuLysSerSerAlaIleGluValIleMetLeuArgSerAsnGlnSerPheThr
MetAspAspMetSerTrpAspCysGlySerGlnAspTyrLysTyrAspValThrAspVal
SerLysAlaGlyHisThrLeuGluLeuIleGluProLeuIleLysPheGlnValGlyLeu
LysLysLeuAsnLeuHisGluGluGluHisValLeuLeuMetAlaIleCysIleValSer (II)
ProAspArgProGlyValGlnAspAlaLysLeuValGluAlaIleGlnAspArgLeuSer
AsnThrLeuGlnThrTyrIleArgCysArgHisProProProGlySerHisGlnLeuTyr
AlaLysMetIleGlnLysLeuAlaAspLeuArgSerLeuAsnGluGluHisSerLysGln
TyrArgSerLeuSerPheGlnProGluAsnSerMetLysLeuThrProLeuValLeuGlu
ValPheGlyAsnGluIleSer

FIG. 2. Amino acid sequence of receptor deduced from the nucleotide sequence. The cDNA insert from the recombinant phage was excised by digesting the DNA with *Eco*RI. Two fragments of 1784 and 761 base pairs were subcloned into pUC-18, where they were sequenced by the technique of Maxam and Gilbert (15). The amino acid sequence derived from the long open reading frame of the 1784-base-pair insert is shown. (I) The amino acids underlined are identical to the amino acids predicted from the chicken intestinal cDNA. (II) The amino acids underlined are identical to the amino acids determined by sequencing the porcine intestinal protein. The two amino acids marked by ··· were not obtainable by protein sequencing.

terone, estradiol, testosterone, cortisol, or aldosterone. The ovalbumin-producing extract did not bind 1,25-$(OH)_2$[26,27-^{3}H]D_3, ruling out the possibility that an *E. coli* protein other than the fusion protein could be responsible for the specific binding of hormone.

The binding of hormone to pig intestinal receptor results in a form that is capable of binding to DNA (7). We determined whether the recombinant protein is also capable of binding to DNA.

E. coli-produced fusion protein was incubated for 2 hr with hormone to allow possible activation reactions to occur. This protein was then incubated for 1 hr with DNA-cellulose and the amount of receptor bound to DNA was determined. A sample of pig nuclear extract was treated similarly as a positive control.

The porcine intestinal receptor clearly has an active DNA-binding domain. However, the receptor produced in *E. coli* did not have an active DNA-binding domain (Table 2). This is consistent with the sequence data presented below.

Amino Acid Sequence. DNA from the recombinant phage was isolated and then cut with *Eco*RI to excise the cDNA inserts. This produced two cDNA fragments of 1784 and 761 base pairs. Both of these fragments were subcloned into pUC-18 and sequenced. Neither sequence contained a poly(A) tail. Analysis of the cDNA sequences showed a long open reading frame spanning base pairs 1 to 1101 of the 1784-base-pair insert. This open reading frame did not have the initiation codon. The deduced sequence of the 367 amino acids nearest the carboxyl terminus and containing approximately one-half of the putative DNA-binding domain is shown in Fig. 2.

Comparison of this amino acid sequence to a partial amino acid sequence of receptor isolated from porcine intestine (T. Brown, J. Prahl, and H.F.D., unpublished data) shows that there is complete agreement between the sequences. Of the amino acids that have been compared, all are identical in these species.

Further analysis of the deduced amino acid sequence shows almost complete agreement with the previously published partial sequence of the receptor derived from the avian cDNA. In the compared region, 33 amino acids are identical. Two amino acids in this region have not been conserved (Fig. 2).

The computer program of Wolf *et al.* (16) predicts the hydrophobic and hydrophilic domains of the receptor (Fig. 3). The receptor has two large hydrophobic regions near the carboxyl terminus. The putative DNA-binding region is much less hydrophobic.

DISCUSSION

We have isolated a cDNA encoding a major section of the rat 1,25-$(OH)_2D_3$ receptor. Protein encoded by this cDNA contains epitopes reactive to the monoclonal antibodies IVG8, VD2, and VIIID8 but not to the pig-only antibody XVIE10 or the control antibody MOPC21 (7). Reactivity by three antibodies that recognize distinct epitopes of the 1,25-$(OH)_2D_3$ receptor provides strong preliminary evidence that the cDNA for the receptor has been isolated.

To confirm the authenticity of the cDNA, the cDNA was used to produce a β-galactosidase–receptor fusion protein in *E. coli* that was capable of binding 1,25-$(OH)_2D_3$. 1,25-$(OH)_2$[26,27-^{3}H]D_3 can be displaced with excess nonradioactive 1,25-$(OH)_2D_3$ but is not displaced by estrogen, progesterone, aldosterone, cortisol, or testosterone. The ability of this protein to bind steroid suggests that the steroid-binding domain does not appear to require any specific modifications to become active, or if it does, these modifications can occur in *E. coli*. The steroid-binding domain also must be localized in the carboxyl terminus of the protein, since the isolated cDNA does not contain the amino termi-

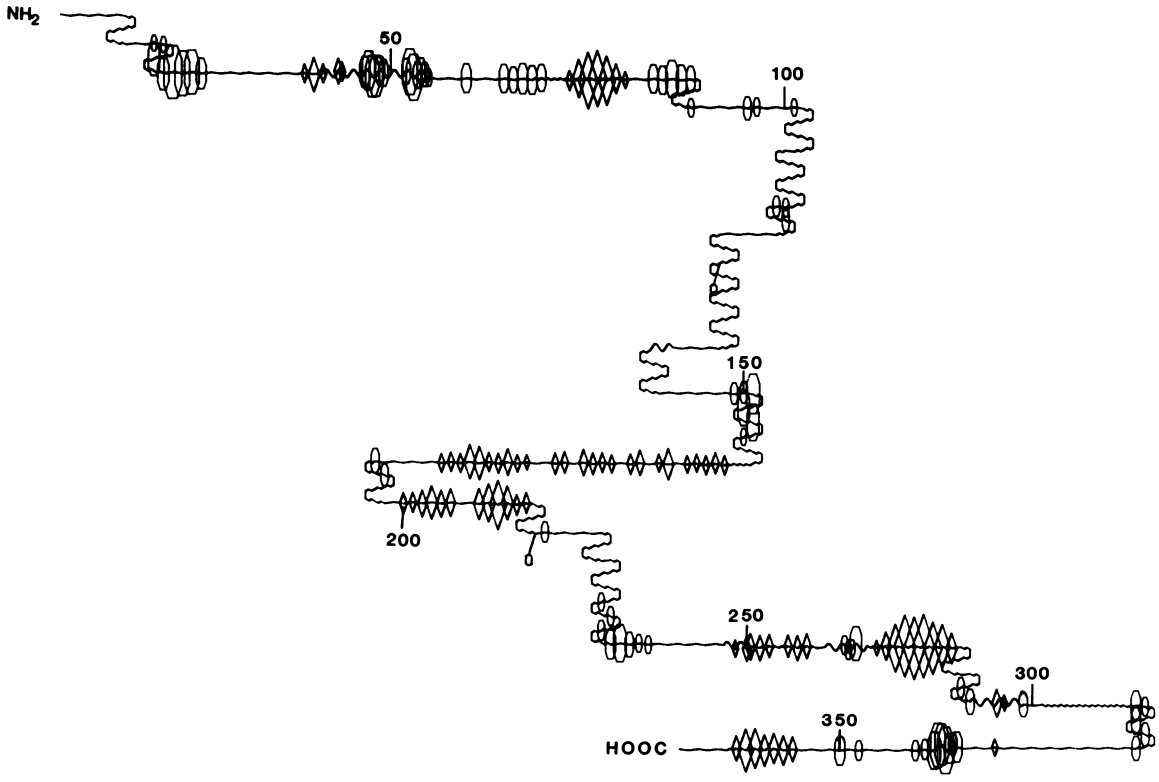

FIG. 3. Hydrophobicity plot of the rat 1,25-$(OH)_2D_3$ receptor. Hydrophobic regions of the receptor are represented by ◊ and hydrophilic regions are represented by ◯. The amino terminus is positioned at the top left. The numbers indicate the position of the corresponding amino acids.

nus. To our knowledge, this is the first report in which steroid-binding activity has been shown by an *E. coli*-synthesized steroid hormone receptor. Analysis of the predicted amino acid sequence shows that the carboxyl region is mainly hydrophobic. These amino acids probably form the hydrophobic pocket responsible for binding the hormone. Since the protein produced in *E. coli* is capable of binding steroid, it should be possible to produce large quantities of the active steroid-binding domain for structural analysis of the receptor's interaction with steroid.

The deduced amino acid sequence derived from the rat receptor cDNA is in complete agreement with 15 amino acids that have been determined unambiguously by sequencing purified porcine intestinal receptor. The deduced sequence is almost identical to the predicted sequence for the avian receptor putative DNA-binding domain. In a 35-amino acid region, only 2 amino acids have not been conserved between rat and chicken. In this region, the receptor contains the conserved cysteine residues that are thought to form a finger that could contact specific DNA sequences.

The receptor cDNA should prove to be an important tool in studying the mechanism of action of 1,25-$(OH)_2D_3$. It can be used to isolate a full-length cDNA and as a probe to investigate regulation of receptor expression and the nature of the receptor gene itself. When a full-length cDNA is obtained, it will be possible to predict the complete amino acid sequence of the 1,25-$(OH)_2D_3$ receptor. It will also be possible to produce large quantities of the receptor for physical and biochemical studies of the hormone- and DNA-binding sites.

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