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Cloning of a Functional Vitamin D Receptor from the Lamprey (*Petromyzon marinus*), an Ancient Vertebrate Lacking a Calcified Skeleton and Teeth

G. KERR WHITFIELD, HOPE T. L. DANG, SAMUEL F. SCHLUTER, RALPH M. BERNSTEIN, TARA BUNAG, LORI A. MANZON, GRACE HSIEH, CARLOS ENCINAS DOMINGUEZ, JOHN H. YOUSON, MARK R. HAUSSLER, AND JOHN J. MARCHALONIS

Departments of Biochemistry and Molecular Biophysics (G.K.W., H.T.L.D., T.B., G.H., C.E.D., M.R.H.) and Microbiology and Immunology (S.F.S., R.M.B., J.J.M.), College of Medicine, University of Arizona, Tucson, Arizona 85724; Department of Zoology and the Division of Life Sciences (L.A.M., J.H.Y.), University of Toronto at Scarborough, Ontario, Canada M1C 1A4

The nuclear vitamin D receptor (VDR) mediates the actions of its 1,25-dihydroxyvitamin D_3 ligand to control gene expression in terrestrial vertebrates. Prominent functions of VDR-regulated genes are to promote intestinal absorption of calcium and phosphate for bone mineralization and to potentiate the hair cycle in mammals. We report the cloning of VDR from *Petromyzon marinus*, an unexpected finding because lampreys lack mineralized tissues and hair. Lamprey VDR (lampVDR) clones were obtained via RT-PCR from larval protospleen tissue and skin and mouth of juveniles. LampVDR expressed in transfected mammalian COS-7 cells bound 1,25-dihydroxyvitamin D_3 with high affinity, and transactivated a reporter gene linked to a vitamin D-responsive element from the human CYP3A4 gene, which encodes a P450 enzyme involved in xenobiotic detoxification. In tests with other vitamin D responsive elements, such as that from the rat osteocalcin gene, lampVDR showed little or no activity. Phylogenetic comparisons with nuclear receptors from other vertebrates revealed that lampVDR is a basal member of the VDR grouping, also closely related to the pregnane X receptors and constitutive androstane receptors. We propose that, in this evolutionarily ancient vertebrate, VDR may function in part, like pregnane X receptors and constitutive androstane receptors, to induce P450 enzymes for xenobiotic detoxification. (*Endocrinology* 144: 2704–2716, 2003)

THE ACTIONS OF 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] are mediated by the vitamin D receptor (VDR) in concert with an obligate heterodimeric partner, the retinoid X receptor (RXR; Ref. 1). Analogous to other members of the nuclear receptor superfamily (2), liganded VDR, together with its RXR dimer partner, binds to specific vitamin D responsive elements (VDREs) adjacent to target genes and activates or represses these genes to mediate the biological effects of the hormonal ligand (1). VDR null mice have provided strong support for a role of liganded VDR in maintaining blood calcium levels by promoting intestinal calcium absorption and permitting the formation of calcified structures, especially after weaning when the animals are deprived of a steady, abundant source of calcium (3, 4). VDR is also crucial for the maintenance of hair in mice, apparently by playing a role in the hair cycle (5).

VDRs have been characterized from mammals (6–8), birds (9), *Xenopus laevis* (10), zebrafish (GenBank accession no. AAF21427), and two VDRs from Japanese flounder, *Paralichthys olivaceus* (11). All of these species represent vertebrates with calcified endoskeletons. True VDRs are unknown in nonchordate species; the currently known nonchordate protein with the highest similarity to VDR is the ecdysone receptor (12), found in insects and crustaceans, with 27–30% amino acid sequence identity to human VDR (hVDR). Before the recent cloning of zebrafish VDR and flounder VDRa and VDRb, it was speculated that the $1,25(OH)_2D_3$-VDR system originated with terrestrial tetrapods (10). Such an origin of VDR, *per se*, has become untenable with the cloning of fish VDRs; also, it is known that a variety of fish species, including a shark (*Prionace glauca*) and lamprey (*Entosphenus japonicus*) (13), contain appreciable levels of plasma $1,25(OH)_2D_3$, ranging from 28–274 pg/ml (0.07–0.66 nM; Refs. 13–17). Nevertheless, in the absence of studies to determine the exact physiologic role of $1,25(OH)_2D_3$ in fish, it is still possible that participation of VDR and $1,25(OH)_2D_3$ in calcium homeostasis may indeed date from a time when animals could no longer access an unlimited supply of calcium from their aquatic environment (10).

Lampreys represent, with hagfishes, jawless (agnathan) fishes with the most ancient lineage among extant vertebrates (18–20). The placement of lampreys in the evolution of vertebrates is not completely clarified, but recent phylogenies, based on new finds of primitive lower Cambrian vertebrates (*e.g.* Ref. 20), suggest that lampreys belong to a group of soft-bodied vertebrates that diverged before the development of calcified structures (19, 20). Given this tentative phylogenetic position, plus the above-cited evidence that

Abbreviations: CAR, Constitutive androstane receptor; CTE, C-terminal extension; cTRα, chicken thyroid receptor-α; CYP, cytochrome P450-containing enzyme; DBD, DNA-binding domain; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; hRAR, human retinoic acid receptor; hVDR, human vitamin D receptor; lampVDR, lamprey vitamin D receptor; ONR, orphan nuclear receptor; PXR, pregnane X receptor; RACE, rapid amplification of cDNA ends; RXR, retinoid X receptor; SSPE, sodium chloride/sodium phosphate/EDTA; TFIIB, transcription factor IIB; UTR, untranslated region; VDR, vitamin D receptor; VDRE, vitamin D responsive element; xONR, *Xenopus* ONR.

lampreys contain significant circulating levels of $1,25(OH)_2D_3$, the active hormonal form of vitamin D, the present study was undertaken to determine whether lampreys contain a functional VDR. In this report, we describe three distinct VDR cDNA clones obtained from both larval and juvenile specimens of sea lamprey, *Petromyzon marinus*, that appear to reflect differences in mRNA splicing. In a limited survey of tissues, skin and mouth represent major sites of VDR mRNA expression in juvenile lamprey. Relatively little VDR mRNA was found in juvenile intestine, unlike the situation in mammals and birds. Lamprey VDR (lampVDR) is functional in that it binds $1,25(OH)_2D_3$ with high affinity and transactivates a transfected reporter construct containing a VDRE derived from the human CYP3A4 gene (21), which encodes a cytochrome P450-containing enzyme (CYP) implicated in detoxification of xenobiotics (22). Therefore, we present the first evidence that the appearance of VDR in the vertebrate lineage may actually predate calcified tissues, and we postulate that its original, noncalcemic role might have been, at least in part, the induction of P450 detoxification enzymes.

Materials and Methods

RNA samples and cDNA libraries

Tissues were dissected from a single *P. marinus* larva and four juveniles. The larva was supplied by the Hammond Bay Biological Station of the U.S. Geological Survey (Millersburg, MI) and was killed after anesthesia in 0.05% tricaine methanesulfonate in accordance with a protocol approved by the University of Arizona Institutional Animal Care and Use Committee. Protospleen tissue was collected by scraping the outside of the larval intestine. The four juvenile animals were captured as larvae in spring 2000 and maintained at the University of Toronto at Scarborough (Toronto, Ontario, Canada) under approved conditions. They underwent metamorphosis in the laboratory during the summer and were killed under 0.05% tricaine methanesulfonate in January 2001. Tissues were dissected, stored at −80 C, and used to prepare poly(A)$^+$ RNA using a FastTrack mRNA isolation system (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Total RNA preparations from toad (*Bufo marinus*) and gecko (*Gekko gecko*) kidney tissues were a kind gift from Dr. R. H. Wasserman, Cornell University (Ithaca, NY).

PCR using degenerate primers

First-strand cDNA was made from protospleen poly(A)$^+$ RNA (1 μg) using an RT-PCR kit with supplied oligo(deoxythymidine) primers (Stratagene Corp., La Jolla, CA). For the design of degenerate VDR primers for PCR, two areas of amino acid identity among human, mouse, rat, and chicken VDRs were found near the N and C termini. A sense primer encoding part of the open reading frame near the N terminus, corresponding to codons 33–40 of hVDR (-GFHFNAMT-), was 5′-GGNTTYCAYTTYAAYGCNATGAC-3′ (where N is a mixture of all four bases and Y is a mixture of both pyrimidines). An antisense primer, corresponding to codons 394–401 near the C terminus of hVDR (-NEEHSKQY-), was 5′-TAYTGYTTNGARTGYTCYTCRTT-3′ (abbreviations as above, with R being a mixture of both purines). The PCR contained 2 μl of the first-strand cDNA reaction (described above) along with 500 ng of each degenerate primer and 2.5 U Taq polymerase (Life Technologies, Inc., Rockville, MD) in a 50-μl reaction volume. PCR conditions were as follows: presteps, 94 C for 1 min and 78 C for 3 min, during which the Taq polymerase was added; 40 cycles of 94 C for 30 sec, 55* C for 30 sec, and 72 C for 1 min 20 sec; and a final incubation at 72 C for 10 min, followed by storage at 4 C. The annealing temperature (indicated by an *asterisk*) was decreased from 55 C to 43 C in 0.3 C increments over 40 cycles.

Cloning and sequencing

PCR products were subjected to agarose gel electrophoresis, excised from the gel, and purified using a Qiaex II agarose gel extraction kit (QIAGEN, Inc., Valencia, CA). Purified PCR products (7.5 μl) were cloned into T-vector (Promega Corp., Madison, WI) and sequenced using a T7 Sequenase 2.0 kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Alternatively, preparations of T-vector harboring PCR products were sent to an in-house core facility for automated sequencing.

5′- and 3′-rapid amplification of cDNA ends (RACE)

A cDNA library was constructed from larval protospleen poly(A)$^+$ RNA (1 μg) using a Marathon cDNA amplification kit (CLONTECH Laboratories, Inc., Palo Alto, CA). 5′ and 3′ RACE reactions were then performed using the AP1 primer from the kit in combination with VDR-specific primers designed from partial lampVDR sequence data. The successful 5′ and 3′ primers are underlined in Fig. 1A (the 5′ primer was the antisense complement of the underlined sequence). To identify the lamprey-specific product, 5′-RACE reactions were subjected to agarose gel electrophoresis and then blotted onto an Immobilon Ny+ membrane (Millipore Corp., Bedford, MA) by capillary action. As the hybridization probe, a 210-bp *Nco*I-*Afl*III fragment including 149 bp from the 5′-end of the original lamprey clone was biotinylated using a biotin high prime kit (Roche Molecular Biochemicals, Indianapolis, IN), and then 4 μl of this probe was incubated with the Immobilon blot at 42 C overnight in 10 ml of a solution containing 5× SSPE (1× SSPE = 0.18 M NaCl; 10 mM Na_xPO_4, pH 7.4; 1 mM EDTA), 5× Denhardt's solution, and 50% formamide. Washes were 2 × 5 min at room temperature in 2× SSPE/0.1% sodium dodecyl sulfate and then 2 × 15 min at 65 C in 0.1× SSPE, 0.1% sodium dodecyl sulfate. Detection of hybridizing bands was carried out using 5-bromo-4-chloro-3-indolylphosphate and 4-nitro blue tetrazolium (both reagents from Roche Molecular Biochemicals) as previously published (23).

Cloning of full-length lampVDR

After the 5′ and 3′ ends of the lampVDR cDNA had been obtained by RACE, primers were designed from the 5′- and 3′-untranslated regions (UTRs) for amplification of the entire coding region. The 5′ primer, corresponding to bases −31 to −7, was 5′-gaattCCGGGCAGGTCTTTAGGCTGCTTAG (a restriction site for *Eco*RI was added at the 5′ end and is shown in lower case). The 3′ primer corresponded to bases +1252–1276, with the (antisense) sequence 5′-ggatccGGAGGGCAGCAATCTCTCCACTCG and a site for *Bam*HI (*lowercase*) at the 5′ end. An aliquot of the Marathon cDNA library was diluted 1:150, and 5.5 μl was taken for a PCR using 100 ng each of the above primers in a 50-μl reaction using reagents from an Advantage high-fidelity PCR kit (CLONTECH Laboratories, Inc.). The temperature profile was: prestep at 94 C for 45 sec; 20 cycles of 94 C for 10 sec and 78* C for 3 min 30 sec; 20 cycles at 94 C for 10 sec and 68 C for 3 min 30 sec; and a final incubation at 72 C for 10 min, followed by storage at 4 C. The annealing temperature (*) was decreased from the initial 78 C to 68 C in 0.5 C increments over the first 20 cycles.

PCR products were resolved on agarose gels, excised, purified, and cloned into T-vector as described above. After confirmatory sequencing, the T-vector clone was digested with *Eco*RI and *Bam*HI, and the lamprey insert of 1347 bp, which lacked insert A (see Fig. 1B, *top*), was purified on an agarose gel and cloned into predigested pSG5 vector (24). Site-directed mutagenesis of the pSG5-lampVDR to remove insert B was performed using the oligonucleotide primer 5′-GGCTATCTGCCTCTTCTCTCCCG ACCGGCCTGGCGTACAAGACCG, and its complement, corresponding to 22–23 bases on either side of the insert (see Fig. 1B, *bottom*), in a PCR-based protocol using a QuikChange kit (Stratagene Corp.). The resulting clone is referred to as lampVDR.

Cloning of lampVDR from juvenile tissues

First-strand cDNA was prepared from juvenile intestine, skin, and mouth poly(A)$^+$ RNA as described above for larval protospleen and then subjected to PCR amplification using primers flanking insert B in the larval VDR clones: forward primer 5′-CATTGAAATCATCATCCTCCGC-3′, corresponding to bp 738–759 of the lampVDR cDNA (Fig. 1), and reverse primer 5′-ATCTCTCCACTTCGTCCATCCC-3′, corre-

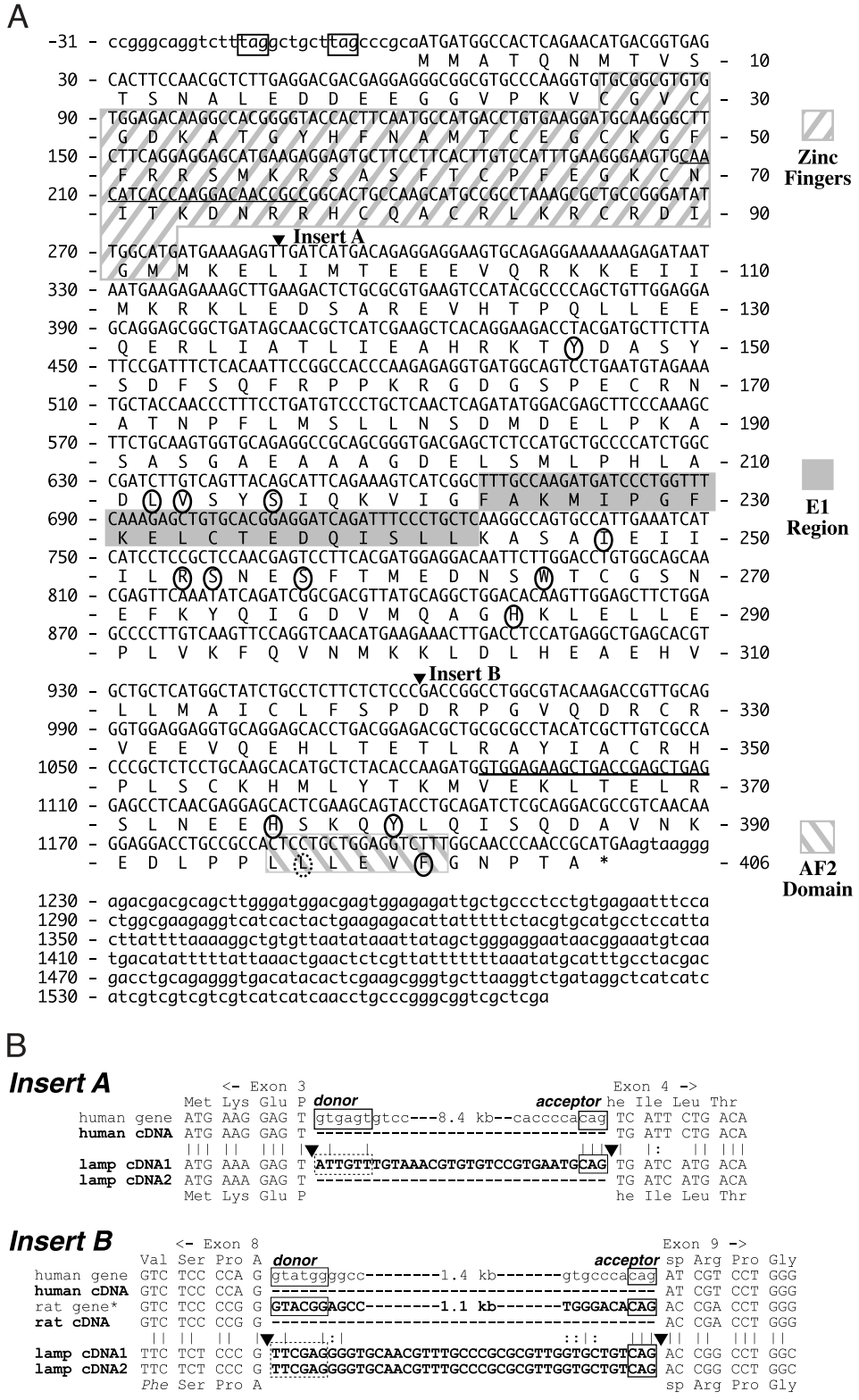

FIG. 1. Deduced amino acid sequence of lampVDR. A, The sequence of VDR cDNA from *P. marinus*, excluding two insertions at intron-exon boundaries. The open reading frame (preceded by two in-frame stop codons, which are *boxed*) predicts a 406 amino acid protein. Regions corresponding to three functional domains in hVDR are *hatched* (the N-terminal DBD), *gray* [for the conserved E1 region implicated in heterodimerization with RXR (77)], or *hatched* [the C-terminal activation function-2, or AF2, domain (78)]. Residues that are identical with ligand contacts in a crystal of the hVDR ligand-binding domain (39) are *circled;* the *dotted circle* around leu-397 indicates that this residue is not identical with the corresponding hVDR amino acid (val). Positions of two out-of-frame insertions are indicated by *solid arrowheads*. Sequences used to design primers for 5′- and 3′-RACE are *underlined*. B, Two insertions (*bold type*) in lamprey cDNA clones relative to known intron-exon boundaries (indicated by *solid arrowheads*) in the human (36) and rat (37) VDR genes. The original lampVDR cDNA clone obtained from larval protospleen contained both insertions; the four other clones obtained to date from this tissue contain only insert B. Clones from juveniles contain neither insert (see Fig. 2). *Boxed* sequences indicate donor or acceptor splicing signals in the mammalian genes.

sponding to the antisense of bp 1244–1264. Likewise, primers flanking insert A were designed, consisting of forward primer 5′-GCAACATCACCAAGGACAACCG-3′, corresponding to bp 237–258, and reverse primer 5′-GCATTTCTACATTCAGGACTGCCATC-3′, corresponding to the antisense of bp 518–543. The absence of inserts A and B was confirmed by sequencing of independent clones from mouth and skin using each primer pair.

Sequence alignments and phylogenetic comparisons

Sequences for VDRs and other related receptors were obtained from GenBank, often by using BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were aligned using Clustal W, version 1.8 (25). Aligned sequences were then used to construct cladograms in Clustal W but also in Puzzle (version 4.0.2; Ref. 26) and in the Protpars routine

from Phylip (version 3.573c; Ref. 27). The insect ultraspiracle protein from *Locusta migratoria* (accession no. AAF00981.1) and the nhr-8 protein from *Caenorhabditis elegans* (accession no. AAB88373.1) were used as outgroups.

Transfection and transactivation assays

The pSG5 plasmids (25–50 ng/well) harboring cDNAs for lampVDR, hVDR, or pSG5 vector without cDNA insert were transfected into COS-7 monkey kidney cells (60,000/1.88 cm^2 well in a 24-well plate), using a calcium phosphate coprecipitation procedure as previously described (28). All transfections included a human GH reporter plasmid (1.0 μg/well) linked to two or more copies of VDREs from vitamin D-regulated genes. VDREs used for this analysis included the rat osteocalcin VDRE (29) as well as two distinct VDREs from the human CYP3A4 gene, namely a distal VDRE (21) of the DR3 type, with the sequence 5′-GGGTCAgcaAGTTCA-3′ (two hexanucleotide half-sites in uppercase), and a proximal VDRE of the ER6 type (21, 30), with the sequence 5′-TGAACTcaaaggAGGTCA-3′ (upstream half-site is everted). Some transfections also contained 50 ng/well of a TIF plasmid expressing *Danio rerio* (zebrafish) RXRα (31). Cells were incubated for 24 h with $1,25(OH)_2D_3$ or ethanol vehicle, and then culture media were assayed for secreted GH using a kit from Nichols Institute (San Juan Capistrano, CA).

Hormone-binding assay

The pSG5 plasmids (10 μg) harboring cDNAs for lampVDR, hVDR, or pSG5 without cDNA insert were transfected into COS-7 cells (3×10^6 cells/150-mm plate) as described above but without the VDRE reporter construct. After a 48-h incubation without hormone, cells were washed twice with PBS, and then cell lysates were prepared as described in Nakajima *et al.* (32). The ability of expressed lampVDR to bind [^{3}H]-$1,25(OH)_2D_3$ was assessed as described in Whitfield *et al.* (28), with the following modifications: 10–30 μl of lysate were combined with 30 μl rat liver nuclear lysate, prepared according to Nakajima *et al.* (33), in 200 μl total volume. [Rat liver contains both RXRα and RXRβ (34) to serve as potential heterodimeric partners for VDR.] The $1,25(OH)_2$-26,27-[^{3}H]dimethyl-vitamin D_3 (Amersham Pharmacia Biotech, original specific activity 163 Ci/mmol) was diluted to 18 Ci/mmol with unlabeled $1,25(OH)_2D_3$ and then further diluted with 90% ethanol/5% isopropanol. After a 15-min incubation on ice, 10 μl of the appropriate dilutions were added to achieve the following approximate final concentrations in nM: 0.1, 0.2, 0.4, 0.8, and 1.6. Binding reactions were incubated overnight on ice. Unbound hormone was removed by incubation for 15 min at 4 C with 80 μl of a 3% suspension of dextran-coated charcoal (Sigma-Aldrich Corp., St. Louis, MO) prepared in 0.15 M NaCl, 0.015 M NaN_3, sodium phosphate buffer, pH 7 (0.085 M Na_2HPO_4 and 0.055 M NaH_2PO_4), and 0.1% gelatin. Samples were centrifuged at 10,000 $\times$ *g* for 2 min, and supernatants (200 μl) were taken for scintillation counting. Data were transformed for Scatchard analysis using LIGAND (35), a program originally written in BASIC and adapted for the Macintosh by Robert E. Williams (Xoma Corp., Santa Monica, CA).

Results

Cloning of lampVDR

PCR using degenerate primers VDR1 and VDR2B yielded an initial 997-bp product from lamprey larval protospleen poly(A)$^+$ RNA. Sequencing revealed an open reading frame whose conceptual translation yielded an amino acid sequence with more than 59% identity to human VDR. However, this reading frame also contained two out-of-frame insertions. Products were also obtained from kidney total RNA samples from cane toad (*B. marinus*) and lizard (*G. gecko*). The toad and gecko sequences were used for phylogenetic comparisons (see below).

A 3′ RACE reaction yielded a 488-bp product containing the 3′ end of the lampVDR coding region, an in-frame stop codon, and 353 bases of 3′-UTR. Numerous 5′ RACE reactions using a variety of primers finally produced a 260-bp product that hybridized to a lampVDR probe (see *Materials and Methods*). This 5′-RACE product contained the 5′ end of the lampVDR coding region and 31 bases of 5′-UTR. In addition, two in-frame stop codons (Fig. 1A, *enclosed in boxes at top*) were found within the 5′-UTR, making it highly likely that the complete coding region of the lamprey cDNA clone had been obtained. High-fidelity PCR was then performed using primers in the 5′- and 3′-UTRs (primer sequences given in *Materials and Methods*). Two independent PCRs each produced a 1349-bp product, which was cloned into T-vector for confirmatory sequencing.

The sequence presented in Fig. 1A represents a composite of nine independent, overlapping PCR products, seven partial sequences, and two high-fidelity cDNAs containing the entire coding region. The open reading frame, discounting the two out-of-frame insertions, predicts a protein of 406 amino acids, compared with a range of 420–453 residues for other known VDRs (6–11). Figure 1B displays the two insertions found in larval clones. Insert A (31 bp), found only in the original clone produced by PCR with degenerate primers, occurred precisely at the junction between exons 3 and 4 in the human VDR gene (36). Insert B (41 bp) occurs at a location exactly matching the junction between exons 8 and 9 in the rat (37) and human (36) VDR genes and was found in all five clones obtained from the original larval protospleen. A minor fraction of rat VDR cDNA has been reported (37) to contain a much larger insertion (retained intron) in exactly the same position as insert B, resulting in expression of a truncated VDR with dominant negative activity.

Expression of lampVDR mRNA in juvenile lamprey tissues

To show that lampVDR transcripts exist in which both insertions are lacking, we prepared poly(A)$^+$ RNA from three tissues dissected from juvenile lampreys. Skin, mouth, and intestinal RNAs were subjected to RT-PCR using two sets of primers (Fig. 2). One primer set matched sequences in lampVDR-flanking insert A (Fig. 2A), and the second primer set flanked insert B (Fig. 2B). PCR products from both reactions were cloned and sequenced. As can be seen in Fig. 2A, the sequences of PCR products from both mouth and skin were nearly identical with the original larval clone except that insert A was missing from both clones; one clone, that from skin, contained a Thr codon in place of a Met codon at position 98 (three codons past where insert A would have occurred). Likewise, the PCR products seen in Fig. 2B also matched the original lamprey clone except that insert B was missing from both clones, and one clone, this time from mouth, contained a different codon at position 371, encoding Gly instead of Ser. Also, both clones differed with the original larval clone at codon 360, which was ACC (Thr) in the larval form but GCC (Ala) in both juvenile clones.

Because the differences at positions 98 and 371 occurred only in single reactions, these were judged to be likely PCR artifacts. However, the discrepancy at position 360 occurred in juvenile clones from both skin and mouth and thus may represent a polymorphic variation among the five different animals used in this study (one larval and four juveniles). It should be noted that both a Thr and Ala are found at this

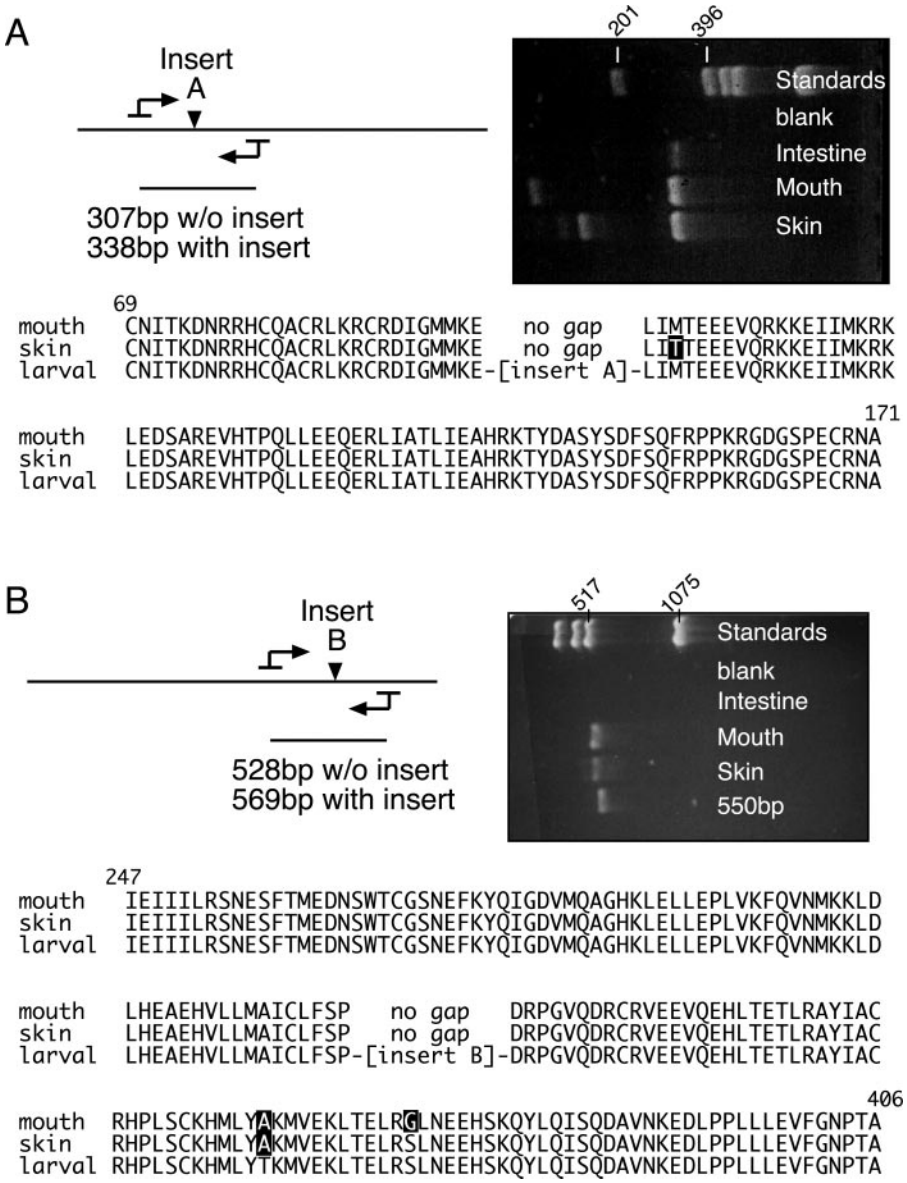

FIG. 2. Juvenile lamprey tissues express VDR mRNAs lacking both inserts. A, Poly(A)$^+$ RNAs from three juvenile tissues were subjected to RT-PCR with primers flanking insert A. Skin and mouth samples yielded a PCR product of approximately 310 bp (see gel photograph at *top right*), but the product from intestine was weak or absent. Products from mouth and skin were subcloned into vector pCR 2.1 (Invitrogen) and sequenced from the M13R and T7 primer sites present in the vector. The cDNA sequence was then conceptually translated to the amino acid sequences shown at the *bottom* of the panel. Amino acids different from the original larval clone are shown with *white text inside a black box.* B, RT-PCR, similar to that in A, was performed with primers flanking insert B. The gel shown at *top right* also contains a 550-bp PCR product, derived from lamprey lactate dehydrogenase, which served to demonstrate that all three samples, including intestine (not shown), contained intact mRNAs. The lack of insert B in mouth and skin was confirmed by sequencing subcloned PCR products. Deduced codons that differ from the original larval sequence are highlighted as in A.

position in VDRs from other species (see Fig. 7, *lower right panel*).

Curiously, poly(A)$^+$ RNA from intestine, a tissue that contains a relatively high level of VDR in higher vertebrates (*e.g.* Ref. 38), did not yield PCR products for lampVDR in a reproducible fashion. This observation does not reflect poor condition of intestinal RNA because a 550-bp PCR product for lamprey lactate dehydrogenase was readily obtained from this RNA (Fig. 2B and data not shown).

General comparison of lampVDR with related nuclear receptors

The deduced amino acid sequence of lampVDR showed an overall amino acid identity of 59–62% with other vertebrate VDRs (Fig. 3). The highest overall identity to lampVDR among other vertebrate VDRs is seen with flounder VDRa (62.2%), and the lowest identity is with *X. laevis* VDR (58.4%). By comparison, human VDR exhibits 67% or greater identity with other vertebrate VDRs (excluding lampVDR). Thus, lampVDR represents the most divergent member of the known VDRs. The amino acids that show identity to other VDRs tend to occur in regions of known functional significance. Accordingly, the zinc finger DNA-binding domain (DBD, *black shading*) in lampVDR contains 58 of 66 residues (87.9%) that are identical with those in human VDR. Also, of 14 residues known to contact the $1,25(OH)_2D_3$ ligand in the hVDR ligand-binding domain crystal (39) (these residues are localized in the *gray shaded areas* in Fig. 3 and are *circled* in Fig. 1A), 13 residues (92.9%) are identical in lampVDR. The heterodimerization interface with RXR (hatched in Fig. 3) is less well defined in VDRs, with the only relevant crystal data being from a heterodimer of mouse RXRα with the all-trans-retinoic acid receptor, hRARα. By analogy to this crystal, the general regions involved in heterodimerization (hatched in Fig. 3) display from 57–63% identity when lampVDR is compared with known VDRs from other vertebrate species (see also Fig. 7, below).

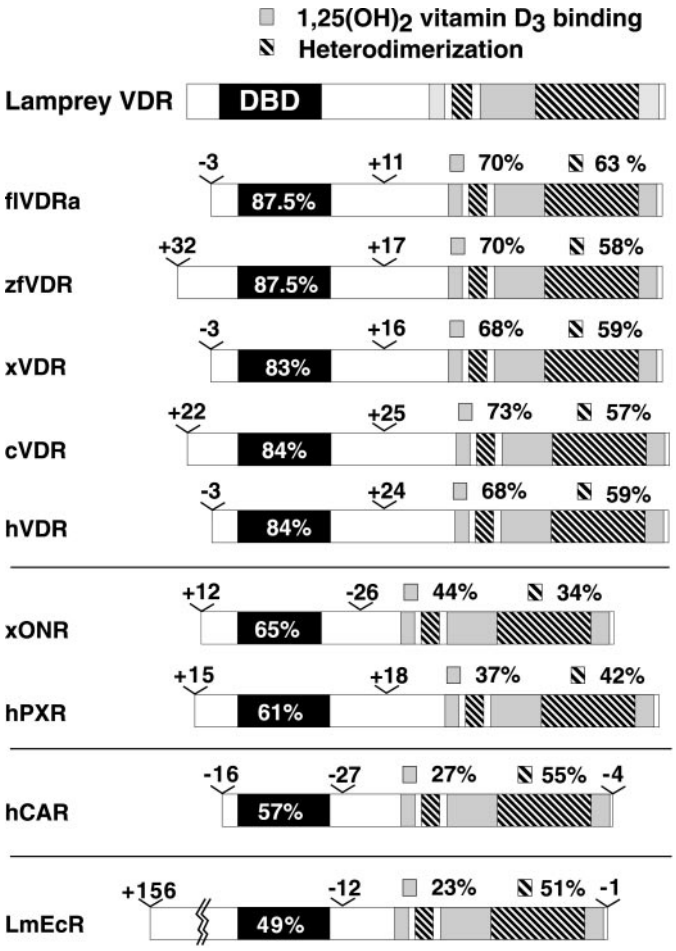

FIG. 3. Amino acid sequence identities between the lampVDR clone (without insertions) and related nuclear receptors. VDRs are from the following species, with GenBank accession numbers in *parentheses*: flVDRa, Japanese flounder (*P. olivaceus*) VDRa (BAA95016); zfVDR, zebrafish *D. rerio* (AAF21427); xVDR, African clawed frog *X. laevis* (U91846); cVDR, chicken *Gallus gallus* (AAB62579.1); hVDR, human (NP_000367). The human PXR (hPXR) sequence was accessed at AF061056, the xONR sequence at CAA53006.1, and the hCAR sequence was taken from NM_005122. The ecdysone receptor from the insect *L. migratoria* (LmEcR), accession no. AAD19828, represents a group 1H receptor used as an outgroup for the cladogram in Fig. 6A. DBD is shown as *black shading;* other functional domains are identified in the key at the top. *Numbers* given above the schematic sequences represent additional (+) or missing (−) amino acid positions relative to the lamprey sequence.

Although lampVDR is the most divergent VDR, compared with other VDRs, it tends to show a higher sequence similarity to the NR1I2 and NR1I3 receptors than do other VDRs. For example, lampVDR has 43.6% identity with the *Xenopus* orphan nuclear receptor (ONR), 43.0% identity to human pregnane X receptor (PXR), and 39.2% to human constitutive androstane receptor (CAR), whereas hVDR possesses amino acid identities of only 42.0%, 39.1%, and 36.2% to human PXR, *Xenopus* ONR (xONR), and human CAR, respectively. NR1I2 and NR1I3 receptors like PXR and CAR are currently thought to function primarily as mediators of xenobiotic ligand detoxification via induction of CYPs and ATP-binding cassette transporters (40).

Assessment of 1,25(OH)$_2$D$_3$ hormone binding by lampVDR

Larval lampVDR cDNAs in a pSG5 expression construct, both containing and lacking insert B, were transfected into VDR-deficient COS-7 cells to test the ability of expressed lampVDR to bind radiolabeled 1,25(OH)$_2$D$_3$, using pSG5-hVDR as a positive control. LampVDR expressed from a cDNA that retained insert B was unable to bind hormone at the tested concentrations of 1,25(OH)$_2$D$_3$ (0.1–1.6 nM), showing only background levels of [^{3}H]1,25(OH)$_2$D$_3$ in the protein-bound fraction, which, in COS-7 cells, typically constitute less than 500 receptors/cell (data not shown). When COS-7 cultures were transfected with either insertless lampVDR or an hVDR positive control; however, the number of high-affinity, saturable receptors was much higher, averaging $5.4 \pm 2.7 \times 10^5$ receptors/cell for lampVDR and $3.0 \pm 0.4 \times 10^5$ receptors/cell for hVDR (±SD, n = 3). Moreover, as illustrated in Fig. 4B, the insertless lampVDR cDNA expressed a protein that bound 1,25(OH)$_2$D$_3$ with high affinity. In three independent experiments, dissociation constants for 1,25(OH)$_2$D$_3$ of lampVDR ranged from 0.1 to 1.4 nM and were typically three times greater than the dissociation constants determined for matched hVDR controls (Fig. 4A). Thus, lampVDR can be considered a bona fide VDR based on its high affinity for the 1,25(OH)$_2$D$_3$ hormonal ligand.

Evaluation of functional transcriptional activation by lampVDR

The ability of lampVDR to activate transcription was examined in transfected mammalian cell lines in a series of experiments using reporter genes linked to three different hormone-responsive elements from two different genes, namely rat osteocalcin and human CYP3A4 (41). Two of these VDREs were imperfect direct AGGTCA repeats separated by a spacer of three nucleotides (DR3), exemplified by the human CYP3A4 VDRE with the sequence GGGTCA-gcaAGTTCA (hexanucleotide repeats are in upper case).

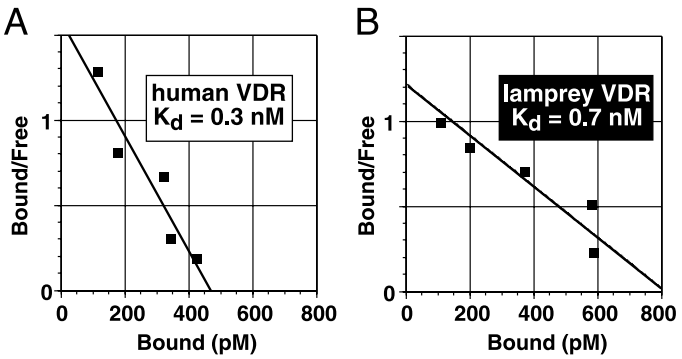

FIG. 4. LampVDR binds 1,25(OH)$_2$D$_3$ with high affinity. Insert B was removed by site-directed mutagenesis (see *Materials and Methods*) to produce lampVDR in the expression plasmid pSG5, which was introduced into COS-7 cells by transfection. Lysates were prepared from these cultures as well as control cultures receiving pSG5-hVDR, pSG5 vector lacking a receptor cDNA, or pSG5 harboring a lampVDR cDNA-containing insert B, and used in saturation binding assays with [^{3}H]-1,25(OH)$_2$D$_3$ (see *Materials and Methods*). Data are shown as Scatchard transformations for hVDR (A) and lampVDR (B). Nonspecific binding was assessed in lysates from cultures containing the pSG5 vector alone (not shown). The results are representative of three independent experiments.

When cultures of COS-7 cells were transfected with insertless lampVDR and a reporter construct containing four copies of the well-characterized rat osteocalcin VDRE, the level of reporter gene expression was only slightly elevated (1.93×) and not significantly higher than that seen in control cells transfected with a pSG5 expression plasmid containing no VDR cDNA insert (1.09×) (Fig. 5A, compare *right two bars* with *left two bars*). In contrast, hVDR mediated a robust (40.8×) transcriptional response from the osteocalcin VDRE (Fig. 5A), as shown in many previous studies (reviewed in Ref. 1). When tested on an everted repeat (ER6) element from the human CYP3A4 gene (Fig. 5B), lampVDR appeared to mediate a small, but not significant, transcriptional response to 10^{-8} M $1,25(OH)_2D_3$, again in contrast to hVDR, which showed a nearly 18-fold induction by $1,25(OH)_2D_3$. However, lampVDR was able to mediate a significant functional response (3.91×) from the CYP3A4 distal VDRE of the DR3 type (Fig. 5C). This response was enhanced to 4.5× (Fig. 5D) by the cotransfection of zebrafish RXRα. In three independent experiments using zebrafish RXRα, the reporter gene up-regulation mediated by lampVDR in the presence of 10^{-8} M $1,25(OH)_2D_3$ was 4.54-, 5.21-, and 4.50-fold, compared with values with the insertless pSG5 expression vector of 1.17-, 1.34-, and 1.44-fold, respectively. LampVDR also appeared to be capable of inhibiting reporter gene transcription in the absence of hormone (*e.g.* Fig. 5D, *white bar* marked with ***).

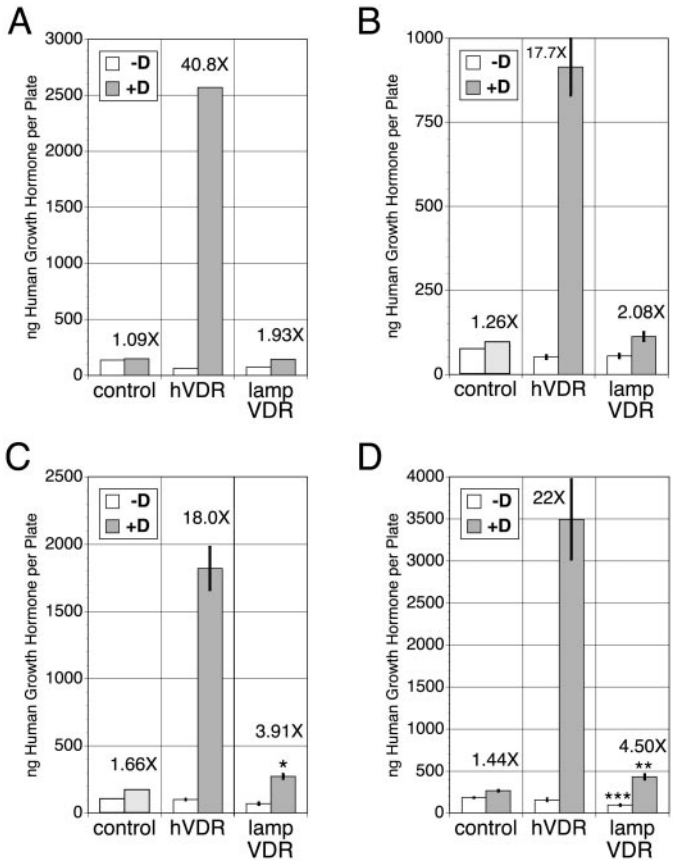

FIG. 5. LampVDR shows a selective ability to mediate $1,25(OH)_2D_3$-dependent transactivation from mammalian VDREs. COS-7 cells were transfected with an empty pSG5 expression plasmid (control), pSG5 plasmid expressing hVDR, or pSG5 vector expressing a lampVDR lacking both inserts. All transfections received a reporter plasmid with a thymidine kinase promoter-driving expression of human GH under the control of various VDREs. A, Four copies of the rat osteocalcin VDRE (29). B, Two copies of the proximal ER6-type element from the human CYP3A4 gene (21, 30). C and D, Two copies of the distal DR3-type element from the human CYP3A4 gene (Ref. 21; see *Materials and Methods* for sequences of these elements). The experiment illustrated in D also included a TIF expression plasmid for zebrafish RXRα (31) to serve as the heterodimeric partner for lampVDR in addition to the monkey RXRα contained in the recipient COS-7 cells. Results are expressed as the amount of human GH secreted in a 24- to 48-h period after transfection. *, Differs from corresponding control value, $P < 0.05$. **, Differs from control, $P < 0.01$. ***, Differs from control, $P < 0.005$. *Bars* (D) represent the average of quadruplicate values ± SD and are typical of three independent experiments.

Phylogenetic comparison of lampVDR with other nuclear receptors

Given the sequence and functional similarity between lampVDR and other VDRs, a phylogenetic analysis was performed to place lampVDR into the context of the complete human group 1 nuclear receptor complement, using an insect group 2 receptor as an outgroup (Fig. 6A). The resulting cladogram confirms that lampVDR is in subgroup NR1I containing known VDRs (1I1) as well as PXRs (1I2) and CARs (1I3). An expanded analysis of lampVDR with a more extensive sampling of group 1I nuclear receptors is shown in Fig. 6B. This analysis includes receptors from mammals, one avian species, one reptile, two amphibians, and three bony fishes. Two VDR sequences, from *G. gecko* (reptile) and *B. marinus* (amphibian), were obtained in our laboratory and are here reported for the first time. In addition, the analysis includes the closest BLAST hits to human and lampVDRs from the genomes of tunicate (*Ciona intestinalis*), fruitfly (*Drosophila melanogaster*), and nematode (*C. elegans*). Among the subgroup 1I receptors, lampVDR unambiguously groups with other VDRs. Numerous other phylogenetic analyses have been performed using different selections of receptors and different regions of the amino acid alignment, but, as shown here, lampVDR always forms the basal member of the VDR grouping and does not group with either the PXRs or CARs (data not shown).

Detailed comparison of functional domains in lampVDR with other VDRs and closely related nuclear receptors

Selected regions of the amino acid sequence from subgroup 1I receptors are shown in Fig. 7. The first of these regions (*left side* of figure) is the N-terminal region that contains a docking site for general transcription factor IIB (TFIIB) in hVDR (42) and the chicken thyroid receptor-α (cTRα; Ref. 43). In both cases, the interacting region contains two or more basic residues (indicated by *circled plus symbols* and enclosed by *brackets* for hVDR and cTRα), flanked on the N-terminal side by a single acidic residue (indicated by a *boxed minus symbol*). The sequence of lampVDR in this region contains only one of the two basic amino acids that are otherwise conserved in all vertebrate VDRs (missing basic residue and other selected sequence divergences highlighted in *yellow*). Also, lampVDR is similar to the PXRs in that the putative TFIIB docking site is flanked on the N-terminal side by an

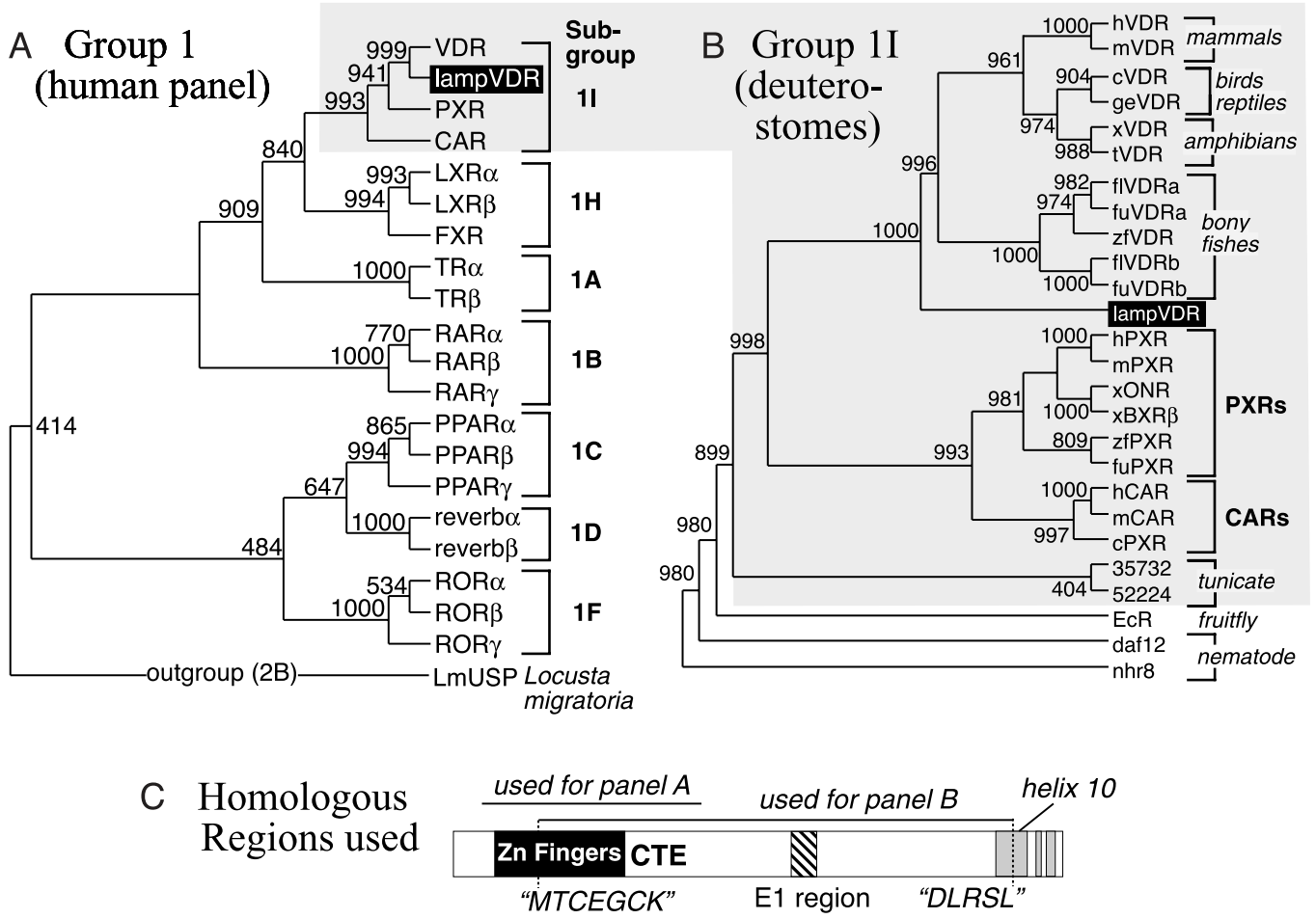

FIG. 6. Phylogenetic relationships between lampVDR and other group 1 nuclear receptors. A, LampVDR groups with hVDR, hPXR, and the human xenobiotic-binding receptor CAR in subgroup 1I, compared with the entire complement of human group 1 receptors. Amino acid sequences of human receptors were obtained from the GenBank database. Sources for hVDR, hPXR, and hCAR are given in the legend to Fig. 3. Other human receptors have the following GenBank accession numbers: liver X receptor-α (LXRα), AAA85856; LXRβ, P55055; farnesoid X receptor (FXR), NP_005114; thyroid hormone receptor-α (TRα), CAA38749; TRβ, TVHUAR; RARα, A29491; RARβ, NP_000956; RARγ, AAA52692; peroxisome proliferator activated receptor-α (PPARα), NP_005027; PPARβ, CAB38629; PPARγ, BAA23354; reverbα, XP_113329; reverbβ, Q14995; RAR-related orphan receptor-α (RORα), U04897; RORβ, Y08639; RORγ, U16997. The ultraspiracle protein from the insect *L. migratoria* (accession no. AAF00981.1) was used as an outgroup for this analysis. A conserved region of 105 amino acids representing the zinc finger DBD and its CTE (see C below) were used to construct this cladogram in Clustal W (25). B, LampVDR is the basal member of known VDRs in subgroup 1I. The phylogenetic position of lampVDR relative to known subgroup 1I receptors from other species was analyzed as in A, except that a 331 amino acid alignment was used, which encompassed approximately two thirds of the DBD (seven of the nine conserved cysteines) and most of the hormone binding domain of VDRs (see C, below). The definitions and accession numbers for hVDR, hPXR, and hCAR are given in the legend to Fig. 3, and the sources of cVDR, xVDR, zfVDR, flVDRa, xONR, and hCAR can be found in the description of Fig. 3 (above). Additional receptors included are: mouse VDR (mVDR), AAH06716; *G. gecko* VDR (geVDR), this study; cane toad (*B. marinus*) VDR (tVDR), this study; pufferfish (*Takifugu rubrides*) VDRa-like protein (fuVDRa), SINFRUP00000073157; Japanese flounder (*Paralichyths olivaceus*) VDRb (flVDRb), BAA95015; mouse PXR (mPXR), AAC39964; *X. laevis* benzoate receptor-β (xBXRβ), AAG24910.1; pufferfish PXR-like protein (fuPXR), SINFRUP00000078207; zebrafish PXR (zfPXR), AAM10635.1; pufferfish VDRb-like protein (fuVDRb), SINFRUP00000064850; mouse CAR (mCAR), AAC53349.1; and chicken xenobiotic receptor (cPXR), AAG18374. Pufferfish sequences were taken from http://scrappy.fugu-sg.org/Fugu_rubripes/blastview. The complete genomes of the tunicate *C. intestinalis*, the fruitfly *D. melanogaster,* and the nematode *C. elegans* were searched using BLAST at the following web addresses: http://genome.jgi-psf.org/ciona4/ciona4.home.html, http://www.ncbi.nlm.nih.gov/blast/, and http://www.wormbase.org/db/searches/blast. Those proteins showing the best matches to the hVDR and lampVDR amino acid sequences were included in the analysis, namely tunicate proteins ci0100135732 (35732) and ci0100152224 (52224), the fruitfly ecdysone receptor (EcR; P34021), and the nematode proteins daf12 (CAC42284) and nhr8 (NP_741445). The cladogram, with bootstrapping, was created in Clustal W (25). C, Schematic diagram of an idealized nuclear receptor showing the segments of amino acid sequence used for the comparisons shown in A and B. E1 and AF2 regions are defined in the legend to Fig. 1A. MTCEGCK and DLRSL refer to conserved sequences in VDRs that define the N- and C-terminal ends, respectively, of the amino acid regions used for the analysis shown in B.

extensive stretch of acidic residues. These differences between lampVDR and other vertebrate VDRs could conceivably impact the transactivation potential of lampVDR when tested in a mammalian system. Indeed, a common polymorphic variant of hVDR has been reported in which the removal of a negatively charged residue from the N terminus enhances transcriptional activity (42, 44).

The second region analyzed is a C-terminal extension (CTE) of the DNA-binding region (Fig. 7, *center*). This region contains clusters of basic amino acids (shown in *red*) that are

conserved between lampVDR and hVDR in a pattern that is distinctive for each subgroup of group 1 receptors (45).

The third region examined in Fig. 7 is a portion of the C-terminal ligand-binding domain that has been shown to form part of the dimerization interface in a cocrystal of hRARα with mouse RXRα (46). Unlike the previously discussed two regions, the sequence of lampVDR in this putative dimer interface is quite divergent from that of other VDRs (differing residues shown in *yellow*) and also related receptors, PXRs and CARs (Fig. 7 and data not shown).

Discussion

The identification of the cDNA cloned in these experiments as encoding a lamprey ortholog to VDR is based on the following: 1) BLAST searches with the lampVDR amino acid sequence return the top 12 matches as vertebrate VDRs with 58–62% identity to lampVDR (most of these are included in the Fig. 6B cladogram); 2) the next closest receptor by BLAST analysis, PXR, shows less than 44% identity to lampVDR; 3) lampVDR binds 1,25$(OH)_2D_3$ with high affinity; and 4) lampVDR is capable of mediating 1,25$(OH)_2D_3$-dependent transactivation from a VDRE-containing reporter gene. However, our data cannot exclude the possibility that the lamprey genome may harbor a second VDR gene, such as is found in pufferfish and flounder.

The present report of a bona fide VDR from an agnathan reveals that this nuclear receptor has a more ancient origin than previously suspected. Complete cDNAs have been reported of apparent VDR homologs from teleost fishes, and, taken together with previous reports of high-affinity 1,25$(OH)_2D_3$ binding in species such as trout (47), eel (16), and cod (17), provide evidence that teleost fishes possess functional VDRs. However, lampreys represent a far more ancient group of fishes than teleosts, having diverged from other vertebrates over 450 million years ago (18–20).

The affinity of lampVDR for 1,25$(OH)_2D_3$ is slightly lower than that of human VDR. This could be due to variation in a residue known to be a ligand contact in the human receptor (39), namely Val-418, which is a leucine in lampVDR. It is perhaps worth noting that the levels of circulating 1,25$(OH)_2D_3$ reported for a Japanese lamprey species, *E. japonicus* (13), are, at 274 pg/ml, approximately 7- to 8-fold higher than the average 1,25$(OH)_2D_3$ levels of 33 pg/ml (48) and 36 pg/ml (49) reported for normal human subjects. Thus, the lower ligand-binding affinity of lampVDR determined in the present study would seem to be compatible with the higher circulating levels of 1,25$(OH)_2D_3$, at least in one lamprey species.

Additional functional testing revealed that lampVDR is capable of modest but reproducible and significant 1,25$(OH)_2D_3$-dependent transactivation from a DR3-type responsive element found in the human CYP3A4 gene (Fig. 5, C and D). LampVDR did not show measurable activity from either the rat osteocalcin VDRE, also of the DR3 type, or ER6-type element in the human CYP3A4 gene. The fact that lampVDR transcriptional activity is of a lower magnitude (5×), compared with hVDR (20×) when tested in a mammalian cell transfection system, could be due to several potential factors. One possibility is that lampVDR may not interact well with mammalian proteins required for transactivation, such as with TFIIB or the endogenous (monkey) RXR isoforms in the host cells used for transfection. As noted above, the amino acid sequence of lampVDR is quite divergent from that of other VDRs in a region corresponding to a dimerization domain in hRARα (Fig. 7, *bottom right*). This possibility is further supported by gel mobility shift analyses, which demonstrated that lampVDR was incapable of forming a detectable complex with human RXRα on either the rat osteocalcin VDRE or mouse osteopontin VDRE (with the sequence GGTTCAcgaGGTTCA) (50), under conditions that allowed for strong complex formation by hVDR (data not shown). That mammalian RXRβ or RXRγ isoforms are also not ideal partners for lampVDR is strongly suggested by transactivation experiments performed in HeLa cells (containing mainly human RXRβ) (51) or HT-29 cells (containing mainly human RXRγ) (52), which also showed no activity with the rat osteocalcin VDRE reporter construct (data not shown). The additional observation that zebrafish RXRα was able to boost transactivation by lampVDR (Fig. 5D) is consistent with the fact that three recently isolated lamprey RXR isoforms show slightly higher amino acid sequence identities to zebrafish RXRα (72%, 73%, and 72%), compared with identities to human RXRα of 70%, 70%, and 69%, respectively (Manzon, L. A., and J. H. Youson, unpublished data).

The evident difficulty in heterodimerization between lampVDR and mammalian RXRs may help explain the selectivity of lampVDR with respect to transactivation. The CYP3A4 DR3 (GGGTCAgcaAGTTCA) (taken from Ref. 21), which supported measurable transcriptional activation by lampVDR (Fig. 5, C and D), represents a nearly perfect high-affinity site for VDR-RXR binding, as determined by binding of hVDR-RXR heterodimers to random oligonucleotides (reported as PuGGTCAxxgPuGTTCA, where Pu is either A or G) (53, 54). Thus, a perfect VDRE may be able to compensate in some way for an imperfect heterodimeric partner, perhaps because of the very high affinity of VDR and RXR for their respective DR3 half-elements.

Another, more speculative explanation relates to the type of gene from which the VDRE was taken. Rat osteocalcin is a Gla-containing, calcium-binding protein expressed in bone that likely has no counterpart in the boneless lamprey. On the other hand, CYP3A4 is an enzyme whose function is believed to be detoxification of xenobiotics. Recent studies have shown that VDR can not only regulate human CYP3A4 in intestine (21, 41) but can also bind to certain toxic ligands such as the secondary bile acid, lithocholic acid, presumably serving as a sensor to activate detoxification of these compounds in mammals (55). This novel activity of VDR is similar to reported actions of PXR and CAR (56), perhaps reflecting the close evolutionary relationship of these receptors (Fig. 6). It has been reported further that a protein in *C. elegans*, nhr-8, whose amino acid sequence shows a similarity to the VDR/PXR/CAR grouping (57), plays a role in detoxification of colchicine and chloroquine in the nematode gut (58). This observation suggests that activation of detoxification pathways may be a very ancient function of group 1I receptors (58).

The present phylogenetic analyses confirm that PXRs and CARs are the closest relatives of VDR. Indeed, a standardized

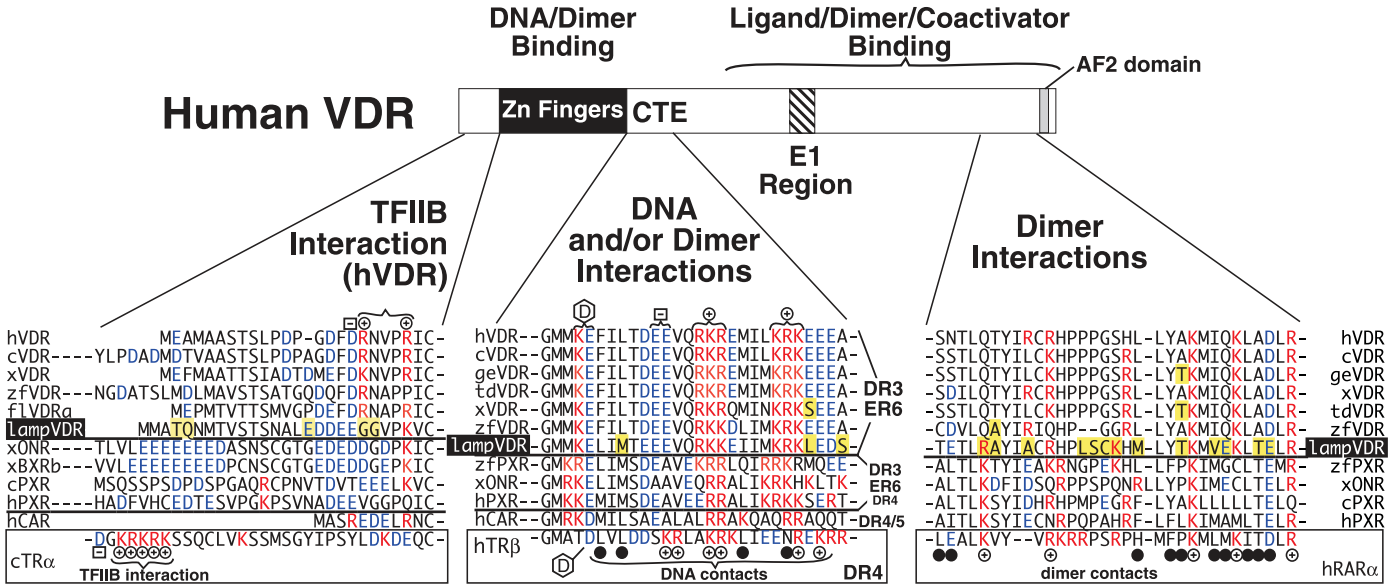

FIG. 7. Sequence comparisons in selected functional domains between lampVDR, other VDRs, PXRs, and related receptors. Accession numbers for most amino acid sequences are given in legend to Figs. 3 and 6. VDR sequences from *G. gecko* and the cane toad, *B. marinus*, are designated as geVDR and tdVDR, respectively, and were obtained as part of this study. *Top panel* shows a schematic representation of functional domains in hVDR, with *lines* indicating those regions that were chosen for analysis. The *left lower panel* contains a comparison of the extreme N terminus of hVDR, compared with selected group 1I receptors, along with the cTRα (accession no. P04625). Residues of particular interest in this region are a pair of basic residues (shown in *red* and indicated with *plus symbols*) that mediate interaction of hVDR with TFIIB (42) and a cluster of basic residues that subserve the same function in cTRα (43). Acidic residues are shown in *blue type.* Those residues in lampVDR that differ significantly from corresponding residues in other VDRs are highlighted in *yellow. Panel at bottom center* compares sequences just C-terminal to the zinc finger DBD, with emphasis on conserved groups of basic (*red*) and acidic (*blue*) amino acids. This region has been shown to contain DNA contacts in a cocrystal of the human thyroid receptor-β (hTRβ) DBD complexed with the same domain of RXRα on a thyroid hormone response element (62). Those known or suspected DNA contacts that are basic charged residues (see text) are indicated by a *circled plus symbol;* other DNA contacts are shown with *solid circles.* Residues shown to be important in dimer formation with RXR on a DR3 (hVDR) or DR4 (hTRβ) are indicated by a *capital D inside a hexagon.* The *right lower panel* illustrates a region near the C terminus that is also involved in dimerization with the RXR coreceptor. The bottom sequence is that of hRARα (accession no. A29491), whose ligand-binding domain has been crystallized as a dimer with the ligand-binding domain of mouse RXRα (46). Residues in hRARα that form part of the dimer interface are indicated by *solid circles* or, if the contact is a basic residue, a *circled plus symbol.* Selected residues in lampVDR, zfVDR, tdVDR, xVDR, or geVDR that differ from other VDRs are shown with *yellow highlighting.*

nuclear receptor nomenclature system places VDRs and PXRs/xONRs as the sister groups NR1I1 and NR1I2 (2, 59). Another striking similarity between VDRs and the PXR/ONR grouping exists as well because these receptors are the only known members of the superfamily to heterodimerize with RXR on a DR3-type responsive element (60, 61). The ability of VDRs and PXRs to preferentially bind DR3-type elements is perhaps reflected in the conservation of the amino acid residues in the DBD. A cocrystal of the human thyroid hormone receptor-β and human RXR-α DBDs on a thyroid hormone DNA response element (62) has revealed that residues just C-terminal to the zinc finger DNA-binding motifs in the thyroid hormone receptor make very significant DNA contacts. Further, of 10 such contacts, six involve positively charged residues. When the corresponding regions of VDRs and PXRs are examined (Fig. 7, *center panel;* see also Ref. 45), it can be seen that clusters of positively charged residues occur in the CTE of the DBD. The conservation of these positively charged clusters between VDRs (including lampVDR) and PXRs is striking and entirely consistent with the above-cited observation that these receptors, alone among known nuclear receptors, bind to DR3-type DNA elements (45, 60–62).

This high degree of relatedness suggests that VDRs (NR1I1) and the PXR/xONR group (NR1I2) diverged relatively recently in evolutionary history, presumably by means of gene duplication (reviewed in Ref. 63). The analysis in Fig. 6B suggests that such a duplication may have taken place before the divergence of vertebrates from nonvertebrate chordates, perhaps during one of two genome-wide duplications proposed to have transpired in early vertebrate evolution (63). This conclusion is based on the presence of separate VDR-like and PXR-like sequences in the pufferfish genome and also on the observation that related sequences from tunicate, fruitfly, and nematode seem to predate the divergence of VDR from PXRs. However, this conclusion must remain tentative because of the uncertainty of whether lampreys or other groups, such as cephalochordates, contain PXR-like sequences. Regarding CARs (NR1I3), the absence of any CAR-like sequences in fish suggests that CAR may be a tetrapod innovation. The exact timing of these divergences must await the analysis of sequences from other taxa. Nevertheless, the present analysis points to an origin of VDR at the dawn of vertebrates. What, then, might have been its ancestral function?

Studies of mice in which the VDR has been ablated support the traditional view that VDR, with its 1,25$(OH)_2D_3$ ligand, plays a crucial role in the formation of calcified structures

(bones and teeth) by acting as a hypercalcemic hormone to promote both calcium and phosphate absorption from the intestine as well as calcium and phosphate retention at the kidney (4, 64). Other studies of VDR null mice have also revealed a role for VDR in skin, mainly in the mammalian hair cycle (5). Translation of these observations in mice to the physiology of lampreys is made difficult by the absence in the latter species of a calcified skeleton and teeth, as well as hair. Nevertheless, related functions may exist in lampreys, or in their ancestors, that may be regulated by VDR.

There are fossil groups of jawless fishes that possessed extensive dermal plates, thought to have contained a calcium phosphate derivative (18, 19). One interpretation (reviewed in Ref. 18) holds that lampreys are descendants of these armored agnathans and that extant lampreys have secondarily lost the ability to form these calcified structures. Two published observations would tend to support this view: an extant lamprey species, *Ichthyomyzon unicuspis*, has been reported to possess calcified cartilage in the head region (65), and living (but not dead) tissues from *P. marinus* adult specimens (head region) have been shown to be capable of calcifying the extracellular matrix when supplied with sufficient calcium and phosphate in organ culture (66).

If it is true that modern lampreys are descended from organisms that had calcified head plates, then the ability to form calcified structures (including, presumably, VDR action to mediate calcium homeostasis) may still be latent in lampreys but not actually expressed. This lack of VDR activity could be due to a lower level of lampVDR expression in key tissues such as intestine and kidney or an inactivation of lampVDR at key times in development. It is conceivable that both of these mechanisms might be at work in *P. marinus* because we have observed both a low level of intestinal expression of lampVDR in the juvenile as well as inactivating insertions in the larval protospleen. A more complete examination of larval juvenile and adult tissues will be required to fully examine this hypothesis.

An alternative interpretation of vertebrate evolution (reviewed in Ref. 19) is that lampreys diverged from other vertebrates before the advent of calcified tissues. If this view is correct, then the occurrence of VDR in a lamprey is evidence that VDR phylogenetically predates calcified structures. This does not totally preclude a role for lampVDR in calcium homeostasis because calcium levels might require regulation for other physiologic reasons. However, a role for VDR in skin differentiation is well established (5), and lampreys, although lacking hair, do have specialized skin structures such as keratinized teeth and mucous glands (67). Whether VDR regulates the development or maintenance of either teeth or mucous glands in lampreys is unknown, although the (qualitatively) high expression of normally spliced lampVDR mRNA in juvenile skin and mouth tissue is consistent with such a possibility.

As introduced above, another candidate function for lampVDR is as a sensor for endogenous or exogenous toxins and an inducer of CYP enzymes to detoxify them. An implication of this idea for VDR is that the toxins themselves, rather than (or in addition to) $1,25(OH)_2D_3$, are ligands for VDR. Indeed, human and rodent VDRs have recently been shown to bind to and be activated by toxic bile salt derivatives, including lithocholic acid and 3-keto lithocholic acid (55). Whether lampVDR binds to the lamprey versions of bile acids or their derivatives has not yet been tested.

Yet a third possibility for a VDR function in lampreys is suggested by many studies documenting a role of the $1,25(OH)_2D_3$-VDR system in the regulation of immune function. In mammals, VDR is involved in differentiation and regulation of certain immune cell types, including T-cells (see reviews in Refs. 68, 69). Although lampreys appear to lack the defining characteristics of the adaptive or combinatorial immune system in higher vertebrates, namely immunoglobulins, T cell receptors, or recombination activator genes (70), larval lampreys do possess cells in either protospleen (71) or gut epithelium (72, 73) that not only morphogenetically resemble lymphocytes but, in the case of the gut-associated cells, also express genes that are known to be crucial for lymphocyte differentiation in higher vertebrates (73–75). Our attempts to isolate lampVDR from juvenile intestine and larval peripheral blood lymphocytes have been either equivocal (intestine) or consistently negative (peripheral blood lymphocytes, data not shown). Also, as noted above, larval intestine yielded lampVDR transcripts that appear to contain inactivating insertions. Nevertheless, it might be worthwhile to examine more thoroughly the possibility of lampVDR expression in larval and/or juvenile lymphocyte-related cells, or in the adult fat column, in which lymphoid-like cells have been shown to reside (76).

In summary, the modern actions of VDR in terrestrial animals relating to a mineralized skeleton for locomotion and hair growth for protection from the sun are clearly not relevant in the sea lamprey. Instead, it must be hypothesized that VDR plays a different and, perhaps, more fundamental role in this ancient vertebrate. A full appreciation of the evolution of the VDR/PXR/CAR nuclear receptor grouping will require further studies in a variety of vertebrates with an ancient lineage as well as in key organisms such as protochordates and echinoderms. The presence of the related nhr-8 receptor in *C. elegans*, as noted above (57, 58), indicates that the evolutionary history of the VDR/PXR/CAR grouping could be very ancient indeed.

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Address all correspondence and requests for reprints to: G. Kerr Whitfield, Department of Biochemistry & Molecular Biophysics, University of Arizona College of Medicine, 1501 North Campbell Avenue, P.O. Box 245042, Tucson, Arizona 85724. E-mail: kerr@medbioc.arizona.edu.

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