Novel retinoic acid receptor ligands in *Xenopus* embryos

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ABSTRACT   Retinoids are a large family of natural and synthetic compounds related to vitamin A that have pleiotropic effects on body physiology, reproduction, immunity, and embryonic development. The diverse activities of retinoids are primarily mediated by two families of nuclear retinoic acid receptors, the RARs and RXRs. Retinoic acids are thought to be the only natural ligands for these receptors and are widely assumed to be the active principle of vitamin A. However, during an unbiased, bioactivity-guided fractionation of *Xenopus* embryos, we were unable to detect significant levels of all-*trans* or 9-*cis* retinoic acids. Instead, we found that the major bioactive retinoid in the *Xenopus* egg and early embryo is 4-oxoretinaldehyde, which is capable of binding to and transactivating RARs. In addition to its inherent activity, 4-oxoretinaldehyde appears to be a metabolic precursor of two other RAR ligands, 4-oxoretinoic acid and 4-oxoretinol. The remarkable increase in activity of retinaldehyde and retinol as a consequence of 4-oxo derivatization suggests that this metabolic step could serve a critical regulatory function during embryogenesis.

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 fetal development (2) and influences limb development and regeneration (for reviews, see refs. 3 and 4). RA is also capable of altering positional identities along the vertebrate anteroposterior axis. Treatment of early *Xenopus* embryos with RA elicits concentration-dependent anterior truncations, which some have interpreted to represent posterior transformations (5). The majority of the effects of RA are likely to result from alterations in gene expression elicited by the two classes of ligand-dependent transcription factors, RA receptors (RARs) and 9-*cis* RA receptors (RXRs) (for review, see ref. 6).

Our interest in the effects of endogenous retinoids in *Xenopus* development led us to undertake a search for naturally occurring compounds capable of activating RARs. In preliminary experiments, we were intrigued by the relative paucity of all-*trans* and 9-*cis* RAs compared with other RAR agonists in the egg and early embryo, leading us to look for other retinoid derivatives. Previous attempts had focused on separating embryonic extracts by HPLC and matching the retention times of components having absorbance at 350 nm with those of known standard retinoids (5, 7–9). To identify potentially novel compounds, we used HPLC fractionation and purification guided simply by biological activity as measured by transcriptional regulation. This strategy does not presume prior knowledge of endogenous retinoids and has the benefit of being able to identify compounds with unusual fractionation and absorption properties.

This approach identified 4-oxoretinaldehyde (4-oxo-RAL) as the major bioactive retinoid in the *Xenopus* egg and early embryo. In addition to being able to bind to and transactivate RARs, 4-oxo-RAL appears to be a metabolic precursor of two other RAR ligands, 4-oxoretinoic acid (4-oxo-RA) (7) and 4-oxoretinol (4-oxo-ROL) (10). Taken together with the nearly complete lack of all-*trans* and 9-*cis* RAs during early development, our results suggest that the oxoretinoids may be the primary retinoids functioning during early *Xenopus* development and that the spectrum of natural bioactive retinoids is much broader than had been suspected. Previous studies have suggested the importance of isomerization to create novel retinoic acid derivatives. This work shows that selective modification of the cyclohexenyl ring may convert retinol and retinal to bioactive hormones.

METHODS

Retinoid Extraction, Fractionation, and Identification. All extraction and other experiments with retinoids were carried out using subdued amber illumination [Sylvania Electric Products (Fall River, MA) F40GO bulb], minimizing exposure to oxygen and heat where possible.

Xenopus ovaries or embryos were homogenized in 4 vol of H_2O, extracted with 0.6 vol of a 1:1 mixture of acetonitrile/1-butanol (containing 10^{-4} M butylated hydroxytoluene as an antioxidant), and 0.3 vol of saturated K_2HPO_4 (11). After centrifugation, the organic phase was analyzed by HPLC. Successive aliquots of the organic phase were loaded onto reversed phase C18 columns equilibrated in buffer A (0.1 M ammonium acetate, pH 6.4) attached to a Waters model 600E pump with a Waters model 996 photodiode array detector; data were collected at 1 spectrum per second with 2.4-nm resolution between 200 and 600 nm. The analytical HPLC profile shown in Fig. 1*A* was obtained with a Vydac (Hesperia, CA) C18 column (201TP54, 0.46 × 25 cm) using a flow of 1.5 ml/min and a mobile phase of 60% buffer A/40% acetonitrile at 1 min, proceeding linearly to 32% buffer A/68% acetonitrile at 35 min, and then to 100% acetonitrile at 36 min (method 1). The major bioactivity in fractions 30–32 coeluted with a compound having a λ_{max} of 384 nm. For scale up, ovaries from 10 frogs were pooled and extracted as above. Successive 2-ml aliquots of the organic extract from three frogs were loaded onto a 2.2 × 25 cm Vydac C18 column (218TP) equilibrated in buffer A. Elution was carried out with a flow rate of 8 ml/min and a linear gradient profile of buffer A/methanol/dichloromethane; 100% buffer A changing to 30% buffer A/70% methanol at 5 min, increasing to 100% methanol at 35 min, and holding at 100% to 40 min, then continuing to 60%
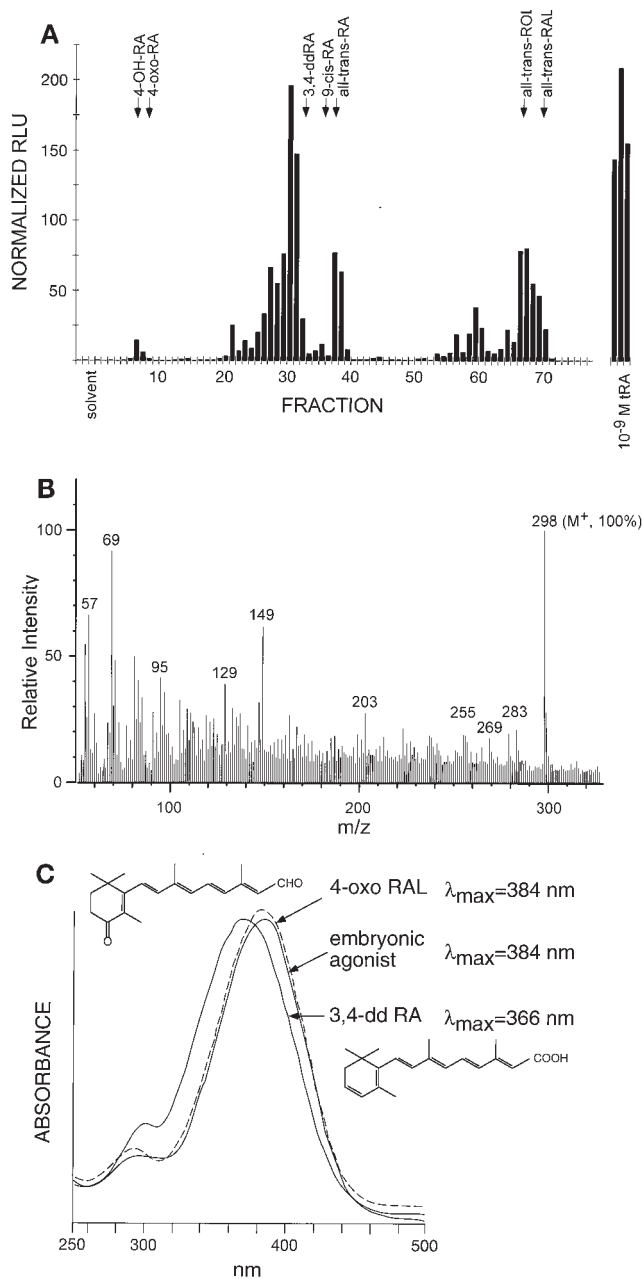
FIG. 1. Identification of bioactive embryonic retinoids. (*A*) *Xenopus* mixed stage embryo extracts were prepared and fractionated by analytical reversed phase-HPLC on a C18 column. Fractions (30 sec) were collected and assayed in their entirety for their ability to activate GAL-hRARα-transfected CV-1 cells. RLUs, relative light units. (*B*) Low-resolution electron impact mass spectrum of the activity from fractions 30–33 after purification to homogeneity. (*C*) Absorption spectra (extracted from three-dimensional contour plots) of 3,4-didehydro-RA (3,4-ddRA), 4-oxo-RAL, and the purified embryonic agonist, indicating that the RAR agonist is identical to 4-oxo-RAL.

methanol/40% dichloromethane at 50 min (method 2). Fractions (1 min) were collected beginning at 10 min and the aliquots were dried and tested for their ability to activate GAL-hRARα or hRXRα (h, human) in the cotransfection assay as described below. Fractions from 16 min to 26 min were pooled and loaded onto a semipreparative Vydac C18 column (201TP, 1.0 × 25 cm) equilibrated in buffer A. Elution was at 2 ml/min with the following gradient profile: 100% buffer A to 50% buffer A/50% acetonitrile at 2.5 min then linearly to 100% acetonitrile at 25 min (method 3). Fractions (30 sec) were collected and the aliquots were dried and tested in the
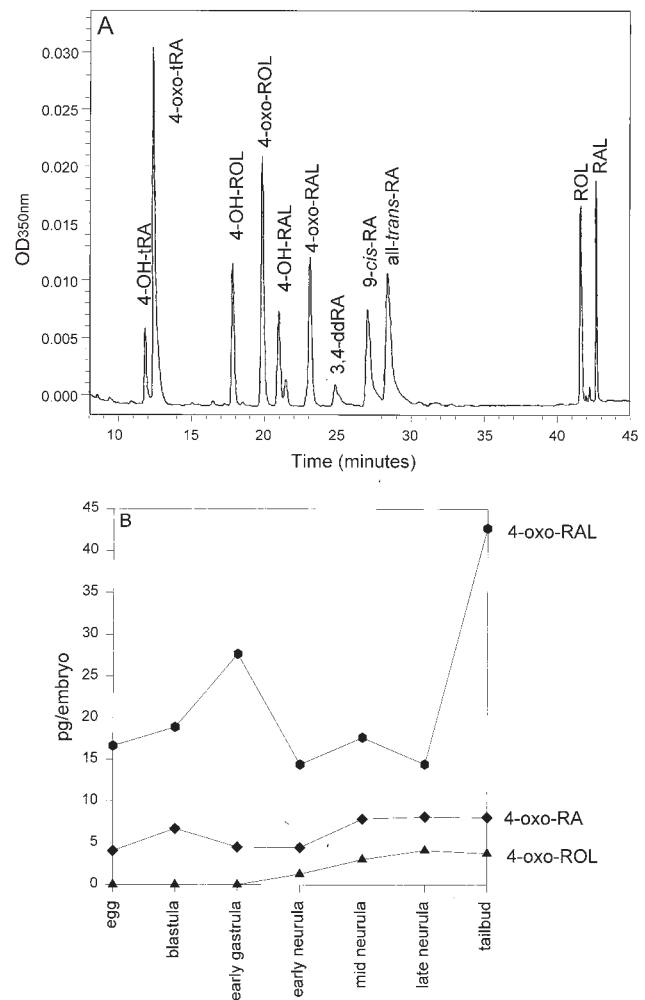

FIG. 2. Identification of endogenous oxoretinoids in staged *Xenopus* embryos. (*A*) Analytical HPLC profile showing baseline separation of a complex mixture of authentic retinoid standards achieved in 45 min. (*B*) HPLC quantitation of retinoid levels from groups of 1000 *Xenopus* embryos. The limit of detection for RA in this system is 0.5 ng (0.5 pg per embryo). Embryos were staged according to Nieuwkoop and Faber (17) and are depicted as follows: blastula (stage 8), early gastrula (stage 11), early neurula (stage 13–14), mid neurula (stage 16–18), late neurula (stage 20–22), and tailbud (stage 26–28). The total amount of each retinoid from a particular stage was normalized to the number of embryos and expressed as pg per embryo.

cotransfection assay. Fractions from 17 to 20 min were pooled and fractionated on an analytical Vydac C18 column (201TP54, 0.46 × 25 cm) developed isocratically with 35% buffer A/27% MeOH/38% acetonitrile (method 4). Fractions (15 sec) were collected and aliquots were tested in the co-transfection assay. Active fractions between 10 and 12 min were pooled and refractionated on the same column with the same mobile phase except that buffer and butylated hydroxy-toluene were omitted. The pure compound has a retention time of 11.4 min.

The high-resolution mass spectrum was measured on a JEOL model JMS-HX100 double-focusing mass spectrometer using a 70-eV electron impact ionization source, 180°C ion source temperature, ≈40°C direct insertion probe temperature, perfluorokerosene as reference, and a resolution of 5000.

Retinoid Quantitation. Pools of 1000 embryos were collected, quick frozen, and stored at −70°C until use. Embryos were thawed, homogenized in 8 ml of H_2O, and extracted with 5 ml of acetonitrile/1-butanol and 2.5 ml of saturated K_2HPO_4. After centrifugation, the organic phase was loaded onto the

semipreparative C18 column equilibrated in buffer A and eluted at 2 ml/min with the following gradient profile: 100% buffer A to 25% buffer A/75% methanol at 2.5 min, linearly increasing to 100% methanol at 20 min, then changing to 60% methanol/40% buffer D (50% methanol/50% $CHCl_3$) at 27.5 min and then to 100% buffer D at 30 min. Fractions between 14 and 26 min were pooled and refractionated on the analytical C18 column using gradient 1. Fractions (30 sec) were collected beginning at 10 min, dried, and resuspended in 25 μl of methanol. Fractions were subsequently tested in their entirety for bioactivity using the cotransfection assay (described below).

For retinoid quantitation, chromatograms were extracted from the three-dimensional photodiode array contour plots at the λ_{max} for each compound in this solvent system, and areas under the peaks were calculated using Waters MILLENNIUM CHROMATOGRAPHY MANAGER software. Absorbance was calculated from the integrated area using the molar extinction coefficient for each compound: 4-oxo-ROL $\varepsilon_{354} = 34,000$, 4-oxo-RAL $\varepsilon_{384} = 42,500$, and 4-oxo-RA $\varepsilon_{360} = 58,220$ $M^{-1}\cdot cm^{-1}$.

Cell Culture and Transfection. CV-1 cells were maintained in DMEM containing 10% resin-charcoal stripped calf bovine serum. Delipidated serum was prepared by stirring heat-inactivated serum for 3 hr with an equal volume of *n*-heptane (12) and then allowing the phases to separate. The serum phase was then re-extracted as above with *n*-heptane and the aqueous phase collected. The heptane treated serum was then resin-charcoal extracted twice and stored at 4°C until use. This treatment removed 99% of serum retinol; other retinoids were undetectable by HPLC analysis. Liposome-mediated transient transfections were performed using DOTAP reagent (Boehringer Mannheim) at a concentration of 5 μg/ml in DMEM containing 10% delipidated fetal bovine serum in 96-well format using a Beckman model Biomek 1000 laboratory workstation. In a typical experiment, cells were plated at a density of 5×10^3 cells per well. The next day, DNA/DOTAP mixture was added in the ratios 10 ng receptor expression plasmid/50 ng of reporter plasmid/40 ng of CMX–β-galactosidase control plasmid per well. After 12–18 hr, the cells were washed and fresh DMEM/10% delipidated serum containing HPLC fractions or retinoids was added in triplicate such that the solvent volume was <2%. After another 24-hr incubation, the cells were lysed and luciferase reporter gene assays and β-galactosidase transfection control assays were done as described (13). Reporter gene expression was normalized to the β-galactosidase transfection control and expressed as relative light units per OD of β-galactosidase activity per minute to allow comparison of inductions between experiments.

pCMX-GAL-hRARβ and pCMX-GAL-hRARγ were prepared by cloning amino acids 147–448 of hRARβ2 or amino acids 156–454 of hRARγ1 into pCMX-GAL4 (14).

4-oxo-RAL and 4-oxo-ROL were synthesized as described (10, 15). 4-oxo-all-*trans*-RA was a gift of Hoffmann–La Roche (to J. Buck) and 3,4-didehydro-RA was a gift of Dr. David Mangelsdorf (University of Texas Southwestern Medical School, Dallas). Other retinoids were purchased from Sigma. All retinoids were stored in amber glass vials as 10^{-3} M ethanolic solutions containing 10^{-4} M butylated hydroxytoluene at −70°C. Before
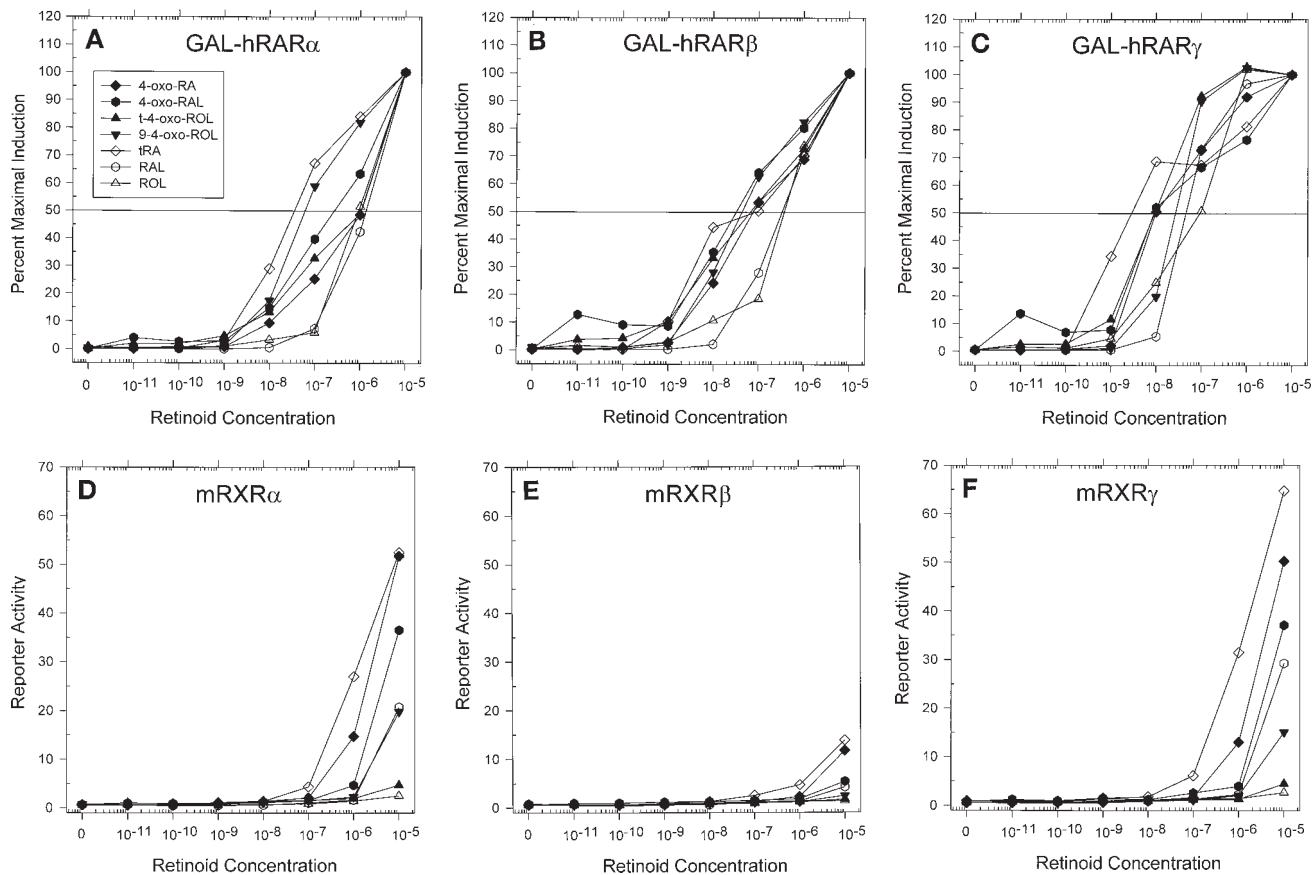

FIG. 3. Oxoretinoids are efficient activators of RARs but not RXRs. (*A–C*) Activation of RARs on tk-(GAL$_p$)$_3$-luc (22) by synthetic retinoids was assayed using chimeric GAL4 DNA-binding domain/RAR ligand binding domain receptors to obviate interference from endogenous receptors (14) (*A*) GAL-hRARα. (*B*) GAL-hRARβ. (*C*) GAL-hRARγ. Data are expressed as percent maximal induction where the maximum response for each retinoid was set to 100%. Similar results were obtained using full-length human and *Xenopus* RARs, although the *Xenopus* receptors were very poor activators in CV-1 cells (data not shown). (*D–F*) Activation of mouse RXRs (23) on tk-(ApoAI)$_4$-luc. (*D*) mRXRα. (*E*) mRXRβ. (*F*) mRXRγ. Data are expressed as normalized relative light units for each compound. tRA, all-*trans*-RA. Symbol assignments are the same in all panels.

storage, the vials were blanketed with argon (ultrapure oxygen-free grade).

Protease Protection and Ligand Binding. ^{35}S-labeled xRARα (x, *Xenopus*) was transcribed and translated *in vitro* using the TNT reticulocyte lysate system (Promega) and [^{35}S]methionine (New England Nuclear). Aliquots of protein were incubated with candidate ligands or solvent control on ice for 30 min and then at 20°C for 10 min. Typically, 5 μl of protein–ligand mixture was added to 5 μl of trypsin (Boehringer Mannheim) diluted in H$_2$O. Incubation was carried out on ice for 7 min and then at 30°C for 12 min. Digestion was terminated by the addition of 10 μl of SDS/loading buffer and boiling for 10 min. The samples were loaded onto an SDS/12% polyacrylamide gel and electrophoresed at 100 V for 15 min followed by 200 V for 25 min. Gels were fixed in 30% methanol/10% acetic acid, sprayed with En^{3}Hance (New England Nuclear), dried, and exposed to x-ray film.

In vitro ligand binding assays used baculovirus-expressed hRARα, -β, -γ, and all-*trans*-[^{3}H]RA or 9-*cis*-[^{3}H]RA and were carried out as described (16).

RESULTS AND DISCUSSION

Lipid extracts were prepared from several thousand mixed-stage embryos and analyzed by reversed-phase HPLC with photodiode array detection between 200 and 600 nm. Fractions were tested for RAR and RXR agonist activity using the cotransfection assay (13). As shown in Fig. 1*A*, we could show several RAR-specific activities that had UV spectra and elution times different from known standard retinoids. The major active fractions 30–32 (Fig. 1*A*) coeluted with a compound having an absorption spectrum maximum at 384 nm, characteristic of a retinaldehyde. Because adult ovaries contained these same fractions, we used them to scale up the production of this retinoid, and to purify it to homogeneity. The high-resolution mass spectrum gave an observed m/z value of 298.1958, calculated for C$_{20}$H$_{26}$O$_2$ = 298.1933, indicating that this compound has two hydrogen atoms less and one oxygen atom more than retinaldehyde. The molecular formula is consistent with 4-oxo-RAL. Indeed, the low-resolution electron impact mass spectrum (Fig. 1*B*) exhibits, in addition to the molecular peak m/z 298, a fragment at m/z 269 corresponding to the loss of a CHO end group. Furthermore, these data exclude 3,4-ddRA, which has the same molecular

Table 1. Teratogenic effects of retinoids in *Xenopus* embryos

	DAI in presence of retinoid			
Retinoid	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M
4-oxo-RAL	ND	2.6 ($n = 42$)	4.1 ($n = 52$)	4.8 ($n = 50$)
RAL	2.7 ($n = 14$)	4.7 ($n = 48$)	5.0 ($n = 48$)	5.0 ($n = 54$)
4-oxo-ROL	ND	2.7 ($n = 28$)	4.6 ($n = 34$)	4.9 ($n = 32$)
ROL	4.2 ($n = 17$)	4.9 ($n = 43$)	5.0 ($n = 56$)	5.0 ($n = 52$)
tRA	1.0* ($n = 11$)	1.0 ($n = 38$)	3.4 ($n = 49$)	4.9 ($n = 48$)
9RA	1.0* ($n = 6$)	1.0 ($n = 15$)	2.5 ($n = 20$)	5.0 ($n = 36$)
4-oxo-RA	1.0* ($n = 13$)	2.3 ($n = 23$)	3.5 ($n = 26$)	4.9 ($n = 51$)

Embryos were treated in groups of 20 at the early blastula stage (stage 8) with retinoids diluted in 20 ml of 0.1× modified Barth's saline and allowed to develop until stage 13 at 18°C. The embryos were subsequently washed three times in 0.1× MBS and incubated until stage 38-40 when they were fixed and scored for development using the DAI (24) as modified for RA by Sive *et al.* (25). Briefly, a DAI of 5 is normal; DAI 4.5, reduced forehead; DAI 4, reduced forehead, slightly cyclopic; DAI 3.5, cyclopic; DAI 3.0, extremely cyclopic, normal or reduced cement gland; DAI 2.5, no eye pigment, some cement gland present; DAI 2.0, no cement gland, beating heart and otic vesicle present; DAI 1.0, no beating heart, no otic vesicle; DAI 0, completely ventralized; and DAI 10, completely dorsalized. Surviving embryos showing normal gastrulation were scored and averaged to yield the values shown. The number of surviving embryos used for scoring is shown in parenthesis. ND, not determined.
*Significant death also observed.

Table 2. Oxoretinoids compete for binding to RARs

	IC$_{50}$, nM		
Retinoid	hRARα	hRARβ	hRARγ
4-oxo-RAL	35,000	38,000	30,000
RAL	NC	NC	NC
4-oxo-ROL	15,000	18,000	15,000
ROL	NC	NC	NC
4-oxo-RA	29	70	98
tRA	24	39	26

IC$_{50}$ values shown are the concentrations at which 50% of prebound ^{3}H-all-*trans* RA is displaced from baculovirus-expressed human RARs. NC, no competition observed.

formula, but whose absorption spectrum ($\lambda_{max} = 366$ nm) and retention time are different (Fig. 1*C*). Synthetic 4-oxo-RAL matches our embryonic activity in retention time, absorption spectrum mass, and biological activity (Fig. 1 *A* and *C*). The identification of the major embryonic RAR agonist as all-*trans*-4-oxo-RAL raised the issue of whether it was acting directly, by conversion to the previously described 4-oxo-RA (7) or to another derivative.

To address this question, we first determined which oxo-retinoids were present at important developmental stages. Lipid extracts were prepared from pools of 1000 staged embryos and fractionated first by semipreparative and then by analytical reversed phase chromatography using C18 columns and an acetonitrile/ammonium acetate gradient. Under these conditions, baseline resolution of a complex mixture of retinoid standards is achieved in 45 min (Fig. 2*A*). Fractions from the analytical column were collected and tested in their entirety for agonist activity. Activities were correlated with the elution profiles and UV spectra of the fractions to unambiguously identify RAR agonists. The use of photodiode array detection between 200 and 600 nm allowed individual chromatograms to be produced at the λ_{max} for each compound of interest and calculation of concentration from the extinction coefficients and absorbance values. The limit of detection in this system is 0.5 ng of RA with the generation of an unambiguous absorption spectrum and 0.1 ng without. The results are summarized in Fig. 2*B*. Levels of 4-oxo-ROL, 4-oxo-RAL, and 4-oxo-RA differ during early development, implying specific functions for each. 4-oxo-RAL is present at high levels in the egg, reaches a maximum at gastrulation, decreases throughout neurulation, and then increases sharply during the tailbud stages. We could also detect significant quantities of 4-hydroxy-RAL, a potential precursor for 4-oxo-RAL, throughout early development, at levels parallel those of 4-oxo-RAL (data not shown). In contrast, 4-oxo-RA levels are minimal when 4-oxo-RAL is maximal at gastrulation and then later increase slightly during tailbud stages; moreover, the absolute levels are only about one-third those of 4-oxo-RAL (Fig. 2*B*). 4-oxo-ROL shows a striking change during development in that it is undetectable before neurulation and then steadily increases thereafter. The differential temporal distribution of the three major oxoretinoids and the lack of a simple correlation between their levels argues against conversion to the 4-oxo-RA as generating the only, or even the major, embryonic RAR agonist. This is especially significant because we could not reproducibly detect all-*trans*-RA or 9-*cis*-RA, even with this highly sensitive detection system, during the same developmental time period. Although, as previously described (8, 9), there were compounds with UV absorbance at 350 nm that comigrated with authentic all-*trans* and 9-*cis* RAs, photodiode array analysis showed them to have UV absorption spectra distinct from all-*trans* and 9-*cis* RAs (data not shown).

Because the temporal and spatial patterns of xRAR and xRXR subtypes and isoforms differ during early *Xenopus*
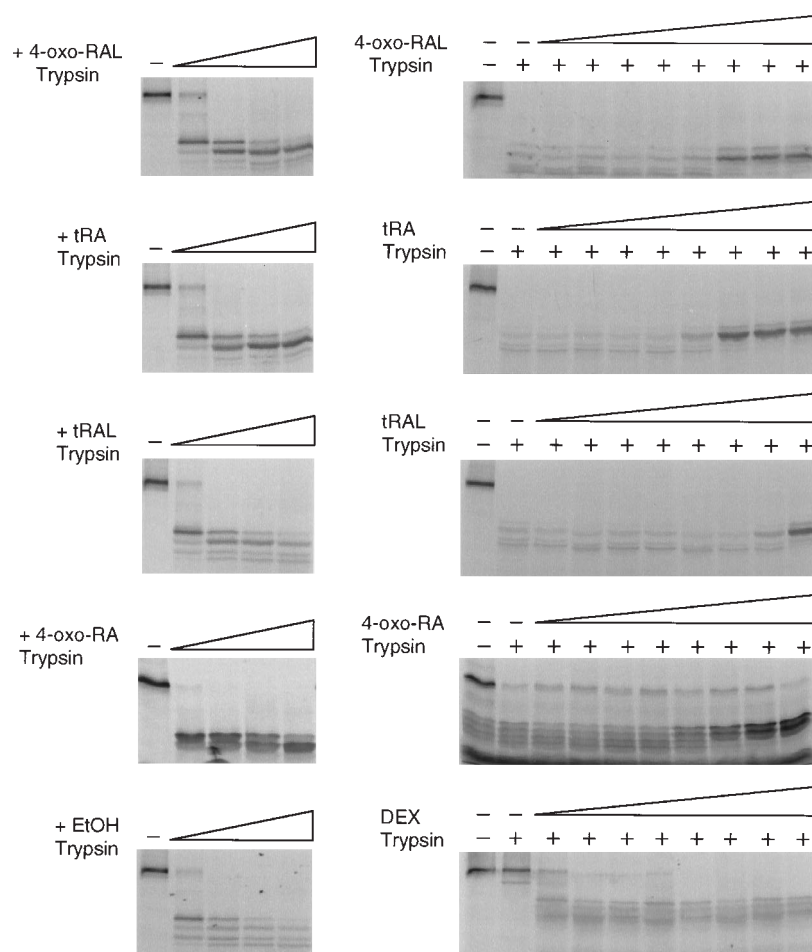
FIG. 4.   4-oxo-RAL protects xRAR from protease digestion. *In vitro* transcribed/translated xRARα was treated with fixed concentrations of retinoid (10^{-6} M) or solvent control and increasing concentrations of protease (*Left*) or with fixed protease and increasing concentrations of retinoids or dexamethasone control (*Right*). The dose of added compounds ranges in 10-fold increments from 10^{-12} to 10^{-5} M.

development (18–21), it was of interest to know whether the oxoretinoids showed any differences in their ability to activate these receptors. Fig. 3 shows a representative profile for the transcriptional response of hRARα, -β, and -γ (Fig. 3 *A–C*) and mouse RXRα, -β, and -γ (Fig. 3 *D–F*) to different doses of retinoids. 4-oxo-RAL and 4-oxo-ROL were typically much better RAR activators than RAL and ROL, respectively (Fig. 3 *A–C*). RARs were activated ≈10-fold better by 4-oxo-ROL than by ROL and by 4-oxo-RAL than by RAL. Conversion of 4-oxo-RAL and 4-oxo-ROL to 4-oxo-RA is an improbable explanation for the observed activity since the potency of these three compounds is nearly the same. In fact, at lower doses (10^{-8} to 10^{-7} M) 4-oxo-RAL is typically more potent. In addition, all-*trans* RA is a more potent activator than 4-oxo-RA whereas 4-oxo-RAL is a more potent activator than RAL. If the aldehydes act primarily by conversion to RAs, then one would expect RAL to be more potent than 4-oxo-RAL because all-*trans* RA is a much better activator at all concentrations than is 4-oxo-RA; moreover, we would expect to see a shift in the dose-response reflective of the need for conversion as is the case for ROL and RAL. Rather, we believe that the strong activation elicited by 4-oxo-RAL reflects primarily native activity and secondarily its potential to be converted into two other efficient activators, 4-oxo-ROL and 4-oxo-RA.

The spectrum of oxoretinoid activity is markedly different on the RXRs than the RARs. As shown in Fig. 3 *D–F*, all-*trans*-4-oxo-ROL and ROL are inactive on all three RXRs whereas 4-oxo-RAL and RAL are weak activators; all-*trans* RA and 4-oxo-RA are the most potent activators. Gudas and

colleagues (10) have obtained similar results with all-*trans*-4-oxo-ROL. Interestingly, 9-*cis*-4-oxo-ROL is a weak RXR activator (Fig. 3 *D–F*) whereas 13-*cis*-4-oxo-ROL was inactive on RXR (data not shown). All three oxo-ROL isomers were equally active on the RARs (Fig. 3 *A–C* and data not shown). Again, these results are not simply explained by conversion of 4-oxo-ROL and RAL to 4-oxo-RA.

The effect of the oxoretinoids on *Xenopus* embryos paralleled the activation assays described above. *Xenopus* embryos were treated at the early blastula stage over a range of concentrations with oxoretinoids or nonoxoretinoids and then allowed to develop until stage 40. When scored for the extent of anterior development using the dorsoanterior index (DAI) (24), it was again observed that 4-oxo-ROL and 4-oxo-RAL were much more potent than RAL or ROL in perturbing normal development (Table 1). Taken together with the receptor transactivation assays, these results suggest that 4-oxo-RAL and 4-oxo-ROL are good agonists, and are likely to be biologically relevant ligands for the xRARs.

The binding of 4-oxo-RAL to RARs and RXRs was determined using purified hRARα, -β, -γ, and hRXRα. We measured the ability of unlabeled oxoretinoids to displace prebound [^{3}H]RAs in standard competition binding assays (16). In accord with the activation data, 4-oxo-RAL and 4-oxo-ROL were unable to compete with 9-*cis*-[^{3}H]RA for binding to hRXRα (data not shown) whereas both competed with all-*trans*-[^{3}H]RA for binding to hRARα, -β, and -γ (Table 2). We note that the amount of oxoretinoids required to displace 50% of all-*trans*-[^{3}H]RA binding was several hundred-fold higher

than the amount required for significant receptor activation (Fig. 3). However, for reasons that are not obvious, receptor activation profiles occur over an exceptionally large range, yielding activation curves substantially more sensitive than predicted by the binding data. These data suggest either that 4-oxo-RAL and 4-oxo-ROL bind to the receptors somewhat differently *in vitro* than *in vivo* or that conversion to a more active form such as 4-oxo-RA is required for the observed activation.

To further investigate this discrepancy between binding and activation, we used a protease protection assay (26, 27). This assay measures the ability of a compound to alter receptor three-dimensional structure and thus its susceptibility to limited proteolysis. The susceptibility to protease cleavage has been shown to be modified by ligand binding. ^{35}S-labeled, *in vitro* transcribed, translated xRARα was incubated with increasing concentrations of various proteases in the presence of solvent carrier or retinoids. As shown in Fig. 4, 4-oxo-RAL and 4-oxo-RA provide similar degrees of protection from trypsin digestion as does all-*trans* RA and much greater protection than does RAL. This result is both consistent with the activation assays shown in Fig. 3 and ligand binding data shown in Table 2 and further supports the idea that 4-oxo-RAL can directly bind to the RARs, resulting in transcriptional activation. The rank order of potency for activation is similar to the protease cleavage and displacement binding data. The discrepancy between the large amount of 4-oxo-RAL compared with RA required to displace prebound all-*trans* RA from RAR and the approximately equal amounts of 4-oxo-RAL and RA required to protect the receptors from limited proteolysis can be explained by a high off rate for binding to the receptors. The protease protection assays are always done under equilibrium conditions and would be relatively insensitive to high off rates, whereas the last step of the displacement assay, and of most other direct binding assays, is the separation of free from receptor-bound retinoids in which a fast off rate may be reflected in the binding results. Furthermore, the protease protection assay measures alteration of the receptor's three-dimensional structure, which may be a more reliable indicator of ability to activate than binding (27). Thus, a key feature of the 4-oxo series of retinoids is their ability to achieve receptor binding at low concentrations ($\approx 10^{-8}$ M) in the absence of the terminal carboxylic acid moiety previously thought to be essential for high affinity binding of retinoids to RARs and RXRs (1).

While there has been ongoing controversy regarding the precise role of endogenous RA in *Xenopus* development, the potent biological effects of exogenously added retinoids together with the variety of retinoids present during development strongly supports the idea that retinoids play key roles in embryogenesis. As a requisite step in understanding the role of retinoids during *Xenopus* development, we sought to identify the endogenous compounds capable of activating xRARs and xRXRs, the primary mediators of RA's developmental effects. We identified the novel RAR ligands 4-oxo-RAL and 4-oxo-ROL in addition to the previously described 4-oxo-RA (7). The oxoretinoids constitute the majority of the embryonic RAR agonists that, taken together with the nearly complete lack of all-*trans* and 9-*cis* RA, identifies 4-oxo-ROL, 4-oxo-RAL, and 4-oxo-RA as the predominant bioactive retinoids during early *Xenopus* development. Rather than representing degradative products of "normal" retinoids, we propose that members of the 4-oxo series should be considered authentic signaling molecules used for discrete purposes during development. Thus, we infer that oxidative enzymes associated with the synthesis and metabolism of these bioactive retinoids will be important regulatory targets during development and that

their identification will be critical to understanding the role of retinoids in pattern formation.

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