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Multiple retinoid-responsive receptors in a single cell: Families of retinoid "X" receptors and retinoic acid receptors in the *Xenopus* egg

(morphogen/transactivation/nuclear receptor)

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ABSTRACT **In a search for nuclear hormone receptors expressed in early development we found that *Xenopus laevis* eggs contain mRNAs from two retinoic acid receptor genes (xRARα and xRARγ) and two retinoid "X" receptor genes (xRXRα and xRXRγ). We also show that RXRs are members of a family of at least three genes, thus expanding the number of genes encoding retinoic acid-responsive transcription factors to six. With the exception of xRXRγ, these maternal mRNAs are degraded before gastrulation. The RXRs isolated are differentially activated by retinoic acid and by 3,4-didehydroretinoic acid. Considered together, these four receptors provide a molecular basis for the pleiotropic effects of retinoic acid on early development, and their pattern of expression suggests a role for retinoic acid at the earliest stages of embryonic determination.**

Retinoic acid (RA) is a vitamin A metabolite that has profound effects on growth, differentiation, and development (1). RA is a potent teratogen in human embryogenesis (2) and influences pattern formation in the limb (reviewed in refs. 3 and 4). There is also considerable evidence that RA provides positional information during development. Treatment of early *Xenopus* embryos with RA produces a concentration-dependent truncation of anterior structures (5, 6) through its influence on the embryonic mesoderm and ectoderm (7, 8). By the use of various position-specific molecular markers, it has been demonstrated that RA increases the expression of some posterior markers, while inhibiting the expression of some anterior markers (6, 9). RA also potentiates the induction of a posterior marker by the posteroventral inducer basic fibroblast growth factor (9) and antagonizes the induction of the anteriorly expressed, primary-response homeobox gene goosecoid by the anterodorsal inducer activin (10). A simplistic interpretation of these results is that RA posteriorizes the embryonic axis; however, neither the quantity nor the distribution of another posterior marker, *Xhox3*, is affected by RA, either in whole embryos or in the animal cap assay (11), suggesting that posterior patterning may require more than one pathway.

The biological effects of RA are thought to be primarily modulated by receptors that are members of the nuclear receptor superfamily (12). One response pathway is mediated by a family of RA receptors (RARs) consisting of three isoforms (RARα, RARβ, RARγ) (13–16). Recently, a second RA response pathway has been identified and shown to be mediated by a receptor, RXR (for retinoid "X" receptor) (17). The RXR differs from RARs in primary sequence and in its pharmacological response to retinoids, although the cognate ligand is not yet known.

In this report we demonstrate that a single cell, the *Xenopus laevis* egg, contains RA-responsive transcription factors derived from four different genes. In addition to *Xenopus* homologs of the previously described receptors, RARα, RARγ, and RXRα, we have identified another RXR, xRXRγ. Interestingly, xRXRα differs from xRXRγ in its response to RA and also to 3,4-didehydroretinoic acid (ddRA). We show that the xRARs and the xRXRs are differentially expressed during early development. This early expression of retinoid receptors is consistent with the view that RA has a role in developmental signaling processes and may have a significant role in anterior–posterior axis specification in addition to its demonstrated role in the axial polarity of limb development. We suggest that many of the effects of RA on early development are mediated by RARs and RXRs present at the earliest developmental stages.

MATERIALS AND METHODS

RNA Preparation and Analysis. RNA was prepared from fertilized *X. laevis* eggs and staged embryos using the LiC/urea method (18) and stored as ethanol precipitates at −20°C. Aliquots of staged *X. laevis* total RNA were hybridized with appropriate ^{32}P-labeled antisense RNA probes prepared as described (19) and analyzed by RNase protection (20). Briefly, ^{32}P-labeled antisense probes were purified by agarose gel electrophoresis, and the major product was excised, transferred to a Spin-X cartridge (Costar), and frozen at −20°C for 15 min. The labeled RNA was recovered by centrifuging for 5 min at room temperature and used directly in the hybridization reaction. Approximately 400,000 cpm were used for each hybridization, which consisted of target RNA, probe, Pipes buffer (40 mM Pipes, pH 6.8/0.4 M NaCl/1 mM EDTA), and 50% deionized formamide in a final volume of 40 μl. The hybridizations were heated at 100°C for 5 min, then transferred to 68°C, and incubated overnight. After slow cooling to room temperature, 300 μl of digestion buffer was added (10 mM Tris·HCl, pH 7.4/5 mM EDTA/0.3 M NaCl/40 μg of RNase A per ml/1000 units of RNase T1 per ml) and the mixture was incubated for 15 min at 37°C. The RNase was removed by adding $NaDodSO_4$ to 1% and proteinase K to 0.2 mg/ml and incubating for 30 min at 37°C. Two micrograms of tRNA (Sigma; type X) was added, and the reaction mixture was extracted twice with phenol/chloroform, extracted once with chloroform, and precipi-

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Abbreviations: RA, retinoic acid; RAR, RA receptor; RXR, retinoid "X" receptor; XOR, *Xenopus* orphan receptor; CAT, chloramphenicol acetyltransferase; ddRA, 3,4-didehydroretinoic acid; RARE and RXRE, RA and RX response elements.

tated with ethanol for 30 min on ice. The RNA was recovered by centrifugation for 30 min at room temperature and resuspended in 3 μl of H_2O, and 5 μl of formamide loading buffer was added. After incubation at 80°C for 20 min, the denatured RNA was electrophoresed in 6% polyacrylamide/8 M urea DNA sequencing gels, and the protection size was determined precisely by comparison with a known DNA sequence ladder. Autoradiography was performed at −70°C with two intensifying screens. The amount of RNA used at each stage was adjusted so that the elongation factor 1α protection was approximately equal. Elongation factor 1α is a ubiquitous transcript that is representative of total poly(A)$^+$ RNA (21). The actual amounts of RNA used from each stage were the following: ovary, 10 μg; egg and stages 2, 7, and 8, 40 μg; stage 10, 10 μg; stage 11, 8 μg; stages 13–45, 4 μg.

cDNA Library Construction and Oligonucleotide Screening. Oligo(dT)-primed first-strand cDNA was prepared from 150 μg of total RNA from unfertilized *Xenopus* eggs (to obviate possible bias due to underpolyadenylylation of certain mRNAs) as described (22). After methylation with *Eco*RI methylase, the cDNA was made blunt-ended and *Eco*RI linkers were added. After *Eco*RI digestion and chromatography over Sepharose CL-4B, the cDNA was ligated to λZAPII arms (Stratagene) and packaged *in vitro* with Gigapack II Gold (Stratagene). This library contains 4×10^6 independent clones and was screened after amplification. A second library was made from 3 μg of poly(A)$^+$ egg RNA, first-strand cDNA was primed with an adaptor, ACTAGTGCGGCCGCCTAGGCCTCGAG$(T)_{15}$, and an oriented cDNA library was prepared (22). This library contains 10^8 independent clones and was screened without amplification.

The libraries were screened in duplicate with a ^{32}P-labeled synthetic oligonucleotide (TGYGARGGNTGYAARGGNTTCTT) under low-stringency conditions [1 M NaCl/0.05 M Tris·HCl, pH 8.0/5 mM EDTA/150 units of heparin per ml/0.05% sodium pyrophosphate/100 μg of yeast RNA per

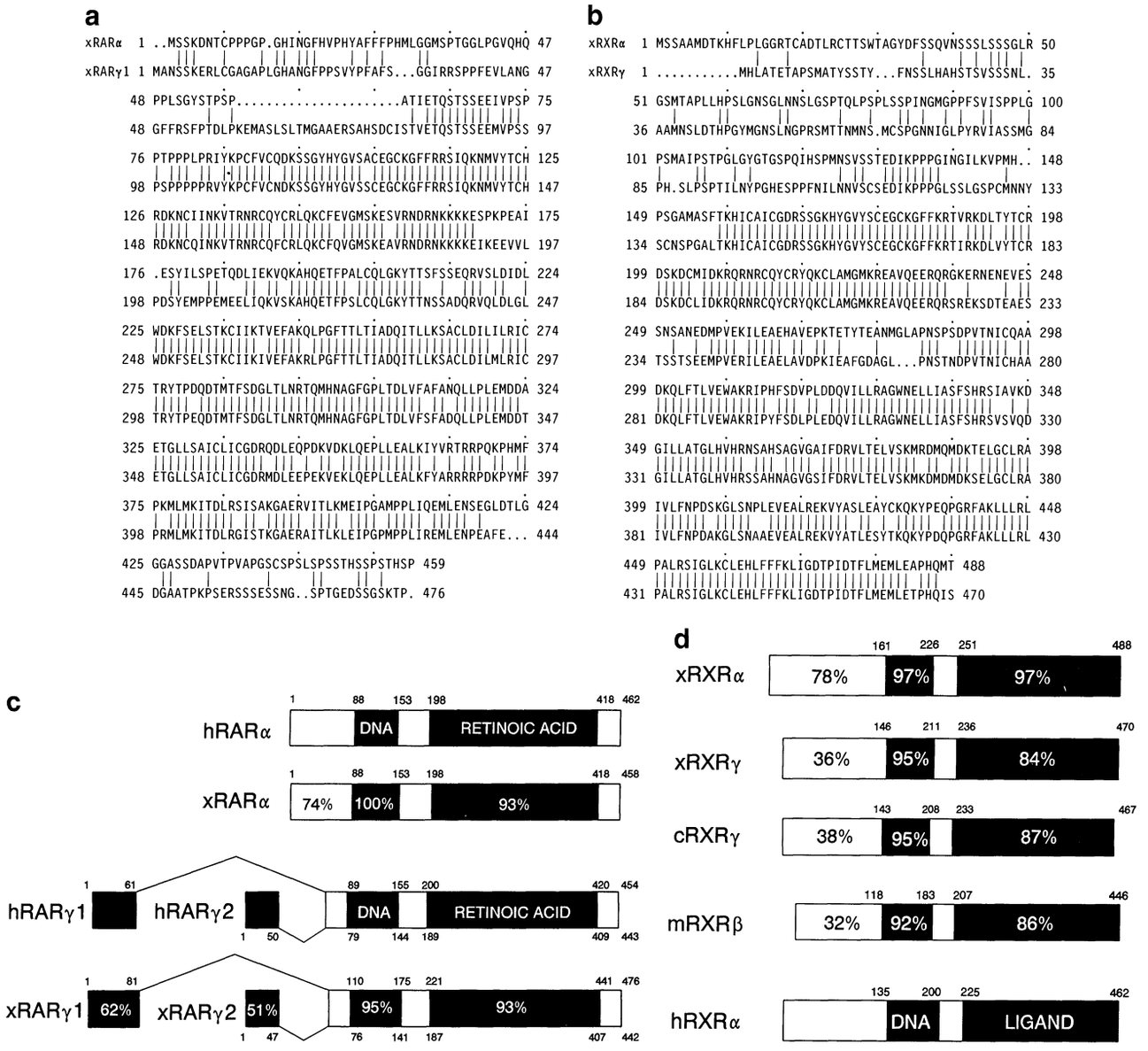

FIG. 1. Retinoid receptor proteins encoded in cDNAs present in the *Xenopus* egg. (*a*) Sequence of the xRARα and xRARγ1 proteins. The sequence of xRARγ2 has been published (30). (*b*) Sequence of the xRXRα and xRXRγ proteins. (*c*) Schematic comparison between the *Xenopus* RARs and their human homologs. Comparisons between different domains of the proteins are expressed as percent amino acid identity. (*d*) Schematic comparison of the members of the RXR gene family, compared with hRXRα. The nucleotide sequences corresponding to these protein sequences have been deposited in the GenBank data base.

ml/0.1% (wt/vol) NaDodSO$_4$ at 46°C] and washed at high stringency (58°C, in 3 M tetramethylammonium chloride/0.05 M Tris·HCl, pH 8.0/0.2 mM EDTA) essentially as described (23). This oligonucleotide is a mixture of all possible DNA sequences encoding the amino acid sequence CEGCKGFF. Positive clones were converted to plasmids by the automatic excision process (24) and sequenced using the screening oligonucleotide as a primer. Clones showing similarity to the conserved cysteine-rich DNA-binding domain of known steroid hormone receptors were further characterized.

Appropriate restriction fragments were subcloned into pBluescript KSII+ (Stratagene) and sequenced using T7 DNA polymerase (Pharmacia) and the dideoxy method. DNA sequences were compiled and analyzed using the programs of Staden (25), Univeristy of Wisconsin Genetics Computer Group (26), and Feng and Doolittle (27). Computer anlaysis showed that there were two RA receptors, xRARα and xRARγ, and two orphan receptors, designated XOR-3 and XOR-5. XOR-3 and XOR-5 appeared related and were later identified as RXRs by sequence similarlity with the human RXRα (17).

Cell Culture and Transfection Studies. The mammalian expression vectors pRS-xRXRα and pRS-xRXRγ were constructed by introducing the cDNA inserts of XOR-3 and XOR-5 into pRS as described (17). Construction of the reporter plasmid Δ MTV-TRE$_p$-CAT has also been described (28). CV-1 cell culture, cotransfections, and chloramphenicol acetyltransferase (CAT) assays were performed as outlined (17). All experiments involving retinoids were conducted in subdued light. Retinoids were dissolved in ethanol and delivered to cells [0.1% (vol/vol) of solvent in media] 36 hr before harvesting cells for CAT assays.

RESULTS

We reasoned that one way to identify potential small, diffusible, hydrophobic morphogens, analogous to RA, was to first isolate candidate nuclear hormone receptor homologs from developmental stages of interest and then attempt to identify the corresponding ligand. Therefore, to identify signaling systems that utilize such morphogens during early development, cDNA libraries were constructed from *Xenopus* egg mRNA and screened with a mixture of oligonucleotides designed to recognize all possible DNA sequence variations of a conserved amino acid sequence (CEGCKGFF), found in members of the thyroid/retinoid/vitamin D family of the nuclear receptor superfamily (12). We isolated cDNA clones arising from eight different nuclear receptor genes. Four corresponded to putative receptors for which the ligand is unknown, so-called "orphan receptors," and will be described elsewhere. The other four encoded retinoid receptors.

Vertebrates contain a family of "canonical" RA receptors consisting of three genes (RARα, RARβ, RARγ) (reviewed in ref. 29). cDNAs derived from two members of this family were isolated from the egg library (Fig. 1*a*). Both are quite similar to their human counterparts and are thus designated xRARα and xRARγ (Fig. 1*c*). Alternatively spliced *Xenopus* RARγ cDNAs, capable of encoding two different receptors, xRARγ1 and xRARγ2, exist and are similar to their mammalian counterparts (Fig. 1*c*) (31, 32). The xRARγ2 cDNA is identical in sequence to a recently reported *Xenopus* RAR (30). The evolutionary conservation of alternatively spliced *Xenopus* RARγ isoforms could be interpreted as reflecting a specific function for each (33).

The identification of a second family of retinoid receptor, the RXR, has provided an alternative pathway for RA-mediated developmental effects (17). RXRs differ considerably from RARs, in primary sequence and in dose–response to retinoids. The relatively high concentrations of RA needed to elicit RXR activation suggest that the RXR ligand might be a metabolite of RA (17). We isolated two types of clones related to RXRs (Fig. 1*b*). One can encode a 488-amino acid protein that is 90% identical with human RXRα (17) and is denoted as xRXRα. The second can encode a 470-amino acid polypeptide that is only 71% identical to human RXRα (17) and 69% identical to mouse RXRβ (34); therefore we designate it xRXRγ (Fig. 1*d*). This classification of another RXR subtype is supported by the finding of chicken (35) and murine homologs (46), extending the RXR family to three genes.

RNase protection assays were employed to study steady-state levels of receptor mRNAs over a developmental time sequence. xRXRγ and xRARα are synthesized during oogenesis and persist in the cleaving embryo at approximately constant levels until they are degraded just before gastrulation (stage 10) (Fig. 2). This expression pattern is reminiscent of maternal mRNAs such as Vg-1 (37), which are accumulated during oogenesis, presumably for use during the rapid cleavage stages of early development. xRXRα and xRARγ mRNAs are synthesized during oogenesis and persist during early cleavage (Fig. 2). The quantity of xRXRα mRNA drops sharply before gastrulation (stage 10), remains low until the tailbud stage (stage 26), and then increases until the latest stage analyzed (stage 45). In contrast to xRXRα, xRARγ mRNA begins to accumulate at gastrulation (stage 10), peaks in quantity during neurulation (stage 17), then drops to a low level after stage 26.

To show that the *Xenopus* RXRs were functional retinoid receptors, full-length cDNAs were subcloned into the expression plasmid pRS (17) and cotransfected with the reporter gene ΔMTV-TRE$_p$-CAT into CV-1 cells (28). We first investigated the rank order of xRXR response to several natural vitamin A metabolites and found that both showed a similar order of retinoid response to that of hRXRα (17)—i.e., RA > retinaldehyde > 4-hydroxyretinoic acid > retinyl acetate > retinol (data not shown). Interestingly, both xRXRs responded to the morphogenetic signal ddRA (38) as do RARs (D.J.M. and R.M.E., unpublished results).

We next analyzed the dose–response of xRXRα and xRXRγ to RA and ddRA (Fig. 3). Similar to what has been reported for hRXRα (17), the response of the *Xenopus* RXRs was not yet saturated at 10^{-5} M RA (Fig. 3*a*). xRXRα appears to be more responsive than xRXRγ to RA (Fig. 3*b*); thus, RXR isoforms, like RAR isoforms, exhibit differential acti-

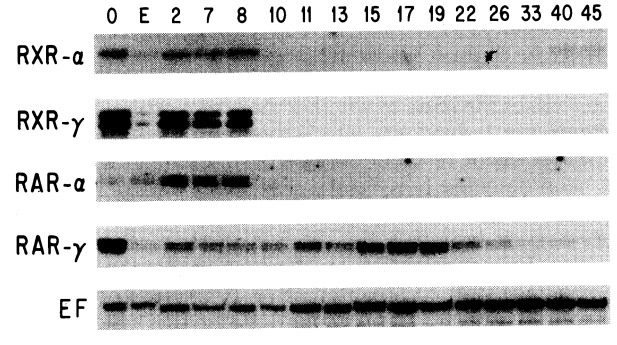

FIG. 2. Analysis of *Xenopus* retinoid receptor mRNA expression. The probes used are the following: xRXRα [nucleotides (nt) 723–1069], xRXRγ (nt 668–823), xRARα (nt 1–196), xRARγ (nt 1413–1734), EF-1α (*X. laevis* elongation factor 1α, nt 790–1167). Stages are according to Nieuwkoop and Faber (36) and are abbreviated as follows: O, total ovary; E, unfertilized egg; 2, two-cell; 7 and 8, blastula; 10 and 11, gastrula; 13–22, neurula; 26 and 33, tailbud; 40 and 45, swimming tadpole. The underrepresentation of receptor mRNAs in unfertilized egg total RNA as compared with later cleavage stages is currently unexplained.

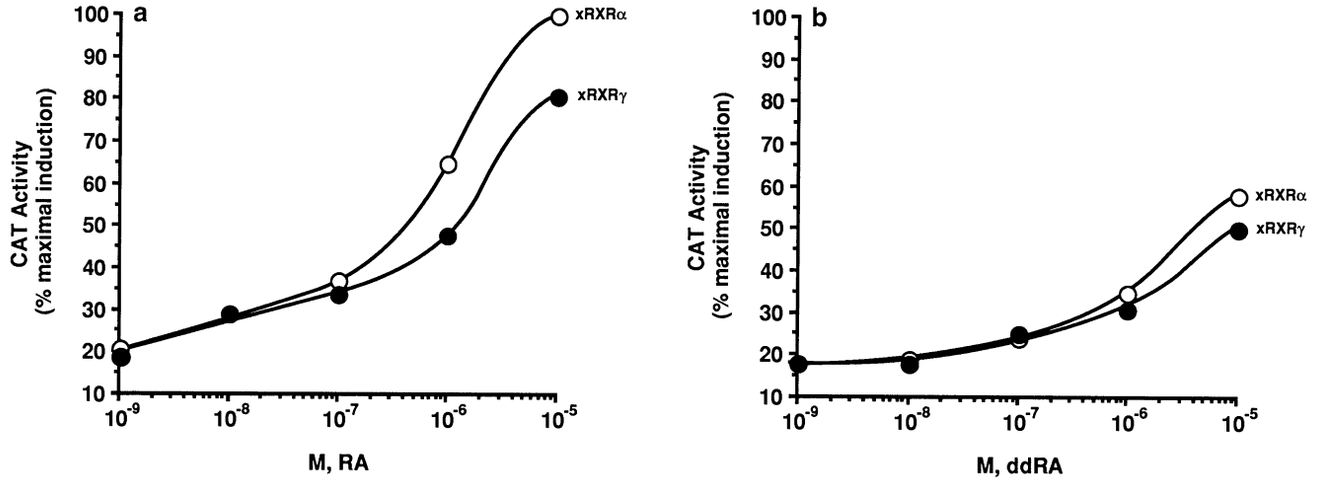

FIG. 3. Comparison of xRXRα and xRXRγ transactivation in mammalian CV-1 cells. (*a*) xRXRα and xRXRγ dose–response to RA. (*b*) xRXRα and xRXRγ dose–response to ddRA. The levels of induced CAT activity for RS-xRXRα and RX-xRXRγ are plotted as percentages of the average maximal response observed in two experiments. CV-1 cells were cotransfected with the reporter ΔMTV-TRE$_p$-CAT and either pRS-xRXRα or pRX-xRXRγ. After transfection cells were incubated with increasing amounts of RA or ddRA and harvested for CAT assays. CAT activities are expressed as percentages of the maximal response observed in this experiment. There was no CAT activity seen with any receptor in combination with the control reporter ΔMTV-CAT (data not shown).

vation by RA (16). Both xRXRs appear to be more sensitive to RA than to ddRA (Fig. 3*b*). In this respect it will be interesting to determine whether *Xenopus* embryos contain ddRA or other retinoid metabolites that may be high-affinity ligands for the RXR.

DISCUSSION

What is the significance of having RXRs and RARs in a single cell? The answer could involve the differential activation of receptor isoforms by ligand. These receptors can respond to RA over a broad range [$\approx 10^{-9}$ M (RARs; ref. 16) through 10^{-5} M (RXRs; ref. 17) (Fig. 3*a*)], which may have potential developmental consequences. For example, the effects of even a shallow gradient of RA could be magnified by the differential activation of RARs and RXRs. RXRs would require the highest levels of RA for activity, whereas RARs would be active at lower RA levels. Alternatively, the availability of a ligand for RXR may be enzymatically regulated by RA, which is already known to regulate its own metabolism in mammalian cells (39, 40). It has been suggested that the RXR ligand may be a metabolic derivative of RA (17). *Xenopus* embryos contain various retinoids (5), one of which is likely to be an RXR ligand.

That RARs and RXRs are apparently universal in vertebrates raises the issue of whether these two retinoid response systems are redundant, interactive, or independent. RXR homologs occur in invertebrates (41), although RARs have not been detected beyond the vertebrate lineage. This suggests that the RXR-mediated retinoid response system is the more ancient type and that the two systems might be independent. However, recent results suggest that the two retinoid response systems are interactive. Both types of receptors are able to activate transcription of identical reporter gene constructs containing thyroid or estrogen response elements (17). RARs have specific, high-affinity DNA target sequences, RAREs (42–44), as do the RXRs, RXREs (39).

It is potentially significant that hRXRα can activate reporter gene constructs containing a RARE; moreover, hRARα can repress hRXRα-dependent transactivation of reporter gene constructs containing a RXRE (39). Thus, although both types of receptor have their own high-affinity target genes, each will probably affect the other's target genes when both types are expressed in the same cell (39). Recent evidence suggests that the inhibition of RXR-dependent transactivation from an RXRE caused by hRARα is mediated via the binding of heterodimers between hRARα and hRXRα to the RXRE (unpublished results). hRXRα forms heterodimers with other members of the steroid receptor superfamily, yielding transcriptional effects dependent on the particular heterodimer formed, the presence of the corresponding ligands, and the response element (45). Interactions between receptors that can be influenced by the presence of the ligands, and the particular target gene, greatly increase the complexity and flexibility of the resulting regulatory networks and may have important developmental consequences.

In conclusion, we have demonstrated that the recently described RXR is a member of a family of at least three genes and that, together with the three previously identified RARs, there are now six known retinoid receptors. At least four of these (xRXRα, xRXRγ, xRARα, and xRARγ) are expressed in a single cell, the *X. laevis* egg. Their differential response to retinoid ligands and the expression pattern of their mRNAs during early development are consistent with each playing a specific developmental role. Moreover, the temporally restricted expression of xRXRα, xRXRγ, and xRARα mRNAs leads us to suggest that these transcription factors are required during the critical period of early development when the future body plan is first specified.

Note Added in Proof. Several recent reports have identified 9-*cis*-RA as a high-affinity RXR ligand (47, 48). Thus the *X. laevis* egg contains specific receptors for two biologically active retinoids, all-*trans*-RA (RARs) and 9-*cis*-RA (RXRs).

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