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A novel nuclear receptor superfamily member in *Xenopus* that associates with RXR, and shares extensive sequence similarity to the mammalian vitamin D3 receptor

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ABSTRACT

We report the isolation of xONR1, a novel member of the nuclear receptor superfamily from *Xenopus laevis*. xONR1 shares a high degree of amino acid sequence identity with the mammalian receptor for 1α, 25-dihydroxy vitamin D3, particularly within the DNA-binding domain, although it does not bind this ligand. xONR1 DNA binding is stimulated by association with retinoid X receptor gamma (RXRγ).

INTRODUCTION

The nuclear receptor superfamily is a group of structurally-related, ligand-modulated transcription factors which mediate the actions of a number of steroid hormones (glucocorticoid, mineralocorticoid, estrogen, progesterone, androgen, ecdysone) and non-steroid hormones (thyroid hormone, retinoids, 1α, 25 dihydroxy vitamin D3) (1, 2, 3). The family also includes a growing number of members for which the ligand has yet to be identified, the so-called orphan receptors (4).

A subset of the nuclear receptor superfamily require heterodimerisation with an accessory protein for efficient DNA binding and transcriptional activation (5, 6). Retinoid X receptor (RXR; the receptor for 9-cis-retinoic acid) acts as an accessory protein for retinoic acid receptors (RARs), thyroid hormone receptors (TRs), peroxisome proliferator receptors (PPARs), 1α, 25-dihydroxy vitamin D3 receptor (VDR), and ecdysone receptor (EcR).

The administration of retinoids to vertebrate embryos causes a range of developmental abnormalities, and it has been proposed that retinoids may have morphogenetic roles in normal development, acting via nuclear receptors (7, 8). As part of a project to analyse the functions of retinoids in the early embryogenesis of *Xenopus laevis* we have screened embryonic cDNA libraries for retinoid receptors, and receptors interacting with RXRs. The use of a human VDR cDNA probe (9), at low stringency, detected a novel nuclear receptor family member, which we have termed xONR1, for *Xenopus* orphan nuclear receptor.

METHODS

Isolation of xONR1 cDNA

The plasmid HVDR1/3, containing an approx. 2kb fragment of the human 1α, 25 dihydroxy vitamin D3 receptor cDNA (hVDR) (9) which encompasses the entire coding region in the vector pGEM7Z (Promega), was a gift of D.P.McDonnell (Ligand Pharmaceuticals Inc.). The 2kb fragment (released by *Eco* RI digestion) was labelled by nick translation and used to screen a stage 13 *X.laevis* embryonic cDNA library in λgt11 (a gift of K.Richter, ref. 10) using standard methods (11). Low stringency hybridisation and washing conditions were 6×SSC/50% formamide/0.1% SDS at 37°C and 2×SSC/0.1% SDS at 50°C, respectively.

One of the cDNA clones isolated was released from the vector as 3 *Eco* RI fragments, that is the cDNA had 2 internal *Eco* RI sites. The 5'-most 2 *Eco* RI fragments were cloned, together, into pBluescript KS+ (Stratagene), and this region was later shown to encompass the entire coding region. The cDNA insert of this subclone is referred to as xONR1 cDNA. xONR1 cDNA was sequenced, by dideoxy chain termination, from subclones made in M13mp18 and M13mp19. The 3'-most approx. 650bp *Eco* RI fragment, encompassing 3' untranslated region, was not sequenced.

Isolation of xRXRγ cDNA

xRXRγ cDNA was isolated by RT-PCR using primers made to the published xRXRγ cDNA sequence (12). The 5' primer was a 17mer including the initiating ATG codon (5'AGGATGCATC-TTGCTAC3'), and the 3' primer was a 17mer extending from the stop codon (5'TCATGAGATCTGGTGAG3'). Total nucleic acid was prepared from *X.laevis* oocytes as described previously (13), and polyadenylated mRNA selected from this by standard methods (11). cDNA was synthesised in 100μl of PCR buffer (Promega) containing 1μg A^+ RNA, 200μM 4 dNTPs, 10 units AMV reverse transcriptase and 200ng of 3' primer at 37°C for 30 minutes. 200ng of 5' primer and 3 units of Taq DNA polymerase (Promega) were added to this and PCR amplification carried for 30 cycles of 94°C 1 min/50°C 1.5 min/72°C 1.5 min. The amplified cDNA fragment was subcloned into the vector

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pSP64T (14), which contains 5′ and 3′ untranslated regions from *X.laevis* β globin cDNA, at the *Bgl*II cloning site.

Receptor expression in oocytes and embryos

For expression in *X.laevis* oocytes and embryos the xONR1 cDNA was subcloned into the vector pSP64T (by blunt ligation into the filled-in *Bgl*II cloning site). Capped synthetic mRNA was transcribed from xONR1, xRARγ and hVDR cDNA-containing plasmids (linearised with *Sal*I, *Sst*I and *Xba*I, respectively) with SP6 RNA polymerase (essentially as described in ref. 15). These transcripts are referred to as xONR1, xRARγ and hVDR mRNA. Working mRNA stocks were approx. 0.5mg/ml.

Oocytes were injected into the cytoplasm with approx. 20nl of mRNA and maintained at 16°C in Barth-X (16) for 16 hours. For protein labelling oocytes were maintained in media containing ^{35}S-methionine (1mCi/ml).

Embryos were obtained and cultured as described previously (17) and injected with approx. 20nl of mRNA, at a stage when the first cleavage furrow was forming but not necessarily complete, and maintained at 16°C until stage 9. For protein labelling embryos were injected, into the blastocoel, at stage 6 with approx. 20nl ^{35}S-methionine (15μCi/μl).

Labelled oocyte and embryo extract was prepared as described below for band shift assay extract, and analysed on 12% SDS-polyacrylamide gels.

1α, 25-dihydroxy vitamin D3 binding assays

1α, 25-dihydroxy vitamin D3 binding assays were performed by a method modified from (18). Pools of 12 uninjected and mRNA-injected oocytes were homogenised in 50μl per oocyte of binding buffer (120mM KCl, 8mM Tris.HCl pH7.5, 8% glycerol, 4mM DTT, 0.5mM PMSF, 5mM $MgCl_2$) on ice, the homogenate microfuged for 10 minutes and the supernatant transferred. Oocyte extract (equivalent to 0.4 to 2 oocytes) was mixed, on ice, with binding buffer in a total volume of 1ml, and 0.02μCi of radiolabelled 1α, 25-dihydroxy vitamin D3 (1α, 25-dihydroxy [26,27-methyl-^{3}H] vitamin D3; 172 Ci/mmol; Amersham) added, with or without 'cold' competitor 1α, 25-dihydroxy vitamin D3 (a gift of the Roche Research Centre). When added, competitor 1α, 25-dihydroxy vitamin D3 was used in 100-fold molar excess. The mixture was incubated at 2°C for 6 hours. 0.5ml of 3% w/v dextran-coated activated charcoal (prepared as described in ref. 18) was added to adsorb unbound 1α, 25-dihydroxy vitamin D3, and the mixture incubated on ice for 15 minutes. Following microfugation for 10 minutes the supernatant was transferred, added to scintillant (Optiphase-Safe; LKB), and the bound radiolabelled 1α, 25-dihydroxy vitamin D3 counted in a scintillation counter. High affinity 1α, 25-dihydroxy vitamin D3 binding in was determined by subtraction of the counts bound in the presence of competitor, from the counts bound in the absence of competitor.

Band shift assays

Extracts were made from pools of 12 uninjected and mRNA-injected embryos at stage 9 as described previously (19). Band shift assays (20μl) contained 10mM Tris.HCl pH8, 40mM KCl, 0.05% nonidet P-40, 6% glycerol, 1mM DTT, 0.5mM PMSF, 0.2μg poly(dI-dC), 2μg salmon sperm DNA, and 6μl embryo extract (equivalent to 1.2 embryos). Assays were incubated on ice for 10 minutes, 0.25ng of ^{32}P-end labelled duplex oligonucleotide added, and incubation continued for 15 minutes. DNA–protein complexes were resolved on a 4% polyacrylamide gel (29:1 bis) in Tris–borate buffer (45mM Tris–borate pH8, 1mM EDTA) at 200 volts for 75 minutes. Gels were fixed, dried and autoradiographed. The duplex oligonucleotide was 5′ AGC-TTCAGGTCAAGGAGGTCAGAGAGCT 3′, which contains the DR-3 response element (AGGTCA AGG AGGTCA) (20).

Northern blot

Polyadenylated RNA was prepared from *X.laevis* embryos (staged according to ref. 21) as described above for oocytes. 5μg of polyadenylated RNA was electrophoresed on a 1.5%

A.

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xONR  MMKVQETLVLEEEEEEEDASNSCGTGEDEDDGDPKICRACGDRATGYHFN
hVDR  M------------EAMAASTSLPDPGDFDRNVPRICGVCGDRATGFHFN

xONR  AMTCEGCKGFFRRAVKRNLRLSCPFQNSCVINKSNRRHCQACRLKKCLDI
hVDR  AMTCEGCKGFFRRSMKRKALFTCPFNGDCRITKDNRRHCEACRLKRCVDI

xONR  GMRKELIMSDAAVEQRRALIKRKHKLTKLPPTPPGASLTPEQQHFLTQLV
hVDR  GMMKEFILTDEEVQRKREMILKRKEEEALKDSLR-PKLSEEQQRIIAILL

xONR  GAHTKTFDFNFT-FSKNFRP----------------------------
hVDR  DAHEKTYDPTYSDFCQ-FRPPVRVNDGGGSHPSRPNSRHTPSFSGDSSSS

xONR  ----------IRRSS---------DPTQEPQATSSEAFL-MLPHISDLVT
hVDR  CSDECITSSDMMDSSSFSNLDLSEEDSDDPSVTLELSQLSMLPHLADLVS

xONR  YMIKGIISFAKMLPYFKSLDIEDQIALLKGSVAEVSVIRFNTVFNSDTNT
hVDR  YSIQKVIGFAKMIPGFRDLTSEDQIVLLKSSAIEVIMLRSNESFTMDDMS

xONR  WECGP--FTYDTEDMFLAGFRQLFLEPLVRIHRMSRKLNLQSEEYAMGAA
hVDR  WTCGNEDMKNKVSDVTKACHSLELIEPLIKFQVGLKKLNLHEEEHVLLMA

xONR  LSIFASDRPGVCDMEKIQKLQEHIALTLKDFIDSQRPPSPQNRLLYPKIM
hVDR  ICIVSPDRPGVQDAALIEAIQDRLSNTLQTYIRCRHPP-PGSHLLYAKMI

xONR  ECLTELRTVMDIHSKQLLEIWDIQPDA----TPLMREVFGSP-E
hVDR  QKLADLRSLNEEHSKEY-RCLSFQPECSMKLTPLVLEVFGNEIS
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B.

	% AMINO ACID IDENTITY TO xONR C-DOMAIN	E-DOMAIN	ALIGNMENT OF FIRST ZINC FINGER SEQUENCES
xONR			CRACGDRATGYHFNAMTCEGCKGFFRR
hVDR	71	40	-GV-------F----------
dEcR	59	28	-LV-----S---Y--L----------
xTRα	55	27	-VV-S-K-----YRCI----------
xPPARβ	52	28	-KI-----S-FYGVEA----------
xRARγ	48	27	-FV-N-KSS---YGVSS---------
xRXRγ	48	18	-AI----SS-K-YGVYS--------K-
xCOUP	52	21	-LV---KSS-K-YGQF------S--K-
hNF4	55	24	-AI-------K-YG-SS-D--------
hERR2	51	24	-LV---I-S---YGVAS--A--A--K-
hEAR1	50	25	-KV---V-S-F-YGVEA---------
dKnr1	55	21	-KV--EP-A-F--G-F------S--G-
dE75	50	23	--V---K-S-F-YGVES---------

Figure 1. Amino acid sequence comparison of xONR1 to other receptors. **(a)** Sequence alignment of xONR1 and hVDR putative amino acid sequences. Sequences were aligned with the Clustal V program (23). Vertical lines indicate identical amino acids, and dots conservative amino acid differences. Regions of sequence enclosed by lines are the putative C (DNA binding) and E (ligand binding) domains, respectively. **(b)** Percentage identity of the C and E domains of xONR1 to some other members of the nuclear receptor superfamily, and amino acid sequence alignment of C-domains in the region of the proposed first zinc finger. Location of the C and E domains of xONR1 is illustrated in (a). Amino acid sequences were aligned with the Clustal V program to calculate percentage identities. In the sequence alignment a dash represents an identical amino acid. hVDR, human 1α, 25-dihydroxy vitamin D3 receptor (9); dEcR, *Drosophila* ecdysone receptor(24); xTRα, *Xenopus* thyroid hormone receptor α (25); xPPARβ, *Xenopus* peroxisome proliferator receptor β (26); xRARγ, *Xenopus* retinoic acid receptor γ (27); xRXRγ, *Xenopus* retinoid X receptor γ (12); and the orphan receptors most closely related to xONR1: xCOUP-TF (28), hNF4 (29), dKnrl (30), dE75 (31) , hEAR1 (37) and hERR2 (38).

formaldehyde agarose gel, the gel Northern blotted, and the filter hybridised using standard methods (11). The gel was stained with ethidium bromide to check the consistency of loading between different samples, and to determine the position of the bands of residual ribosomal RNA. The probe used was the xONR1 cDNA *Eco* RI fragment between nucleotides 472 and 1633 (see figure 1a) labelled by nick translation. Hybridisation and washing conditions were 6×SSC/50% formamide/0.1% SDS at 37°C and 0.2×SSC/0.1% SDS at 65°C, respectively.

RESULTS

xONR1, a novel member of the nuclear receptor superfamily isolated by homology to the human 1α, 25-dihydroxy vitamin D3 receptor

A probe encompassing the entire coding region of hVDR (1α, 25-dihydroxy vitamin D3 receptor) was used to screen a *Xenopus laevis* stage 13 embryonic cDNA library (details in methods). One of the clones isolated, termed xONR1, consisted of 3 *Eco* RI fragments. The 5'-most 2 *Eco* RI fragments, comprising 1633 nucleotides and containing the entire putative coding region of xONR1, were sequenced. The predicted xONR1 protein has a molecular weight of approx. 45kD, which is good agreement with the estimated size of the translation product observed on expression in oocytes and embryos that had been injected with synthetic mRNA (see later).

Comparison of the predicted xONR1 amino acid sequence to hVDR and other members of the nuclear receptor superfamily demonstrates that xONR1 is a new member of the superfamily (figure 1). The xONR1 putative DNA binding domain contains the P box (believed to determine response element half-site recognition, 20, reviewed in 22) sequence CEGCKG in the first zinc finger, and is therefore a member of the sub group of receptors of which the main members are retinoid receptors, thyroid hormone receptors, 1α, 25-dihydroxy vitamin D3 receptor and peroxisome proliferator receptors (some others are listed in figure 1b).

xONR1 is much more closely related to hVDR than any other member of the receptor superfamily (figure 1). xONR1 and hVDR share significant sequence similarity in the conserved C (putative DNA binding) and E (putative ligand binding) domains: 71% and 40% identity, respectively. In these regions identity to other members of the superfamily ranges between 42% and 59% for the C domain, and between 16% and 28% for the E domain. The superfamily members to which xONR1 is most closely related are listed in figure 1b. The first zinc finger region of the C domain of xONR1 and hVDR is particularly well conserved (figure 1b). The amino acids in the variable region preceding the P box (FNAMTCEGCKG) are conserved between xONR1 and hVDR, and seem characteristic of these two receptors. It is this first zinc finger which is believed to determine target sequence selection. The N-terminal A/B domain, and the D domain (see ref. 1 for domain designation) of xONR1 display little homology to the corresponding regions of hVDR.

The level of identity between xONR1 and hVDR is lower than would be expected, cross-species, for homologous receptors. For example, human and *Xenopus* RARγ share 95% identity in the C domain and 93% identity in the E domain (12). Consequently, xONR1 is most likely not the true homologue of hVDR, and does not in fact bind 1α, 25-dihydroxy vitamin D3 (see later).

xONR1 does not bind 1α, 25-dihydroxy vitamin D3

For 1α, 25-dihydroxy vitamin D3 binding assays xONR1 and hVDR were expressed in *Xenopus laevis* oocytes by injection of synthetic mRNA. Expression in oocytes was confirmed by labelling translation products with ^{35}S-methionine, and resolving proteins in oocyte extract on a SDS-polyacrylamide gel (figure 2a). Protein bands corresponding to translation of the injected mRNAs are seen, relative to uninjected embryos. xONR1 and hVDR are expressed at similar levels.

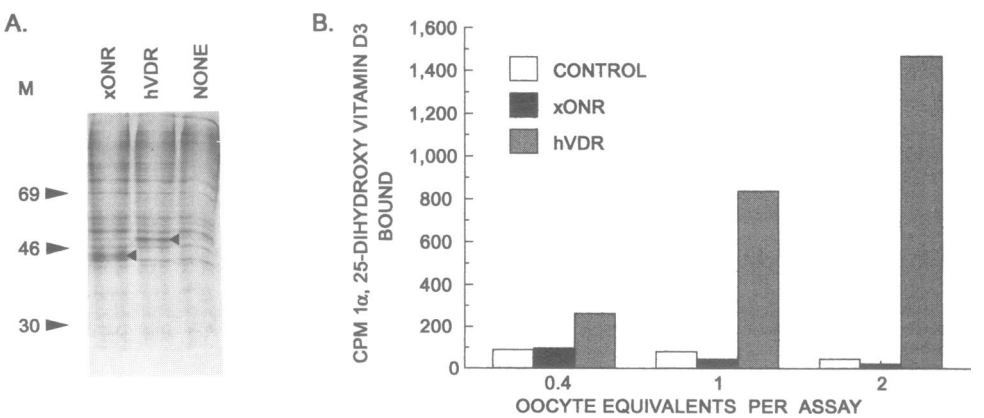

Figure 2. xONR1 does not bind 1α, 25-dihydroxy vitamin D3. **(a)** Expression of xONR1 and hVDR in oocytes. *Xenopus laevis* oocytes were injected with the synthetic mRNAs indicated (xONR1 or hVDR), and some of these oocytes along with uninjected oocytes labelled with ^{35}S-methionine (see methods). Extract equivalent to 0.25 oocytes was electrophoresed on a 12% SDS-polyacrylamide gel, the gel dried and labelled translation products visualised by autoradiography. Labelled protein bands (indicated by arrowheads) corresponding to the translation products of xONR1 mRNA and hVDR mRNA are seen relative to control oocytes. MW size markers (M) are in kD. **(b)** 1α, 25-dihydroxy vitamin D3 (D3) binding assays. xONR1 mRNA-injected, hVDR mRNA-injected, and control (uninjected) oocytes, from the same batches which were labelled to check expression in (a), were used to make extracts for binding assays (see methods). The different amounts of extract indicated were incubated with radiolabelled D3, unbound D3 adsorbed with charcoal, and the residual bound radiolabelled D3 counted. Duplicate assays were set up with and without a 100-fold molar excess of unlabelled D3. The value given represents the amount of specific binding, that is the counts per minute (cpm) bound in the absence of competitor minus the cpm bound in the presence of competitor.

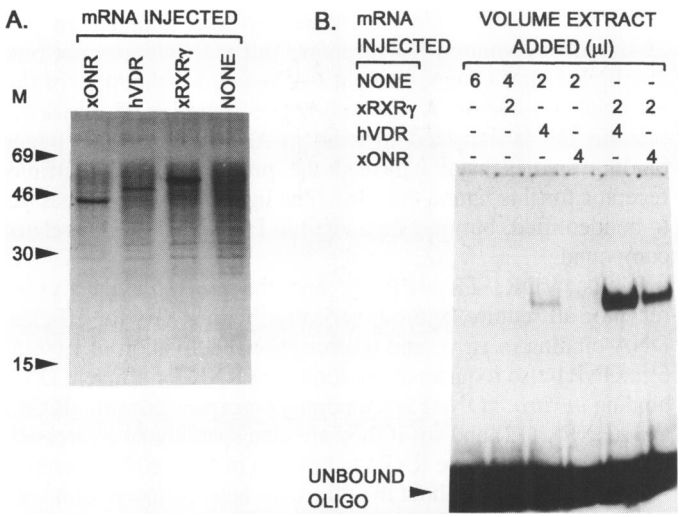

Figure 3. xONR1 binds to a DR-3 DNA element in association with RXR. **(a)** Expression of xONR1, hVDR and xRXRγ in embryos. *Xenopus laevis* embryos were injected with synthetic mRNAs, and some of these embryos along with control (uninjected) embryos labelled with ^{35}S-methionine (see methods). Extract equivalent to 0.25 embryos was electrophoresed on a 12% SDS-polyacrylamide gel, the gel dried and labelled translation products visualised by autoradiography. Labelled protein bands corresponding to the translation products of xONR1 mRNA, hVDR mRNA, and xRXRγ mRNA are seen relative to control embryos. MW size markers (M) are in kD. **(b)** Band shift assays. mRNA-injected embryos, and control (uninjected) embryos, from the batches which were labelled to check expression in (a), were used to make extract for band shift assays. Band shift assays contained a total of 6μl of embryo extract (equivalent to 1.2 embryos) and 0.25ng of end-labelled duplex DR-3 oligonucleotide (see methods). Embryo extracts were mixed in the assays as indicated. Protein−DNA complexes were resolved on a 4% polyacrylamide gel, and the gel dried and autoradiographed.

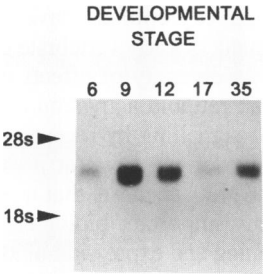

Figure 4. Temporal expression of xONR1 transcripts in early embryogenesis. Polyadenylated RNA from early *Xenopus laevis* embryos of the stages indicated (staged according to 21) was resolved by electrophoresis on a 1.5% formaldehyde-agarose gel. The consistency of RNA loading was checked by staining the residual ribosomal RNA bands with ethidium bromide. The gel was Northern blotted and the resultant filter hybridised to a restriction fragment of xONR1 (nucleotides 472 to 1663) radiolabelled by nick translation. The final wash stringency was 0.2×SSC/0.1% SDS at 65°C. The positions of 18s and 28s ribosomal RNA bands is indicated.

xONR1 mRNA-injected and hVDR mRNA-injected oocytes, and uninjected oocytes, from the same batches that were labelled with ^{35}S-methionine to confirm expression, were used to make extract for 1α, 25-dihydroxy vitamin D3 binding assays. Different amounts of oocyte extract were incubated with radiolabelled 1α, 25-dihydroxy vitamin D3, unbound 1α, 25-dihydroxy vitamin D3 adsorbed with charcoal, and the residual bound 1α, 25-dihydroxy vitamin D3 measured. For each assay, a second duplicate assay containing a 100-fold molar excess of unlabelled competitor 1α, 25-dihydroxy vitamin D3 was carried out. Specific 1α, 25-dihydroxy vitamin D3 binding was determined by subtraction of the counts bound in the presence of competitor from the counts bound in the absence of competitor. The results are shown in figure 2b. As expected, extract from hVDR expressing oocytes displays specific 1α, 25-dihydroxy vitamin D3 binding. However, extract from oocytes expressing xONR1 (at a level similar to the hVDR expression) displays no specific 1α, 25-dihydroxy vitamin D3 binding above control (uninjected oocyte extract) levels. The experiment shown in figure 2 was repeated on 2 other occasions with essentially the same result.

Despite the significant identity between xONR1 and hVDR in the putative ligand binding domain, 1α, 25-dihydroxy vitamin D3 is not a ligand for xONR1. The ligand for xONR1 is, therefore, unknown and xONR1 is a novel orphan nuclear receptor superfamily member (hence the name xONR1, for *Xenopus* orphan nuclear receptor).

xONR1 binds to a DR-3 DNA element in association with RXR

Nuclear receptors of the retinoid/thyroid/vitamin D3 group loosely obey the 3−4−5 rule for the preference of the DNA response element to which they bind (20, 32, reviewed in 5). That is, they bind response elements containing two direct repeat copies of a sequence related to AGGTCA (the P box CEGCKG specifying recognition of this half-site), the spacing between the repeats determining which receptor (as a heterodimer in conjunction with RXR) a particular response element interacts with. A response element with a spacing of 3 base pairs is a 1α, 25-dihydroxy vitamin D3 receptor response element, with 4 base pairs a thyroid hormone receptor response element, and with 5 base pairs a retinoic acid receptor response element. These response elements are described as DR-3, DR-4 and DR-5, respectively.

Bearing in mind the similarity between xONR1 and hVDR in the putative DNA binding domain, particularly in the region of the first zinc finger (which is involved in target sequence recognition) we tested xONR1 binding to an artificial DR-3 element (AGGTCA AGG AGGTCA, ref. 20). The results are shown in figure 3.

xONR1, hVDR and xRXRγ (the cDNA of which was isolated by RT-PCR from a published sequence, ref. 12) were expressed in *Xenopus laevis* embryos by injection of synthetic mRNA. Expression in embryos was confirmed by labelling translation products with ^{35}S-methionine, and resolving proteins in embryo extract on a SDS-polyacrylamide gel (figure 3a). Protein bands corresponding to translation of the injected mRNAs are seen, relative to uninjected embryos. xRXRγ is expressed at a somewhat higher level than the other two receptors.

mRNA-injected embryos and uninjected embryos, from the same batches that were labelled with ^{35}S-methionine to confirm expression, were used to make extract for band shift assays. Band shift assays contained end-labelled DR-3 duplex oligonucleotide and a total of 6μl of embryo extract (equivalent to 1.2 embryos). Different embryo extracts were mixed as indicated in figure 4b. When present in an assay, 4μl of hVDR mRNA-injected, 4μl xONR1 mRNA-injected, and 2μl of xRXRγ mRNA-injected embryo extract were used.

Extract from uninjected embryos gives two weak retarded bands (which are not apparent in the exposure shown in figure

3b) corresponding to DNA–protein complexes (the origins of which are unknown). Relative to uninjected embryo extract, extract from embryos injected with xONR1 or xRXRγ mRNA shows no additional retarded bands. Extract from embryos injected with hVDR mRNA shows an additional retarded band. Efficient binding of hVDR to the DR-3 response element requires RXR as an accessory protein (32, 33, 34). The observed binding activity is most probably a consequence of the interaction between hVDR and endogenous *Xenopus* RXRs. *Xenopus* RXRα and RXRγ transcripts are known to be expressed at the embryonic stage when the extract was prepared (stage 9; see ref. 12). hVDR associates with xRXRγ to bind DNA, since the hVDR binding activity is dramatically stimulated by mixing extract from hVDR mRNA and xRXRγ mRNA-injected embryos. When extract from xONR1 mRNA-injected and xRXRγ mRNA-injected embryos was mixed an additional retarded band was seen (although alone these extracts showed no additional retarded bands). This binding activity most probably represents the binding of xONR1 and xRXRγ together to the DR-3 response element. (The lack of any observed xONR1 binding as a consequence of interaction with endogenous RXRs was most likely a problem of sensitivity.)

In summary, hVDR DNA binding to the DR-3 element is stimulated by association with xRXRγ. This demonstrates cross-species compatibility of the receptor regions necessary for interaction. xONR1 binds to the DR-3 element in association with xRXRγ, but does not detectably bind alone.

This experiment was repeated on two other occasions with essentially the same results. A similar result was obtained with the natural DR-3 vitamin D3 response element associated with the mouse osteopontin gene (20) (data not shown), although xONR1 (in association with xRXRγ) binding to this element is somewhat weaker than to the synthetic DR-3 element.

Temporal expression of xONR1 transcripts in early embryogenesis

Polyadenylated RNA from early *Xenopus laevis* embryos of a range of developmental stages was Northern blotted, and the resultant filter hybridised to a radiolabelled fragment of xONR1 DNA (figure 4). The region of xONR1 DNA used as probe extends from sequences encoding the beginning of the second zinc finger (in the C-domain) to the end of the cloned cDNA, some 233 nucleotides downstream of the stop codon. The consistency of RNA loading was confirmed by staining the residual ribosomal RNA bands with ethidium bromide after electrophoresis. The filter was washed at high stringency.

The probe detects a single xONR1 transcript. The transcript lies between the 18s (1.9kb) and 28s (4.5kb) ribosomal bands, which is in good agreement with the total size of the cDNA isolated (approx. 2.3kb). xONR1 transcripts are present at stage 6, levels peak at stage 9, and then levels decline to lower amounts later in embryogenesis. Zygotic transcription in *Xenopus* begins at the mid blastula transition (stage 8), and so the transcripts present at stage 6 are maternal.

DISCUSSION

xONR1 is a novel member of the nuclear receptor superfamily, and (on the basis of DNA binding domain similarity) belongs to the group of receptors which includes retinoid receptors (RARs and RXRs), thyroid hormone receptors (TRs), 1α, 25-dihydroxy vitamin D3 receptor (VDR), and peroxisome proliferator receptors (PPARs).

xONR1 is most closely related to the mammalian 1α, 25-dihydroxy vitamin D3 receptor. But xONR1 does not bind this ligand, and is therefore not the *Xenopus* orthologue of this mammalian receptor. A true homologue of the 1α, 25-dihydroxy vitamin D3 is expected to exist in *Xenopus*, because ligand binding studies have indicated the presence of high affinity receptor for this ligand (35, 36). The ligand for xONR1 has yet to be identified, but may be a vitamin D3 metabolite, or related compound.

RARs, VDR, TRs, PPARs and the *Drosophila* ecdysone receptor all require heterodimerisation with RXRs for efficient DNA binding *in vitro*, and transcriptional activation *in vivo* (5, 6). xONR1 also requires association with RXR for efficient DNA binding *in vitro*. xONR1 is temporally co-expressed with xRXRα and xRXRγ (12) and so, if they are also spatially co-expressed, may interact with these RXRs to function in early embryogenesis. As yet we not know about the effects of heterodimerisation with RXR on the ability of xONR1 to activate transcription. Studies of transcriptional activation will be aided if the ligand for xONR1 can be identified. The functional reasons for RXRs interacting with this wide range of receptors remain unclear (various possibilities are reviewed in ref. 5).

Conservation between xONR1 and hVDR is particularly high in the first zinc finger of the DNA binding domain. This region is believed to be involved in the selectivity of DNA binding site recognition. xONR1 and hVDR both bind to DR-3 response elements (artificial and natural) in association with RXR. Consequently, they may regulate an overlapping set of target genes.

xONR1 is temporally co-expressed in early *Xenopus* embryos with RXRs (12), and probably functions *in vivo* in association with these RXRs. In addition to xONR1, RARs (12), PPARs (26) and COUP-TF (28) are expressed in early *Xenopus* embryos, and are able to associate with temporally co-expressed RXRs (5, 37). Exogenously supplied retinoids have complex, multiple effects on the development of early vertebrate embryos, including *Xenopus* (7, 8). Such a diversity of effects may, in part, be a consequence of ectopic retinoid activation of RARs and RXRs also leading to altered signalling by receptors for several other ligands, whether known or unknown at present.

The discovery of xONR1 suggests that a presently unknown ligand, with potentially important biological activities, awaits identification. The cloning and expression of xONR1 are the first steps in identifying this ligand.

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