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The avian vitamin D receptors: Primary structures and their origins

(steroid receptor cDNA/expression system/chorioallantoic membrane)

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ABSTRACT **cDNA clones encoding Japanese quail chorioallantoic membrane and chicken kidney 1,25-dihydroxyvitamin D_3 receptors were isolated and the total 448-amino acid (aa) sequence was deduced. The sequences of the chicken and quail receptors are identical. The nucleotide and deduced amino acid sequences of the avian receptors are similar but not identical to the reported rat or human receptor sequences. There is a 78% similarity in the nucleotide sequences and 98.5% and 87.5% similarities in the amino acid sequences of the DNA-binding and ligand-binding domains, respectively. Two avian receptor proteins (58 and 60 kDa) arise from a single mRNA transcript by alternate initiation of translation. The avian 1,25-dihydroxyvitamin D_3 receptors were produced using a bacterial expression system. Form A receptor was expressed from a cloned cDNA that contains the first translation signal (ATG) and corresponds with the 60-kDa avian receptor protein, and form B receptor was initiated from the third ATG on the same mRNA transcript to give rise to the 58-kDa protein. The cysteine-rich DNA-binding domain is almost conserved among human, rat, and avian receptors. The position of the nine cysteines was conserved in all three sequences. The avian receptor differs in the second zinc finger domain, where a methionine replaces a leucine, a serine replaces an asparagine, and a lysine replaces an arginine at aa 77, 83, and 87, respectively, of the avian sequence. The increased length of the avian receptor results from a 20-aa extension of the N-terminal region. RNA hybridization indicates there is a single mRNA species of ≈2700 bp for both the chicken and quail receptors compared to 4400 bp for the rat transcript. Surprisingly, the translated avian sequence is larger (448 aa) than the 423-aa rat receptor protein. Therefore, our results confirm that despite the difference in molecular mass between different receptor proteins, there is a similarity in gene organization such that the DNA-binding and hormone-binding domains are positionally conserved from the C terminus.**

Vitamin D_3 receptors (VDRs) mediate the action of their ligand 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], the hormonal form of vitamin D, by altering the expression of genes whose protein products influence calcium (Ca) homeostasis (1). An outstanding example of this mechanism is the chorioallantoic membrane (CAM) of the avian embryo where 1,25-$(OH)_2D_3$ causes the resorption of eggshell and the subsequent calcium transport to the embryo (2). Because of the controlling role of the VDR in the action of vitamin D, intense effort has been placed on the cloning of these receptors. Full coding sequences have been determined for rat (r) (3) and human (h) (4) VDRs, and the VDR is now known to be a member of the steroid/thyroid and retinoid family of receptors (5).

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The chicken (c) VDR apparently has the largest molecular mass of the VDR proteins (6). The cVDR exists as a doublet of 60 kDa and 58 kDa (7). Similarly analysis of the porcine, mouse, r-, monkey, and hVDRs showed them to be 55, 54.5, 54, 52, and 52 kDa, respectively (6). These results are consistent with the finding that mammalian VDRs sediment more rapidly during sucrose density gradient analysis (8). The existence of two proteins for the avian VDR is unique among the VDRs characterized to date. It was suggested that the 58-kDa polypeptide represents a proteolyzed form of the native 60-kDa cVDR (7). Another report from the same group (9), based on *in vitro* translation, suggested that the VDR protein is not synthesized as a large (≫60 kDa) parent molecule that is subsequently processed into a smaller protein. They concluded that the presence of the lower molecular mass protein could be a translation product of multiple transcripts, as is the case for glucocorticoid receptors or represents post-translationally modified species (9). Pike *et al.* (7) showed that both A and B forms of cVDR bind similarly to DNA and, thus, are not analogous in properties to the A and B polypeptides of the avian progesterone receptor (10). The considerable debate over the origin of the B form of the avian VDR and the suggestion that this 58-kDa form may arise by proteolysis of the larger A form receptor protein led us to isolate the full-length cDNA encoding the avian VDRs.* We present evidence to demonstrate that the A and B forms of the avian VDR are derived by alternate initiation of translation from two in-phase ATG initiation signals located on a single mRNA transcript.

MATERIALS AND METHODS

Identification of Japanese Quail (jq) VDR cDNAs. An oligo(dT)-primed and random-primed λgt11 cDNA library made from 5 μg of jqCAM poly(A)$^+$ mRNA was screened using 358- and 1783-bp fragments from the coding region of the rVDR (3). For the first round of screening, recombinant phages were plated at a density of 15,000 plaque-forming units per 150-mm plate on a lawn of *Escherichia coli* y1090. Lifted Hybond-N nylon filters were prehybridized for 12 h at 42°C in 50% (vol/vol) formamide/5× SSPE/5× Denhardt's solution/0.1% SDS containing denatured salmon sperm DNA (100 μg/ml) (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA). Nick-translated probe was added to the filters (1×10^6 cpm) (11). After hybridization for 30 h at 42°C, the filters were washed successively in 2× SSC/0.1% SDS at room temperature for 15 min, 1× SSC/0.1% SDS at 65°C for 1 h, and finally, if necessary, in 0.1× SSC/0.1% SDS at 65°C for 1 h. Filters were then exposed to

Abbreviations: VDR, vitamin D_3 receptor; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; CAM, chorioallantoic membrane; hVDR, human VDR; rVDR, rat VDR; cVDR, chicken VDR; jqVDR, Japanese quail VDR; PNE, pig nuclear extract.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. U12641).

Kodak X-Omat AR film at −70°C with an intensifying screen. Hybridization-positive phage were isolated and two subsequent rounds of purification were undertaken. Insert cDNAs were excised and subcloned into the *Eco*RI site of plasmid vector pUC18 (11).

DNA Sequence Analysis. Recombinant plasmid DNA prepared through polyethylene glycol precipitation (11) was sequenced in both directions by the dideoxynucleotide method with the Sequenase enzyme (United States Biochemical) using synthetic oligonucleotide primers. The resulting sequence data were compared with previously published sequences by using the facilities of the University of Wisconsin Genetics Computer Group (Madison) (12). The cloned jqVDR cDNA containing the open reading frame was used as a probe similarly to screen a chicken kidney library and resulted in the isolation and cloning of the cVDR cDNA. Northern blot analyses of rat, chicken, and quail mRNAs were carried out by electrophoresis using a 1% agarose gel containing 6.6% formaldehyde in 20 mM Mops (pH 7.0). The RNA was transferred to nylon membranes with 20× SSC overnight. mRNA (2–5 μg) was loaded in each lane. The filter was hybridized to nick-translated probes from r-, c-, or jqVDR cDNAs (1×10^5 cpm/ml) by using the conditions described above.

Amplification of Coding Sequences of jqVDR. We used the following primers to amplify the coding sequence of the avian VDR by PCR techniques (13). The 5′ oligonucleotides using the first, second, or third possible initiation codons were as follows, respectively: 5′-TAGTATAATGCCATATGGTCTCCATCTCTGC-3′, 5′-TAGTATAATGCCATATGCCCTGCTGCTGTGA-3′, and 5′-TAGTATAATGCCATATGGAGACACCTGCAGT-3′. The underlined sequence represents the *Nde* I restriction site. The 3′ oligonucleotide used was 5′-TAGCTACGTGGATCCTCAGGAGATCTCGTTGCCAAA-3′. The underlined sequence represents the *Bam*HI restriction site.

The jqCAM VDR full coding sequence in a pUC18 plasmid was linearized and amplified using only one of the 5′ oligonucleotides at the first, second, or third ATG in the sequence along with the 3′ oligonucleotide, *Taq* polymerase, and PCR buffer (50 mM KCl/10 mM Tris·HCl, pH 8.3/1.25 mM $MgCl_2$). The products were electroeluted, treated with *Nde* I and *Bam*HI restriction endonucleases, and ligated into the pET3a plasmid vector (Novagen) that had been cut with the same enzymes. *E. coli* JM109 cells were transformed (11) with the chimeric plasmid. After amplification of plasmid clones containing one of three insert sizes, the jqVDR pET-3a chimeric plasmids were used to transform *E. coli* BL21 (DE3/pLysS) cells (14).

Expression of jqVDR Protein in *E. coli* BL21 and Its Purification. Typically, 200 ml of M9ZB medium was used [10 g of N-Z-Amine A (ICN), 5 g of NaCl, 1 g of NH_4Cl, 3 g of KH_2PO_4, 6 g of Na_2HPO_4 in 1 liter of H_2O at pH 7.0 and sterilized by autoclave]. After cooling, M9ZB was brought to 0.02 M glucose and 1 mM $MgSO_4$, ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) were added, and the mixture was inoculated with BL21 (DE3/pLysS) cells that had been transformed with one of the three jqVDR-pET-3a constructs. The culture was grown at 37°C until an OD_{600} value of 0.6 was attained. The expression of the protein was initiated by adding isopropyl β-D-thiogalactoside to 0.4 mM. Incubation was continued at 37°C for another 6 h. Induction of the VDR protein was assayed by SDS/gel electrophoresis. The cells from each of the construct cultures of *E. coli* [BL21 (DE3/pLysS)] were collected, and the procedure of Towers *et al.* (15) was followed for the purification of the protein. Expression of the hVDR in *E. coli* in the system described by Kumar *et al.* (16) was beneficial in undertaking this part of the present study.

Gel Electrophoresis and Western Blot Analysis. Total soluble protein extracts with sample buffer (17) added were boiled for 1 min and electrophoresed on SDS/7% polyacrylamide gels. The gels were either stained with Coomassie brilliant blue or transferred to nitrocellulose for Western blot analysis. After immobilization of proteins for Western blot analysis, the filters were blocked with Tris-buffered saline/Tween 20 (TBST) containing 5% (wt/vol) nonfat dry milk and then incubated with primary antibody for 90 min. The filters were washed extensively in TBST and then incubated with a secondary alkaline phosphatase-conjugated goat anti-mouse IgG antibody. The color was developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate using the ProtoBlot AP system according to the manufacturer's specifications (Promega). The anti-receptor monoclonal antibody preparation has been described (18).

Measurement of VDRs. The 1,25-$(OH)_2$[26,27-^{3}H]D_3 binding activity in total soluble protein extracts from *E. coli* BL21 (DE3/pLysS) transformed with either receptor construct in pET-3a plasmid was determined by a hydroxylapatite binding assay as described (19). Total VDR was determined by an ELISA assay that was developed in our laboratory (A. Uhland-Smith and H.F.D., unpublished data) by a modification of the IRMA assay (20). Protein content of the extracts was remeasured by the Bradford method (21).

Vitamin D Compounds. 1,25-$(OH)_2D_3$ was made by Tetrionics (Madison, WI). 1,25-$(OH)_2$[26,27-^{3}H]D_3 (160 Ci/mmol, 1 Ci = 37 GBq) was produced by DuPont/NEN as described (22).

RESULTS

Identification of Avian VDR cDNA Clones. Two rVDR cDNA fragments of 358 and 1783 bp (3) were used to screen a jqCAM library, and three positive clones were identified in the first round. DNA from the recombinant phage of these three clones was isolated and cut with *Eco*RI to release the cDNA inserts. The excised cDNA fragments were subcloned into pUC18 and sequenced. Only one clone contained the whole translated region of the transcript. The open reading frame was 1344 bp. It had three possible ATG initiation codons. The deduced sequence of 448 amino acids (aa) contained both DNA-binding domains (Fig. 1) (I and II) and ligand binding domain. The calculated molecular weights of the jqVDR receptor proteins were 58,768 and 55,752. This agrees well with previous reports (7) taking into account post-translational modifications. When comparing the avian nucleotide sequences to either the human or rat sequence by the BEST FIT or GAP ANALYSIS programs of GCG, the similarity was 78% and 65%, respectively. RNA blot hybridization analysis of rVDR, cVDR, and jqVDR is shown in Fig. 2. The predominant mRNA species for the rat was ≈4.4 kb, while both chicken and quail mRNA species were ≈2.7 kb. Only one RNA species was observed for the avian receptor, unlike a previous report that showed two mRNA species of 2.6 and 3.2 kb (23).

Both human and rat transcript sizes are ≈4.4 kb, whereas the avian transcript is ≈2.7 kb. Paradoxically, the coding sequence of the avian receptor is longer than the mammalian coding sequence. The functional significance of such an untranslated region in mammalian species is unknown, although it is similar for other mammalian steroid receptors (24).

After obtaining the cDNA for jqVDR that contained all of the translated sequences, a chicken kidney library was screened using the jqVDR cDNA and six positive clones were identified. Since the sequence for either the quail or the chicken cDNA contains an internal *Eco*RI site, the cVDR insert, when excised from the phage, was cut into two fragments. Both fragments were subcloned into pUC18 and

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-34 accggccgggtcggggagggatgtgctggcagccatggtctccatctctgcatcgggggg
                                    M  V  S  I  S  A  S  G  G
 27 gtacgccatgccctgctgctgtgaagtcccaggagctgcagagctcagacatggaagcacc
    Y  A  M  P  C  C  C  E  S  Q  E  L  Q  S  S  D  M  E  T  P
    10                            20
 87 tgcagtaggaacgcccgaattcgaccgcaacgtgccccgaatctgtggggtctgtgggga
    A  V  G  T  P  E  F  D  R  N  V  P  R  I  C  G  V  C  G  D
    30                            40
147 cagagccactggattccacttcaacgccatgacctgcgagggctgcaagggcttcttcag
    R  A  T  G  F  H  F  N  A  M  T  C  E  G  C  K  G  F  F  R
    50                            60
207 gagaagcatgaagaggaaagccatgttcacgtgtccgttcagcggggactgcaaaatcac
    R  S  M  K  R  K  A  M  F  T  C  P  F  S  G  D  C  K  I  T
    70                            80
267 caaggacaaccggcggcactgccaggcctgcaggctgaaacgctgcgtggacatcgggat
    K  D  N  R  R  H  C  Q  A  C  R  L  K  R  C  V  D  I  G  M
    90                           100
327 gatgaaggagttcatcctgacggatgaggaggtgcagaggaagcgtgagatgatcctgaa
    M  K  E  F  I  L  T  D  E  E  V  Q  R  K  R  E  M  I  L  K
   110                           120
387 gcgcaaagaggaggaagctctgaaggagagcctgaagcccaagctgtcagaggagcagca
    R  K  E  E  E  A  L  K  E  S  L  K  P  K  L  S  E  E  Q  Q
   130                           140
447 gaaagtcatcaacatcctcctcgaggcccatcacaaaaccttcgacaccacatactctga
    K  V  I  N  I  L  L  E  A  H  H  K  T  F  D  T  T  Y  S  D
   150                           160
507 cttcaacaagtttcggccccctgtgagaagcaaattcagcagcagtacagcaacgcactc
    F  N  K  F  R  P  P  V  R  S  K  F  S  S  S  T  A  T  H  S
   170                           180
567 ctcctccgtcgtgtcccaggacttctcctcggaggactccaatgatgtttttggttccga
    S  S  V  V  S  Q  D  F  S  S  E  D  S  N  D  V  F  G  S  D
   190                           210
627 tgccttcggtgcattcccagagcccatggagccccaaatgttttccaacctggacctctc
    A  F  G  A  F  P  E  P  M  E  P  Q  M  F  S  N  L  D  L  S
   210                           220
687 cgaggagagcgatgagagcccctccatgaacatcgagctgccccacctccccatgctccc
    E  E  S  D  E  S  P  S  M  N  I  E  L  P  H  L  P  M  L  P
   230                           240
747 acacctggctgacctggtcagttacagcatacagaaagtgatcggctttgccaaaatgat
    H  L  A  D  L  V  S  Y  S  I  Q  K  V  I  G  F  A  K  M  I
   250                           260
807 ccctggattcagggatctgactgcagaggaccaaattgccctgctgaaatccagcgccat
    P  G  F  R  D  L  T  A  E  D  Q  I  A  L  L  K  S  S  A  I
   270                           280
867 cgaggtgatcatgctgcgctccaaccagtccttcaccatggaggacatgtcatggacctg
    E  V  I  M  L  R  S  N  Q  S  F  T  M  E  D  M  S  W  T  C
   290                           300
927 tggaagcaatgacttcaagtacaaagtcagtgatgtcacccaagctggtcacagcatgga
    G  S  N  D  F  K  Y  K  V  S  D  V  T  Q  A  G  H  S  M  D
   310                           320
987 cctcctcgagccactcgtgaagttccaggtgggcttgaagaagctgaaccttcacgaaga
    L  L  E  P  L  V  K  F  Q  V  G  L  K  K  L  N  L  H  E  E
   330                           340
1047 agagcatgtgctcctcatggccatctgtatcctgtccccagatcgacccggcgttcagga
     E  H  V  L  L  M  A  I  C  I  L  S  P  D  R  P  G  V  Q  D
     350                           360
1107 cacgtctctggtggagtccatccaggaccgtctctctgacaccctccagacctacatccg
     T  S  L  V  E  S  I  Q  D  R  L  S  D  T  L  Q  T  Y  I  R
     370                           380
1167 ctgcagacacccccctcctggcagccgcttgctgtatgccaagatgatccagaagctggc
     C  R  H  P  P  P  G  S  R  L  L  Y  A  K  M  I  Q  K  L  A
     390                           400
1227 cgacctacgcagcctgaacgaggagcactccaagcagtaccgctgcctctccttccagcc
     D  L  R  S  L  N  E  E  H  S  K  Q  Y  R  C  L  S  F  Q  P
     410                           420
1287 cgagcacagcatgcagctcacccctttagtgctcgaggtctttggcaacgagatctcctg
     E  H  S  M  Q  L  T  P  L  V  L  E  V  F  G  N  E  I  S  *
     430                           440
1347 attgcagccgtgggttctgagcacccacgtgtaaagggataagtcagccttgcgcctatg
1407 gaggcactgaggagggctgtgacctgggaggaggttgtatgtttagtttcacactactgc
1467 aggtcctcgtttgttttcttagccacaatgcgcgagttttgctcactgcagacccagatc
1527 tgtgcatttttcccccctcacgagtggttttttccacttgagctgacttatccttta
1587 tggtgttttgaggctgggctgccttgaactacttctttctgaccagaaactgaggctgct
1647 tgggaaggagaggctcaggttctgcagattcatggcactgggccgaattcgtaatcatgt
1707 catagctgtttcctgtgtgaaattgtt 1733
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FIG. 1. Nucleotide sequence and deduced amino acid sequence of the avian (chicken and quail) VDR. Both sequences are numbered with respect to the putative initiating methionine. Oligonucleotides used to amplify the coding sequence of the jqVDR starting at the first, second, or third ATG at the 5′ end and the 3′ oligonucleotide are underlined.

sequenced. The sequence analysis revealed that the quail and chicken nucleotide sequences are identical. The N-terminal portion of amino acid sequences for the quail and chicken was 100% identical to the published partial chicken sequence (23). Therefore, the sequence presented in Fig. 1 represents the sequence for either the chicken or quail and possibly all avian species. The cVDR cDNA probe hybridized only to the chicken kidney mRNA, whereas the rat probe showed slight hybridization to the chicken mRNA (Fig. 2).

Comparison of Amino Acid Sequences of the rVDR, hVDR, and Avian VDRs. Fig. 3 shows a comparison of the rVDR, hVDR, and avian VDR amino acid sequences. Alignment of

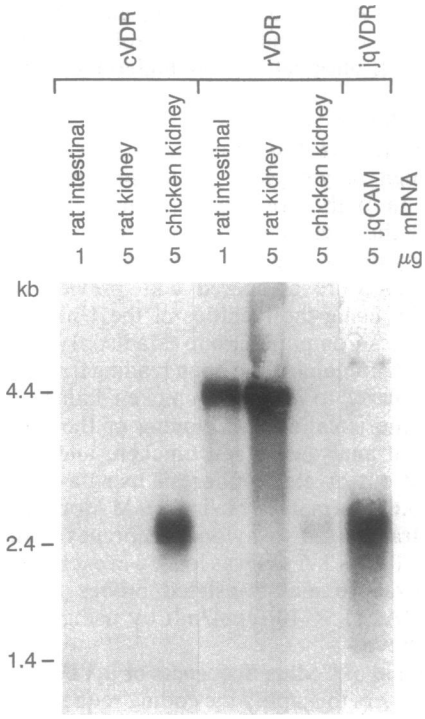

FIG. 2. RNA blot hybridization analysis of r-, c-, and jqVDR transcripts from intestine and kidney of rats, chicken kidney, and jqCAM. Polyadenylylated RNA from rats, chickens, and quail as indicated on top of the blot were electrophoresed in a 1% agarose/2.2 M formaldehyde gel and then transferred to a nylon membrane. The membranes were probed with a nick-translated receptor cDNA for the r-, c-, or jqVDR as indicated. Autoradiography was for 48 h. Positions of molecular size markers are shown at the left.

the sequences indicated that all three are very similar. A cysteine-rich region of the N terminus representing the DNA-binding domain of the receptors is 100% conserved in the first domain of the three species, whereas the second domain of the chicken had some differences. Only 3 aa were different from the rat or human sequence, a methionine instead of leucine, a serine instead of asparagine, and a lysine instead of arginine at aa 77, 83, and 87 of the avian protein sequence, respectively. The position of the nine cysteines was conserved among all three VDRs. The increase in size of the avian receptor is due to additional unconserved N-terminal sequence. Furthermore, the major difference in the avian receptor was the presence of two initiation start sites that produce the known doublet for avian receptors at 60 and 58 kDa.

Expression of the jqCAM VDR. To determine whether both cVDR proteins (60 and 58 kDa) are produced from one transcript by initiation at two in-line initiation sites, the following experiment was undertaken. An *E. coli* expression system (pET) was used to overproduce the jqVDR. Three recombinant plasmids were constructed, containing the first, second, or third ATG of the translated sequence of the jqVDR; thus, the first construct contained the whole amino acid coding segment for the 448-aa receptor. The second construct started from the second methionine codon and contained the sequence for 437 aa, and the third construct was from the third methionine and contained the sequence for 423 aa of the coding segment. The 5′ end for each of the three inserts was subcloned in pET3a plasmid in the *Nde* I site, to express proteins without N-terminal fusions (Fig. 4*A*). When the recombinant plasmids were cut with *Nde* I and *Bam*HI enzymes, the three inserts were released with sizes 1344, 1311, and 1269 bp. After sequencing to confirm that there was

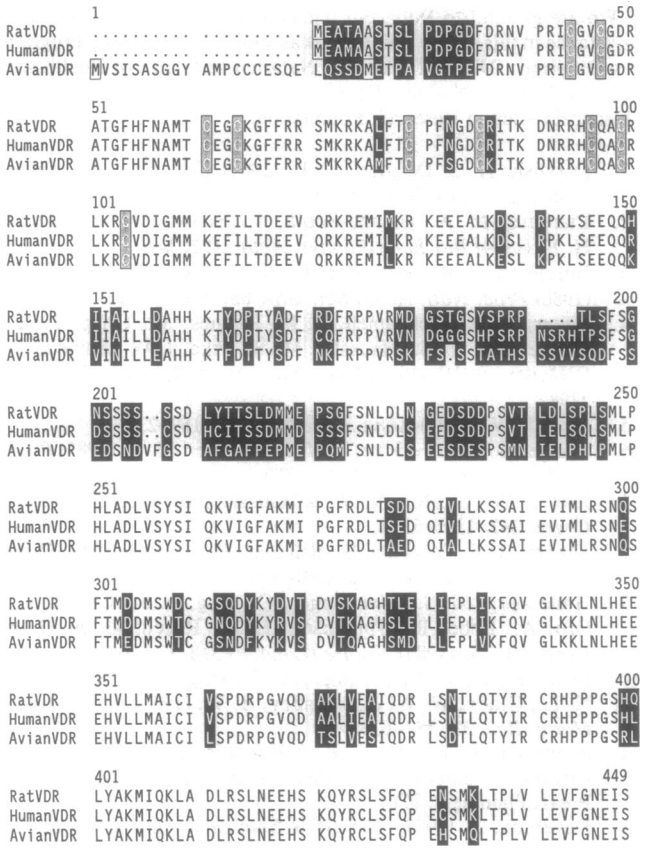

FIG. 3. Comparison of h-, r-, and avian VDR protein sequences. Amino acid sequences were aligned to show similarities in the DNA and hormone-binding domains. The predicted human, rat, and avian translation products were 427, 423, and 448 aa long, respectively. Those regions corresponding to the two DNA-binding domains are underlined in refs. 1 and 11. The positions of the nine cysteines are conserved among all three VDRs (boxed). Boxed also are the initiating methionines, with two for the avian sequence that give rise to the doublet proteins of 60 and 58 kDa.

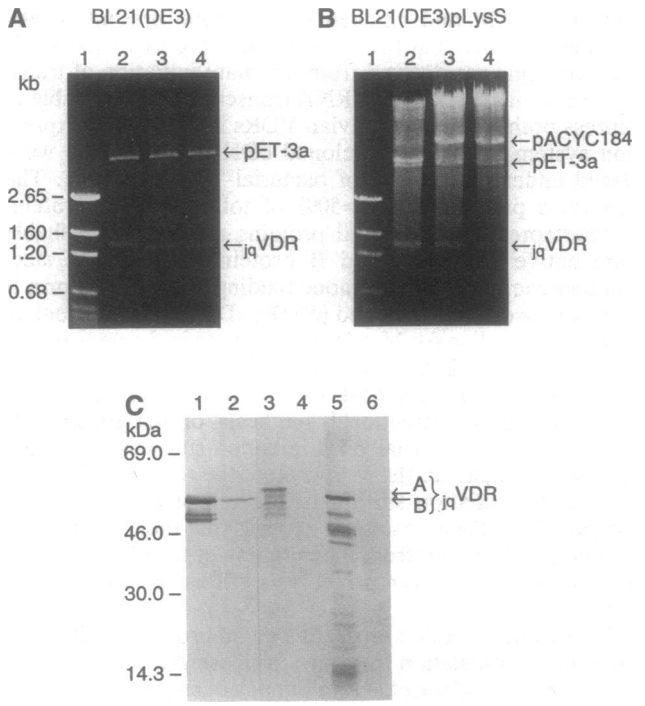

FIG. 4. Cloning of jqVDR coding region inserts 1, 2, and 3 (first, second, and third ATG, respectively) in pET vector and transformation of expression hosts *E. coli* BL21 (DE3) (*A*) or BL21 (DE3pLysS) (*B*). Insert sizes were 1344 (lane 2), 1311 (lane 3), and 1269 (lane 4) bp from first, second, and third ATG possible initiation sites to the end of the coding region. (*C*) Western blot of jqVDR protein overexpressed in the bacterial system after its purification. Protein extracts from recombinant receptor produced by *E. coli* transformed with construct 1, 2, or 3 are shown in lanes 3, 4, and 5, respectively. The cross-reacting monoclonal antibody IVG8C11 was the primary antibody. The PNEs and CAM were the positive control for the VDR (lanes 2 and 6, respectively). We also used the baculovirus-expressed rVDR (lane 1). The molecular masses correspond to the following protein markers, from top to bottom, bovine serum albumin, ovalbumin, and carbonic anhydrase.

no mismatch incorporated during PCR amplification, expression was carried out.

Monoclonal antibodies directed against the VDR were used in a Western blot analysis to confirm the authenticity of the recombinant receptors (Fig. 4*B*). Monoclonal antibody IVG8C11, reported to cross-react with the VDR from pigs, rats, monkeys, humans, and chickens (18), was used in Western blot analysis of purified proteins from *E. coli* transformed with construct 1, 2, or 3. Construct 1, which contained the whole coding region, when expressed produced the 60-kDa receptor protein. Construct 2 did not produce a receptor, and construct 3 produced the 58-kDa receptor protein. The CAM, pig nuclear extract (PNE), and the baculovirus-expressed rVDR were used as VDR-positive controls. The native CAM nuclear extract showed the known doublet bands at ≈60 and 58 kDa. Both the PNE and recombinant rVDR showed a band at 55 kDa.

A more precise determination of the recombinant VDR was performed using the hydroxylapatite ligand binding assay and the ligand-independent ELISA (Table 1). An average of 6268 and 8103 fmol of steroid binding per mg of protein was measured from constructs 1 and 3, respectively, using the hydroxylapatite assay. CAM and PNE, which served as controls, were 88 and 2352 fmol/mg of protein, respectively. No measurable receptor was obtained from construct 2. Using the ELISA measurement, the average level of total VDR from constructs 1 and 3 was 15,668 and 48,819 fmol/mg of protein, respectively. Nuclear extracts from CAM and pig averaged 250 and 3431 fmol/mg of protein, respectively. By the ELISA measurement, the level of receptors was determined to be nearly 62 and 195 times greater in the bacterial-expressed system from constructs 1 and 3, respectively, than in the CAM nuclear extract.

DISCUSSION

This report presents the nucleotide sequence of a cDNA containing the complete open reading frame for the VDR from the jqCAM and from chicken kidney. The nucleotide sequence data from the avian, r-, and hVDRs revealed an overall 78% homology between the avian and the mammalian VDRs. Two forms of the avian VDR with apparent molecular masses of 60 kDa and 58 kDa have been found in approximately equimolar amounts in the chicken intestine (7) and

Table 1. Measurement of VDR

Sample	Ligand binding assay, fmol/mg of protein	ELISA, fmol/mg of protein
Expressed jqVDR		
Using first ATG	6268 ± 129	15,668 ± 225
Using second ATG	0	0
Using third ATG	8103 ± 178	48,819 ± 426
CAM nuclear extract	88 ± 24	250 ± 41
PNE	2352 ± 227	3,431 ± 104

now in jqCAM and chicken kidney. Our present results provide evidence that the avian VDR A (60 kDa) and B (58 kDa) proteins are derived from alternate initiation of translation of a single ≈2.7-kb mRNA transcript. We were able to express both forms of the avian VDRs in a bacterial expression system in which the cloned cDNA for jqVDRs were placed under the control of bacterial T7 polymerase. The expressed proteins were >50% of total bacterial protein. Furthermore, the expressed proteins are indistinguishable from native jqVDR A and B proteins in terms of size, immunoreactivity, and hormone binding properties. Form B was expressed from a cloned jqVDR cDNA starting from an ATG signal coding for Met-26 and resulted in the appearance of a protein indistinguishable from the natural jqVDR form B in its electrophoretic migration and hormone binding properties. Therefore, functional synthesis of the receptor B protein from the internal ATG initiation site was confirmed by deletion of part of the 5′ portion of the receptor cDNA to remove the initiation site for translation of the receptor A protein. From these results, it appears that the two forms of the avian VDR result from the initiation of translation at two ATG sites and not from proteolysis of form A, as has been suggested (7).

The synthesis of two forms of a given protein by alternate initiation of translation from two in-phase ATG signals on a single mRNA transcript is rare but has been reported for the chicken progesterone receptor (25). A 9-nt consensus sequence [CC(A/G)CCATGG] optimal for initiation of translation of eukaryotic mRNA has been demonstrated by Kozak (26). It has been suggested that if the most 5′ ATG codon occurs in a sequence context that is less conserved than the Kozak consensus sequence, then a second internal ATG codon translation may reinitiate at the more favorable initiation signal to provide two proteins from a single mRNA (27). With regard to the jqVDR, we had three possible ATGs where initiation of translation could occur, but it was the first and third ATGs that produced the A and B forms of protein. The second ATG does not appear to be in a favorable sequence for translation.

The deduced amino acid sequence derived from the avian receptor cDNA showed 98.5% similarity with both the h- and rVDR in the DNA binding domain. The position of the nine cysteines in this region was conserved among all three. There was 87.5% homology in the ligand binding domain. Crossreactivity of the anti-porcine receptor monoclonal antibody with the avian and rat receptors further demonstrates that the VDR is a highly conserved molecule. However, it is of some interest that the DNA binding domain in the avian receptor is not identical to the rat and human receptors. Perhaps not all of the vitamin D-responsive genes in the avian species will bind to the human or rat receptors but may require the avian receptor to be responsive.

Despite the fact that the transcript size of the avian receptor is ≈2.7 kb and that of the rat and human receptors is ≈4.4 kb, the translated part of the transcript is larger in the avian VDR (448 aa) than in the rVDR (423 aa) or the hVDR (427 aa). Although there is a high homology between receptor species, there is a trend showing receptor mass has decreased as the organism has evolved.

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