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Metabolism to a response pathway selective retinoid ligand during axial pattern formation

W. W. M. PIJNAPPEL*†, G. E. FOLKERS*, W. J. DE JONGE*, P. J. E. VERDEGEM‡, S. W. DE LAAT*, J. LUGTENBURG‡, H. F. J. HENDRIKS§, P. T. VAN DER SAAG*, AND A. J. DURSTON*¶

*Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands; ‡Leiden Institute of Chemistry, Gorlaeus Laboratories, P.O. Box 9502, 2300 RA Leiden, The Netherlands; and §Netherlands Central Organization for Applied Scientific Research, Nutrition and Food Research Institute, P.O. Box 360, 3700 AJ Zeist, The Netherlands

Edited by Pierre Chambon, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France, and approved October 13, 1998 (received for review February 25, 1998)

ABSTRACT **We report identification of 9-*cis*-4-oxo-retinoic acid (9-*cis*-4-oxo-RA) as an *in vivo* retinoid metabolite in *Xenopus* embryos. 9-*Cis*-4-oxo-RA bound receptors (RARs) α, β, and γ as well as retinoid X receptors (RXRs) α, β, and γ *in vitro*. However, this retinoid displayed differential RXR activation depending on the response pathway used. Although it failed to activate RXRs in RXR homodimers, it activated RXRs and RARs synergistically in RAR-RXR heterodimers. 9-*Cis*-4-oxo-RA thus acted as a dimer-specific agonist. Considering that RAR-RXR heterodimers are major functional units involved in transducing retinoid signals during embryogenesis and that 9-*cis*-4-oxo-RA displayed high potency for modulating axial pattern formation in *Xenopus*, metabolism to 9-*cis*-4-oxo-RA may provide a mechanism to target retinoid action to this and other RAR-RXR heterodimer-mediated processes.**

Much evidence implicates retinoids (vitamin A and metabolites) in regulating embryonic pattern formation as well as growth, differentiation, reproduction, metabolism, and homeostasis (1–4). Many important effects of retinoids are mediated by two families of nuclear receptors: retinoic acid (RA) receptors (RARs) and retinoid X receptor (RXRs), each consisting of three types (α, β, and γ), there being several isoforms of each type (5–7). RARs and RXRs are retinoid ligand-dependent transcription factors, which can act via specific DNA response elements consisting of direct repeats of the hexameric motif RG(G/T)TCA. Two main retinoid response pathways are known. RARs and RXRs heterodimerize and then activate transcription via RA response elements consisting of direct repeats (DRs) spaced by 2 (DR-2) or 5 (DR-5) base pairs. Both the RAR and RXR partners of the heterodimer can be ligand activated *in vivo,* resulting in synergistic activation (8–13). RXRs also homodimerize, and this homodimerization contrasts with RAR-RXR heterodimerization in being ligand stimulated (14–17). RXR homodimers activate transcription from retinoid X response elements (RXREs) consisting of DRs spaced by 1 (DR-1) base pair. In addition to these two main pathways, retinoid receptors interact with other signaling pathways, either via heterodimerization between RXR and other nuclear hormone receptors or via crosstalk with AP-1.

Paralleling the multiplicity of retinoid receptors, several natural retinoids are known to act as retinoid receptor ligands (4, 18–23). These fall into two groups. Ligands that activate RARs only include all-*trans*-RA, all-*trans*-3,4-didehydroretinoic acid (ddRA), all-*trans*-4-oxo-retinoic acid (4-oxo-RA), all-*trans*-4-oxo-retinal, and all-*trans*-4-oxo-retinol. These ligands activate only RAR-RXR heterodimers and act via the RAR part of the heterodimer. The second group, including 9-*cis*-RA and 9-*cis*-3,4-didehydroretinoic acid, activates both RARs and RXRs. 9-*Cis*-RA has been shown to activate RAR-RXR heterodimers more efficiently than natural RAR ligands, suggesting that both partners of the heterodimer are activated by this ligand (8, 12). 9-*Cis*-RA also activates RXR homodimers. Here, we identify a natural retinoid ligand, 9-*cis*-4-oxo-RA (for structural formula see Fig. 1*A, Inset*), which exhibits response pathway specificity rather than the receptor specificity shown by other natural ligands.

MATERIALS AND METHODS

Retinoids. 9-*Cis*-4-oxo-RA was synthesized from 9-*cis*-RA by methylation via diazomethane (21), 4-hydroxylation using SeO_2 (24), 4-oxidation via MnO_2 (25), and saponification (25), respectively. Subsequent preparations of 9-*cis*-4-oxo-RA were obtained from photoisomerates of 4-oxo-RA, prepared by irradiating 5 mg of 4-oxo-RA in 70 ml of solution A (hexane/acetronitrile/acetic acid: 97.8:1.1:1.1; adapted from ref. 26) with normal light for 4 hr. Absorption maxima (in solution A) were: 4-oxo-RA, 360 and 280 nm; 9-*cis*-4-oxo-RA, 350 and 280 nm. The molar extinction coefficient of 9-*cis*-4-oxo-RA was estimated at 38,29 by comparing UV absorbance at the absorption maxima with the amount of cpm of ^{3}H-4-oxo-RA and ^{3}H-9-*cis*-4-oxo-RA of the same specific activity. 600.1 MHz ^{1}H NMR of 9-*cis*-4-oxo-RA (in CdCl3): 7.04 (dd, 1H, $^3J_{H11H12}$ 16.5 Hz, $^3J_{H11H10}$ 11.3 Hz, H11); 6.84 (d, 1H, $^3J_{H8H7}$ 16.1 Hz, H8); 6.34 (d, 1H, $^3J_{H7H8}$ 16.1 Hz, H7); 6.31 (d, 1H, $^3J_{H12H11}$ 16.5 Hz, H12); 6.21 (d, 1H, $^3J_{H10H11}$ 11.3 Hz, H10); 5.83 (s, 1H, H14); 2.54 (t, 2H, ^3J_H^{3}H2 6.8 Hz, H3); 2.33 (s, 3H, H20); 2.04 (s, 3H, H18); 1.89 (s, 3H, H19); 1.89 (t, 2H, $^3J_{H3H2}$ 6.8 Hz, H2); 1.20 (s, 6H, H16 + H17) ppm. ^{3}H-4-oxo-RA isomers were prepared by 4-oxidation of ^{3}H-methylretinoate (prepared by methylation of ^{3}H-RA using diazomethane) using sodiumchlorate and sodiumiodide as described (27). Extensive washing was performed with 1 M potassiumiodide to remove iodine. All retinoids were purified by normal-phase HPLC. SR11246 was a gift from M. Dawson (SRI International, Menlo Park, CA), Am80 and Ch55 from K. Shudo, University of Tokyo (to B. van der Burg and P.T.v.d.S., Hubrecht Laboratory). ^{3}H-RA (50 Ci/mmol) was obtained from DuPont/NEN and ^{3}H-9-*cis*-RA (48 Ci/mmol) from Amersham. Other retinoids were gifts from J. Bausch (Hoffmann-LaRoche, Basel).

***In Vivo* Isomerization Assay.** Fifty late-stage 9 *Xenopus* embryos were cultured in 0.5 ml of tap water containing 2 μCi ^{3}H-4-oxo-RA (solvent methanol was evaporated under nitrogen) and 0.01% BSA (essential fatty acid free), as carrier protein, at 21°C for 3 hr (embryos reaching stage 11–11.5). Control incuba-

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Abbreviations: RA, retinoic acid; RAR, RA receptor; RXR, retinoid X receptor; DR, direct repeat.

†Present address: Gene Expression Program, European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany.
¶To whom reprint requests should be addressed. e-mail: tony@niob.knaw.nl.

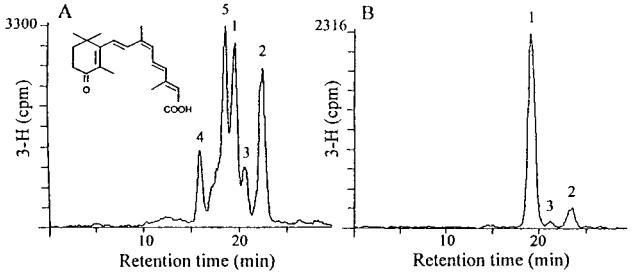

FIG. 1. *In vivo* isomerization of 4-oxo-RA to 9-*cis*-4-oxo-RA. Normal-phase HPLC showing separation of ^{3}H-4-oxo-RA isomers produced from ^{3}H-4-oxo-RA in gastrulating *Xenopus* embryos (*A*) or in control medium (*B*). Peak identification: 1, 4-oxo-RA; 2, 13-*cis*-4-oxo-RA; 3, 9-*cis*-4-oxo-RA. Peaks 4 and 5 represent unassigned 4-oxo-RA isomers. (*Inset*) Structural formula of 9-*cis*-4-oxo-RA.

tions were identical but without embryos. After washing with 0.01% BSA, retinoids were extracted as described (21) by using mixed nonlabeled 4-oxo-RA as internal standards. Retinoids were analyzed by normal-phase HPLC using a Spherisorb S3W silica column and elution with solution A. Detection was via a photodiodearray detector (991 m, Waters) and an on-line radioactivity detector (LB506, Berthold, Nashua, NH) using a Ready Flow III (Beckman) scintillator.

Expression and Reporter Constructs. hRAR and mRXR expression plasmids were gifts from P. Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France). GAL4-hRARβ, GAL4-mRXRα, and GAl4-luc have been described (21, 28). Other GAL4-RAR fusion constructs were made by cloning ligand binding domain containing fragments (when appropriate made blunt by using a large fragment of Klenow enzyme) of hRARα (*Bst*EII–*Stu*I; amino acids begin/end, respectively: 105-end); hRAR (*Bst*EII–*Eco*RI; 176-end); mRXRα (*Hpa*I–*Sma*I; 130-end), and mRXRγ (*Pvu*II–*Pvu*II; 156-end) in the *Sma*I site of pSG424. DR-1-TATAluc was constructed by inserting one copy of an oligonucleotide containing a DR-1G motif (29) (5′-AGCTTAGGGGTCAGAGGTCACTCG-3′) in sense orientation into the *Hin*dIII site of TATA-luc, which contains the E1b TATA box in front of the luciferase reporter gene (28). DR-5-TATAluc was constructed analogously by using the oligonucleotide 5′-AGCTGGAGGTCACTGTCAGGTCACA-3′.

Transfection and Luciferase Assay. DF medium (a 1:1 mixture of DMEM and Ham's F-12, buffered with 44 mM $NaHCO_3$) containing 7.5% fetal calf serum was used in all cell culture experiments (28). Transfections, cell lysis, and assay for LacZ activity were as described (21, 28). Retinoid treatments were 12 hr (for DR-1-TATAluc) or 24 hr (for GAL-luc). Luciferase activity was measured by using the Luclite luciferase reporter gene assay kit (Packard).

Ligand Binding Assay. Competition binding assays were as described (21).

Gel Retardation Assay. Gel retardation was done as described (15) by using *in vitro*-translated RXR protein, via the coupled reticulocyte lysate system (Promega), and ^{32}P-labeled DR-1 probe (see above).

Assay for Reporter Activity in *Xenopus*. *Xenopus zygotes*, 1 hr after fertilization, in 4% Ficoll in tap water were injected with 75 pg of DR5-TATAluc DNA in 4 nl of injection buffer (88 mM NaCl/1 mM KCl/15 mM Tris·HCl, pH 7.5) by using a WPI PV830 pneumatic injector (WPI Instruments, Waltham, MA). Embryos were treated at stage 10 with retinoids at 10 embryos/1 ml of retinoid containing tap water for 5 hr at 18 C. Ten embryos were lysed in 400 μl of reporter lysis buffer (Promega) by vortexing for 1 min. After centrifuging 10 min at 14,000 rpm at 4°C, supernatant was assayed for luciferase as above.

Assay for Retinoid Effects on *Xenopus* Axis Formation. *Xenopus laevis* embryos were treated with retinoids continuously from early stage 10 onwards at 10 embryos/1 ml of retinoid containing tap water in a 24-well plate as described (21). Late tadpole larvae were scored for anteroposterior defects by using a dorsoanterior index as described (21).

Measurement of Retinoid Uptake by *Xenopus*. Stage 10 *Xenopus* embryos were treated in a small volume (10 embryos/1 ml as above) or in a large volume (10 embryos/5 ml) 10^{-6} M retinoid containing tap water at 20°C. After 5 hr, when embryos had reached stage 12, embryos were washed in tap water, and retinoids were extracted by using Ro 10–1670 as internal standard as described (21). RA isomers were measured by reverse-phase HPLC as described (21). 4-Oxo-RA isomers were measured by normal-phase HPLC as above.

RESULTS

Metabolism of 4-Oxo-RA to 9-*Cis*-4-Oxo-RA in Embryos. We and others showed previously that 4-oxo-RA is an endogenous RAR ligand in the early *Xenopus* embryo, where it may participate in patterning the anteroposterior body axis (21, 22). It also is known that the related RAR ligand RA can be isomerized specifically by certain tissues or cell lines to 9-*cis*-RA, a member of the second retinoid ligand family that can bind and activate both RARs and RXRs (18, 19, 30, 31).

We therefore investigated whether isomerization of 4-oxo-RA provides an endogenous pathway to generate a ligand for both RARs and RXRs. We first tested whether all-*trans* to 9-*cis* isomerization of 4-oxo-RA occurs *in vivo* in *Xenopus* embryos. Fig. 1*A* shows normal-phase HPLC of an extract from mid-late gastrula stage *Xenopus* embryos previously treated during gastrulation with a trace amount of ^{3}H-4-oxo-RA. ^{3}H-4-oxo-RA (peak 1) was isomerized extensively into four other main isomers: ^{3}H-13-*cis*-4-oxo-RA (peak 2), ^{3}H-9-*cis*-4-oxo-RA (peak 3), and two unassigned ^{3}H-4-oxo-RA isomers (peaks 4 and 5). In control medium without embryos (Fig. 1*B*), only ^{3}H-13-*cis*-4-oxo-RA and ^{3}H-9-*cis*-4-oxo-RA were formed, but to a far lesser extent than in embryos. These results indicate all-*trans* to 9-*cis* isomerization of 4-oxo-RA in *Xenopus* gastrula embryos.

Binding of 9-*Cis*-4-Oxo-RA to RARs and RXRs. Next, we examined binding of 9-*cis*-4-oxo-RA to RARs and RXRs. Unlabeled retinoids were tested for the ability to compete with ^{3}H-RA for binding to RAR α, β, or γ or to compete with ^{3}H-9-*cis*-RA for binding to RXR α, β, or γ. As a receptor source, we used nuclear extracts from COS-1 cells in which full-length RARs or RXRs were overexpressed. As indicated in Table 1, 9-*cis*-4-oxo-RA competed binding to all three RARs, although less efficiently than RA, 9-*cis*-RA, or 4-oxo-RA. An exception was RAR, which showed similar competitive binding by 9-*cis*-4-oxo-RA as by 4-oxo-RA. Binding to the three RXRs also was competed by 9-*cis*-4-oxo-RA, with affinities lower than those of 9-*cis*-RA. RA and 4-oxo-RA were very poor competitors of RXR binding. We conclude that 9-*cis*-4-oxo-RA is a ligand *in vitro* both for RARs and RXRs.

Differential Activation of GAL4-RARs and GAL4-RXRs by 9-*Cis*-4-Oxo-RA. To test whether 9-*cis*-4-oxo-RA activates both RARs and RXRs, we used chimeric receptors consisting of the

Table 1. 9-*Cis*-4-oxo-RA binds RARs and RXRs *in vitro*

	IC_{50}, nM					
Retinoid	RARα	RARβ	RARγ	RXRα	RXRβ	RXRγ
RA	3.5	1.2	6.3	>5,000	>5,000	>1,000
4-oxo-RA	10.3	4.1	78.4	>5,000	>5,000	>1,000
9-*cis*-RA	13.1	3.1	13.3	65.4	9.7	57.0
9-*cis*-4-oxo-RA	139.8	33.7	55.6	195.7	369.3	255.9

Concentrations required to produce a 50% reduction of specific binding (IC_{50} values) for the ability of indicated retinoids to compete with 0.2 nM ^{3}H-RA for binding to RARs, or with 10 nM ^{3}H-9-*cis*-RA for binding to RXRs. Data are means of at least two experiments. SD was <20%.

GAL4 DNA binding domain coupled to the ligand binding domain of RAR or RXR as described (21). This approach allows activation measurements in which interference from endogenous receptors is reduced. As with binding to full-length RARs, 9-*cis*-4-oxo-RA activated all three GAL4-RARs, but with lower potencies than RA, 9-*cis*-RA, or 4-oxo-RA (Table 2). This finding shows that 9-*cis*-4-oxo-RA is an activating ligand for all three RARs. Surprisingly, despite binding RXRs *in vitro*, 9-*cis*-4-oxo-RA activated GAL4-RXRα, GAL4-RXRβ, and GAL4-RXRγ only weakly, at a similar level as non-RXR ligands RA and 4-oxo-RA (Table 2). This finding suggested a divergent action of 9-*cis*-4-oxo-RA on RXR compared with the classical RXR ligand 9-*cis*-RA. Because RXR has different functions during retinoid signaling, acting as a homodimer via DR-1 retinoid X response elements, or a heterodimer with RAR via DR-5 and DR-2 RA response elements, we investigated activation of RXRs by 9-*cis*-4-oxo-RA in these two pathways.

9-*Cis*-4-Oxo-RA and RXR Homodimer Formation and Activation. Activation curves for 9-*cis*-RA and RA on GAL4-RXRs indicated that the predominant response was via GAL4-RXR homodimers (see also refs. 11 and 32) rather than by GAL4-RXR heterodimers with endogenous RARs in COS cells (see Table 2 and data not shown). Weak activation of GAL4-RXRs by 9-*cis*-4-oxo-RA therefore suggested that 9-*cis*-4-oxo-RA is a poor activator of RXR homodimers. Consistently, 9-*cis*-4-oxo-RA failed to cause a significant activation of a DR-1 retinoid X response element containing reporter via RXRα homodimers (Fig. 2*A*). 9-*Cis*-RA activated this response pathway as reported (33–35). Similar results were obtained with RXRβ and RXRγ, the latter being moderately and similarly responsive to RA and 9-*cis*-4-oxo-RA, and much more responsive to 9-*cis*-RA (data not shown).

To explain the lack of correlation between RXR binding and RXR homodimer activation by 9-*cis*-4-oxo-RA, we considered current knowledge on the mechanism of RXR homodimer activation. This process falls roughly into four events: 1) RXR ligand binding, 2) ligand-induced RXR homodimerization, 3) binding of ligand-bound RXR homodimers to DNA, and 4) transcriptional activation (refs. 14–17 and references therein). Because 9-*cis*-4-oxo-RA is a high affinity ligand for RXRs (event 1; see Table 1), its failure to activate RXR homodimers should reflect failure in a downstream event (events 2, 3, or 4). To test whether 9-*cis*-4-oxo-RA was defective in causing event 2 and/or 3, we used gel retardation to test ability to induce formation and/or DNA binding of RXR homodimers using a ^{32}P-labeled DR-1 probe. As indicated in Fig. 2*B*, a small amount of DR-1-bound RXRα homodimer was detected in absence of ligand. 9-*Cis*-RA strongly induced formation of DNA-bound RXRα homodimers, already detectable at 10^{-8} M. In contrast, RA only caused slight induction of RXRα homodimer formation at 10^{-6} M, probably because of isomerization to 9-*cis*-RA. 9-*Cis*-4-oxo-RA completely failed to induce formation of DNA-bound RXRα homodimers. Similar results were obtained with mRXRγ (data not shown). This finding indicates that 9-*cis*-4-oxo-RA is unable to induce formation and/or DNA binding of RXR homodimers, which may, at least partially, explain why 9-*cis*-4-oxo-RA is a poor activator of RXR homodimers. It is unclear to what extent 9-*cis*-4-oxo-RA is a poor inducer of transcriptional activation via RXR homodimers (event 4), because it is not possible to manipulate event 4 without affecting events 2 and 3 in full-length RXRs, and it is unknown how events 2–4 are interrelated in GAL4-RXR chimeric receptors.

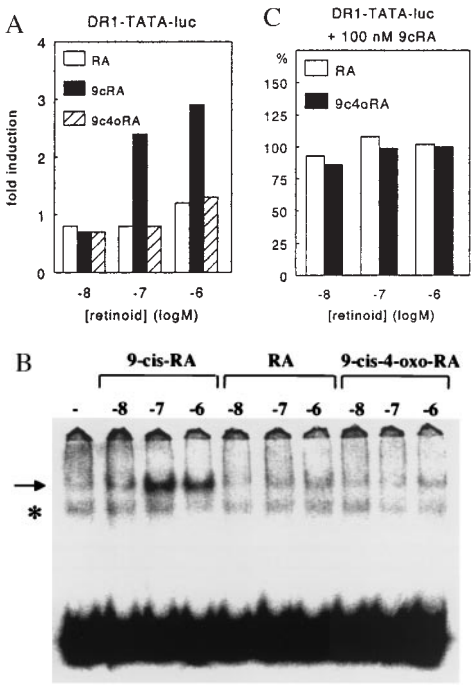

FIG. 2. (*A*) 9-*Cis*-4-oxo-RA (9c4oRA) fails to activate DR-1-TATAluc via RXRα homodimers. COS-1 cells were transfected with DR-1-TATAluc, RXRα, and SV2lacZ. (*B*) 9-*Cis*-4-oxo-RA fails to induce RXRα homodimer formation. Gel retardation assay using ^{32}P-labeled DR-1 probe and *in vitro*-translated RXRα protein. Arrow indicates RXRα homodimers. * indicates nonspecific complex. Retinoid concentrations indicated in log M. (*C*) 9-*Cis*-4-oxo-RA (9c4oRA) fails to compete with 9-*cis*-RA for activation of DR-1-TATAluc via RXRα homodimers. COS-1 cells transfected as in *A*). RA or 9-*cis*-4-oxo-RA were applied together with 10^{-7} M 9-*cis*-RA. Data are percentages relative to activation induced by 10^{-7} M 9-*cis*-RA alone. 9-*Cis*-4-oxo-RA has only 3-fold lower affinity for RXRα than 9-*cis*-RA (see Table 1), but was present in maximal 10-fold excess. Considering the lack of background caused by endogenous RXRs (not shown), we believe our experimental setting was adequate to detect any competition of 9-*cis*-RA by 9-*cis*-4-oxo-RA. We observed no competition whatsoever.

What is the consequence of this special behavior of 9-*cis*-4-oxo-RA on RXR homodimers? Does 9-*cis*-4-oxo-RA counteract activity of a "normal" RXR ligand, e.g., 9-*cis*-RA, or does it not interfere with RXR homodimer activating ligands at all? To answer these questions, we tested the ability of 9-*cis*-4-oxo-RA to

Table 2. Differential activation of GAL4-RARs and GAL4-RXRs by 9-*cis*-4-oxo-RA

	EC_{50}, nM					
Retinoid	GAL4-RARα	GAL4-RARβ	GAL4-RARγ	GAL4-RXRα	GAL4-RXRβ	GAL4-RXRγ
RA	5.9	1.0	1.1	≥800	≥1,000	≥1,000
4-oxo-RA	21.9	5.4	11.8	≥800	≥1,000	≥1,000
9-*cis*-RA	26.3	5.4	14.2	10.4	80.17	67.5
9-*cis*-4-oxo-RA	72.8	33.6	44.9	≥800	≥1,000	≥800

GAL4-receptor chimeras were cotransfected with GAL4-luc reporter and SV2lacZ in COS-1 cells. EC_{50} values are shown and represent concentrations required to produce 50% of the maximal response induced by RA (GAL4-RARs) or 9-*cis*-RA (GAL4-RXRs). At a high concentration (10^{-6} M), RA, 4-oxo-RA and 9-*cis*-4-oxo-RA caused partial activation of GAL4-RXRs (expressed as % relative to maximal activation induced by 9-*cis*-RA): GAL4-RARα, 60.2, 64.7, 59.7; GAL4-RXRβ, 50.4, 19.6, 58.9; GAL4-RXRγ, 48.9, 33.9, 65.3, respectively. Data are means of at least three experiments. SD was <25%.

compete with 9-*cis*-RA for RXR homodimer-mediated DR-1 activation. As shown in Fig. 2*C*, 9-*cis*-4-oxo-RA, like RA, failed to compete with 9-*cis*-RA for activation of the DR-1 retinoid X response element via RXR homodimers. There are two possible explanations: (*i*) 9-*cis*-4-oxo-RA is only defective in causing events 2 and/or 3. Once events 2 and 3 are achieved normally, e.g., by 9-*cis*-RA action, 9-*cis*-4-oxo-RA action proceeds identically as 9-*cis*-RA action. (*ii*) 9-*cis*-4-oxo-RA is unable to bind RXR homodimers bound to DNA. Regardless of the precise mechanism of 9-*cis*-4-oxo-RA action on RXR homodimers, our findings that 9-*cis*-4-oxo-RA failed to activate RXR homodimers or interfere with 9-*cis*-RA-mediated RXR homodimer activation suggests 9-*cis*-4-oxo-RA is a non-RXR agonist rather than an antagonist in the context of RXR homodimers.

9-*Cis*-4-Oxo-RA and RAR-RXR Heterodimer Activation. We investigated whether 9-*cis*-4-oxo-RA is a non-RXR agonist in RAR–RXR heterodimers, using the RXR-specific agonist SR11246 (36). Similarly as reported in investigations in cell lines (10–12), SR 11246 alone failed to cause significant activation of a DR-5 RA response element containing reporter via endogenous RARs and RXRs in early *Xenopus* embryos, but caused synergistic activation when combined with RAR-specific ligands [RA, 4-oxo-RA, or the RARα-specific (at suboptimal concentrations) ligand Am80] (Fig. 3*A*). As expected, 9-*cis*-RA showed no synergism with SR11246, consistent with the idea that 9-*cis*-RA itself is an efficient synergist that activates RAR–RXR heterodimers via both partners (8, 12). 9-*Cis*-4-oxo-RA behaved like 9-*cis*-RA in this respect: it failed to synergize with SR11246. This finding suggests that, in contrast to its inability to activate RXR as homodimer, 9-*cis*-4-oxo-RA activates RXR when present in a RAR–RXR heterodimer, resulting in synergistic activation of both partners of the heterodimer.

Retinoids can cause teratogenesis in vertebrate embryos. Effects are diverse and include microcephaly and tail truncations, which reflect retinoid-induced repatterning of the anteroposterior body axis (4, 21–23, 38–40). We investigated whether 9-*cis*-4-oxo-RA activates RXRs during retinoid axis disruption in *Xenopus*, using a combination with SR11246 as above (Fig. 3*B*). SR11246 alone had no obvious effect on axis formation, suggesting RXR homodimers are not involved. However, SR11246 synergized strongly with RAR-specific ligands, RA, 4-oxo-RA, and Am80, indicating that synergistic activation of RAR-RXR heterodimers efficiently mediates retinoid effects on axial patterning. Both 9-*cis*-RA and 9-*cis*-4-oxo-RA failed to synergize with SR11246. This finding suggests that 9-*cis*-4-oxo-RA, like 9-*cis*-RA, activates RARs and RXRs synergistically during retinoid-induced axis disruption, a process mediated by RAR–RXR heterodimers.

To investigate whether 9-*cis*-4-oxo-RA binds to both partners in RAR–RXR heterodimers, we performed competitive binding using RARβ–RXRα heterodimers, obtained by using nuclear extracts from COS-1 cells in which RARβ and RXRα were co-overexpressed. Fig. 3*C* shows that receptor-specific retinoid ligands only partially competed 10 nM ^{3}H-9-*cis*-RA for binding to RARβ–RXRα heterodimers, indicating that they competed for binding to only one subunit of the heterodimer (RAR in the case of Ch55-a pan RAR agonist and 4-oxo-RA; RXR in the case of SR11246). Both 9-*cis*-RA and 9-*cis*-4-oxo-RA competed ^{3}H-9-*cis*-RA binding fully, indicating 9-*cis*-4-oxo-RA can bind both subunits of RARβ–RXRα heterodimers. Together with the data in Fig. 3 *A* and *B*, these results show 9-*cis*-4-oxo-RA binds and activates both RAR and RXR in RAR–RXR heterodimers.

9-*Cis*-4-Oxo-RA Is a Very Potent Modifier of Axial Patterning. Fig. 3*B* also shows 9-*cis*-4-oxo-RA is a very potent teratogen compared with other known natural retinoid ligands. To investigate this further, we measured dose–response curves (Fig. 4*A*). 9-*Cis*-4-oxo-RA appeared approximately 10 times more potent than RA when external retinoid concentrations were plotted on the *x* axis. Because 4-oxo-RA isomers are more polar than RA isomers, a difference that might cause differential uptake from

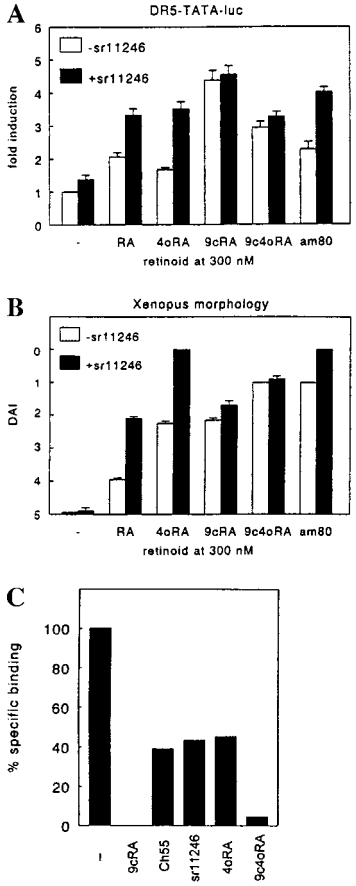

FIG. 3. RXR-specific ligand increases effects of natural and synthetic RAR ligands, but not of 9-*cis*-RA (9cRA) or 9-*cis*-4-oxo-RA (9c4oRA) on DR5-TATAluc activation (*A*) or on axis formation (*B*) in *Xenopus*. (*A*) *Xenopus* zygotes were injected with DR-5-TATAluc reporter. At stage 10, embryos were treated at 10 embryos/1 ml $3 \times 10_{-7}$ M retinoid in the absence or presence of 2.5×10^{-7} M RXR-specific ligand SR11246. 9-*Cis*-4-oxo-RA also proved to be an efficient activator of DR-5 transcription via RAR–RXR heterodimers in COS-1 cells (using cotransfection of RARβ, RXRα, and DR5-TATA-luc), its activity being in between the activities of RA and 9-*cis*-RA (not shown). Data are means ± SEM. (*B*) Stage 10 *Xenopus* embryos treated with retinoids as in *A*. Larvae were scored for anteroposterior defects using a dorsoanterior index (DAI) as described (21). Index 5 represents normal larvae, and index 0 the most ventro-posteriorized larvae. At 3×10^{-7} M, Am80 induced the maximal response, DAI value 0, in the presence of SR11246. At lower Am80 concentrations, synergy with SR11246 was comparable to that of RA or 4-oxo-RA (not shown). Data are means of 10 embryos ± SEM. (*C*) 9-*Cis*-4-oxo-RA binds both subunits of RARβ–RXRα heterodimers. Indicated retinoids (at 10^{-5} M) were tested for competition with 10 nM ^{3}H-9-*cis*-RA for binding to RARβ–RXRα heterodimers. Data are means of two experiments.

the medium, we measured embryonal retinoid concentrations reached after retinoid treatment by using HPLC (Fig. 4*B*). Surprisingly, RA isomers were trapped in embryos to concentrations exceeding the applied concentration (1 μM) by 2- to 18-fold, depending on the RA isomer and total amount of retinoid in the bathing solution. In contrast, 4-oxo-RA isomer concentrations closely resembled applied concentrations and were independent of bathing volume. When embryonal rather than external retinoid concentrations were plotted on the *x* axis, 9-*cis*-4-oxo-RA was seen to be ≈60 times more potent than RA (Fig. 4*C*). One could argue that RA isomers were trapped in embryos in locations where they were not bioactive. That this is not the case was indicated by the finding that RA isomer trapping caused by increasing bathing volume was paralleled by a concomitant increase in teratogenic effect (data not shown). These

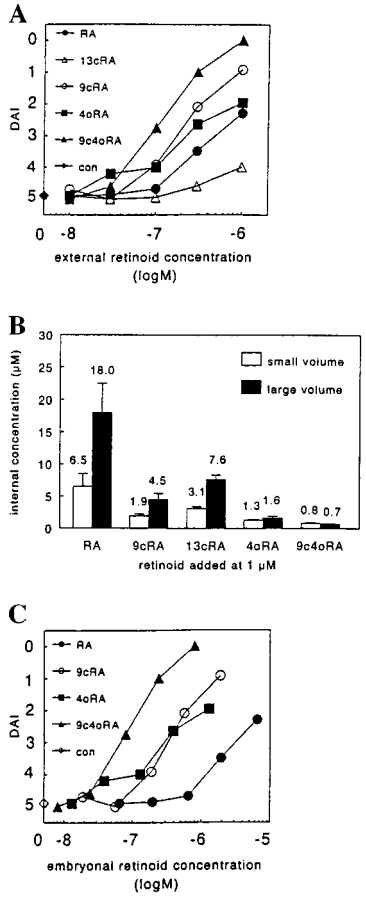

FIG. 4. 9-*Cis*-4-oxo-RA (9c4oRA) disrupts *Xenopus* axis formation. (*A*) Dose–response curves for indicated retinoids, with external retinoid concentrations plotted on the *x* axis, and dorso-anterior index (see Fig. 3*B*) on the *y* axis. Embryos treated at 10 embryos/1 ml retinoid containing tap water from stage 10 (early gastrula) onwards. Data are means of 10 embryos. SD < 10%. con, control. (*B*) Uptake of medium applied retinoids by *Xenopus* embryos. Embryos bathed at 10 embryos/1 ml 10^{-6} M retinoid containing tap water (small volume) or 10 embryos/5 ml 10^{-6} M retinoid containing tap water (large volume) from stage 10–12 (5 hr at 20°C). Embryonal retinoid concentrations then were determined by HPLC. Data are means (above each bar in μM) ± SD. (*C*) Dose–response curves for indicated retinoids, using embryonal retinoid concentrations. Calculated from *A*, using correction factors for retinoid uptake derived from *B*: RA, 6.5; 9-*cis*-RA (9cRA), 1.9; 4-oxo-RA (4oRA), 1.3; 9-*cis*-4-oxo-RA (9c4oRA), 0.8. It is assumed that these correction factors, measured at 10^{-6} M external retinoid concentrations, are also valid for lower external retinoid concentrations. con, control.

findings indicate that 9-*cis*-4-oxo-RA is the most effective natural retinoid metabolite known for disturbing axis formation during *Xenopus* development, suggesting that it may be an important active retinoid ligand *in vivo.* We note that a large part of this effect is likely to be mediated via RAR–RXR heterodimers acting on DR-5 (or DR-2) RA response elements, because, based on the activation levels at embryonal retinoid concentrations rather than external concentrations (compare Figs. 3*A* and 4*B*), 9-*cis*-4-oxo-RA is also a remarkably potent activator of this pathway. A model summarizing the properties of 9-*cis*-4-oxo-RA and other natural ligands for activating retinoid receptor dimers is in Fig. 5.

DISCUSSION

The idea that receptor dimerization can influence responsiveness to ligands is not surprising, because retinoid (and related) receptors contain a strong dimerization interface within the ligand binding domain (6, 7). It is known that ligand binding/activation

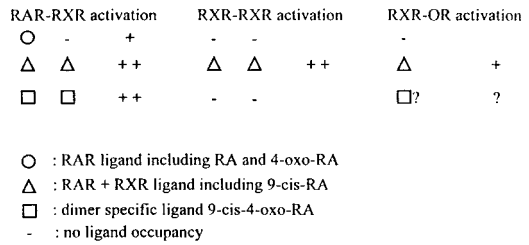

FIG. 5. Model illustrating ligand responsiveness of retinoid receptors in different RXR dimers. Ligands interacting with a receptor partner are indicated below that partner. Level of induced transcriptional activation indicated by number of + symbols. RAR ligands RA and 4-oxo-RA activate RAR–RXR heterodimers via RAR only. 9-*cis*-RA activates both RARs and RXRs in RAR–RXR heterodimers, which generally leads to higher activation levels. 9-*Cis*-4-oxo-RA (9c4oRA) behaves as 9-*cis*-RA in RAR–RXR heterodimers by activating both partners. 9-*cis*-RA, but not 9-*cis*-4-oxo-RA, activates RXR homodimers. Other 9-*cis*-RA-responsive pathways involving heterodimers between RXR and orphan receptors (ORs) remain to be tested for 9-*cis*-4-oxo-RA responsiveness.

and receptor dimerization are intimately related for members of the steroid/thyroid hormone receptor family. A striking example is the insect hormone ecdysone, which binds only to heterodimers between the ecdysone receptor and ultraspiracle, but not to the individual monomeric subunits (41). For retinoid receptors, allosteric inhibition of ligand binding to RXRs was reported under certain *in vitro* conditions for RAR–RXR heterodimers bound to DR-1 or DR-5 elements (42, 43). This finding suggested a possible additional influence of DNA binding on ligand responsiveness. However, *in vivo*, both receptor partners can be liganded, leading to a characteristic response in which liganded RXR is only transcriptionally active when RAR is liganded (9–11). On the other hand, other RXR dimers, including peroxisome proliferator-activated receptor (PPAR)-RXR, liver X receptor (LXR)-RXR, nerve growth factor-induced gene B (NGFI-B)-RXR, and farnesoid X-activated receptor (FXR)-RXR, can be activated independently via either subunit (44, 45).

A further extension of the concept that receptor dimerization and ligand responsiveness are closely connected is provided by the discovery of dimer-dependent receptor ligands. So far, two cases are known in which ligands show specificity for receptor dimers rather than individual receptors, although these cases involve artificial ligands or dimers. Ecdysone and muristerone A differ in activating ecdysone receptor (EcR)-ultraspiracle (USP) and both EcR-USP and EcR-RXR dimers, respectively (41). For retinoid receptors, differential effects, namely activation of RAR–RXR heterodimers and repression of RXR homodimers, were observed by using the synthetic retinoid LG100754 (37). This study suggested the possibility that natural retinoid ligands possessing dimer-specific receptor activation properties also might exist. Our finding that 9-*cis*-4-oxo-RA is RAR–RXR heterodimer specific fulfills this expectation and reveals a natural retinoid metabolite with this property. Other natural retinoids activate either RARs or RARs and RXRs, irrespective of the response pathway used *in vivo*, and can be considered more general retinoid ligands. Whether these known ligands or other yet-to-be-discovered ligands do have some differential pathway discriminating properties *in vivo* is an interesting question for the future. This possibility already is suggested by known differences between ligands with respect to affinities for retinoid receptor types (RA vs. 9-*cis*-RA for RARγ binding) (46), biological activities during retinoid-sensitive processes (RA vs. 4-oxo-RA during anteroposterior axis formation in *Xenopus* embryos) (21), and availability during different developmental stages (all-*trans*-4-oxo-retinol vs. all-*trans*-4-oxo-retinal vs. 4-oxo-RA in early *Xenopus* development) (22).

What is the physiological significance of a ligand that discriminates between RXR homodimers and RAR–RXR het-

erodimers? At present, it is unclear to what extent RXR homodimer signaling, including ligand-induced RXR homodimerization and/or DNA binding, is used *in vivo* (see refs. 3, 5–7, 17, 45, and 47 for discussion). Despite the uncertainties, which require further investigation, several reports propose that ligand-induced RXR homodimers regulate expression of retinoid target genes. These include *hRXRγ* (35), *rGH* (48), *hApoA-I* (33), *hApoA-II* (34), among others (5, 6). We note that none of these genes has a direct role in embryonic pattern formation; they are instead involved in retinoid autoregulation, growth, and metabolism. Our own (Fig. 3*B*) and other (12) results concerning the inability of RXR-specific agonists to cause developmental abnormalities further suggest that RXR homodimers do not mediate retinoid effects during embryonic pattern formation (3). On the other hand, several studies indicate that RAR–RXR heterodimers transduce retinoid signals during embryonic development *in vivo* (3). Putative RAR–RXR heterodimer target genes include the important axial patterning genes *Hoxa-1* (49), *Hoxb-1* (50), and *Hoxd-4* (51). The importance of synergistic activation of RAR and RXR (in the case of 9-*cis*-RA and 9-*cis*-4-oxo-RA) compared with specific activation of RAR (in the case of RA and 4-oxo-RA) in the context of RAR–RXR heterodimers has been suggested to be the enhanced capacity of RAR–RXR synergism to activate target genes at low endogenous ligand concentrations (9).

Interestingly, the different proposed roles of RXR homodimer and RAR–RXR heterodimer signaling parallel the ligand availabilities known so far. We and others detected 4-oxo retinoid metabolites in early *Xenopus* embryos (21, 22), and we show here that 4-oxo-RA can be isomerized to 9-*cis*-4-oxo-RA *in vivo* in these embryos. 9-*Cis*-RA is not present at detectable levels in early *Xenopus* embryos (ref. 22 and our unpublished results), whereas this isomer has been detected in adult liver and kidney, sites that are known to express RXR at high levels (19). Here, it may be involved in processes such as vitamin A and lipid metabolism. To what extent the absence of 9-*cis*-RA during early *Xenopus* development is significant remains to be established. It is, however, tempting to speculate that production of a RAR–RXR heterodimer-specific ligand is a step toward targeting retinoid action to processes mediated via RAR–RXR heterodimers. These processes include diverse aspects of embryonic pattern formation, among which patterning of the *Xenopus* anteroposterior body axis is particularly sensitive to 9-*cis*-4-oxo-RA compared with RA, 4-oxo-RA, or 9-*cis*-RA, arguing for an important role of 9-*cis*-4-oxo-RA during this process.

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