4-Oxoretinol, a new natural ligand and transactivator of the retinoic acid receptors

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(4-hydroxyretinol/vitamin A metabolism/embryonic pattern formation)

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ABSTRACT All-*trans*-retinoic acid (at-RA) induces cell differentiation in a wide variety of cell types, including F9 embryonic teratocarcinoma cells, and can influence axial pattern formation during embryonic development. We now identify a novel retinoid synthetic pathway in differentiating F9 cells that results in the intracellular production of 4-oxoretinol (4-oxo-ROL) from retinol (vitamin A). Approximately 10–15% of the total retinol in the culture is metabolized to 4-hydroxyretinol and 4-oxo-ROL by the at-RA-treated, differentiating F9 cells over an 18-hr period, but no detectable metabolism of all-*trans*-retinol to at-RA or 9-*cis*-retinoic acid is observed in these cells. Remarkably, we show that 4-oxo-ROL can bind and activate transcription of the retinoic acid receptors whereas all-*trans*-retinol shows neither activity. Low doses of 4-oxo-ROL (e.g., 10^{-9} or 10^{-10} M) can activate the retinoic acid receptors even though, unlike at-RA, 4-oxo-ROL does not contain an acid moiety at the carbon 15 position. 4-oxo-ROL does not bind or transcriptionally activate the retinoid X receptors. Treatment of F9 cells with 4-oxo-ROL induces differentiation without conversion to the acid and 4-oxo-ROL is active in causing axial truncation when administered to *Xenopus* embryos at the blastula stage. Thus, 4-oxo-ROL is a natural, biologically active retinoid that is present in differentiated F9 cells. Our data suggest that 4-oxo-ROL may be a novel signaling molecule and regulator of cell differentiation.

Retinoids are derivatives of retinol (vitamin A) that are required for the appropriate functioning of a wide number of cell types involved in processes such as cell differentiation, axial pattern formation in embryogenesis, reproduction, and vision (for reviews, see refs. 1–3). Retinoids also exhibit cancer preventive actions and are used to treat some types of cancers (for review, see ref. 2). The biological and transcriptional effects of retinoids are mediated by their interactions with the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (for review, see ref. 4). RARs and RXRs can heterodimerize and bind response elements in a variety of target genes (for reviews, see refs. 1 and 4). The ligands for these receptors are generally thought to be acid derivatives of retinol; all-*trans*-retinoic acid (at-RA) has been shown to bind to the RARs with high affinity and 9-*cis*-retinoic acid (9-*cis*-RA) binds and activates the RXRs as well the RARs (for reviews, see refs. 4 and 5). The N-terminal portion of the ligand binding domain (E domain) is essential for the recognition of at-RA by various RARs (6).

Much recent research in the vitamin A field has focused on at-RA and its isomers. Retinol has been reported to be metabolized to at-RA in a large number of different cultured cells and in various tissues from developing embryos (3, 5). However, the complexity of the biological actions of vitamin A suggests that retinol derivatives other than at-RA mediate some of these actions. Consistent with this idea, two nonacidic retinol derivatives, 14-hydroxy-4,14-*retro*-retinol and anhydroretinol, have recently been identified as putative intracellular signaling molecules (7, 8).

To search for other novel retinol derivatives, we have used the F9 teratocarcinoma cell line. F9 stem cells share biochemical and morphological characteristics with mouse embryonic stem cells (for review, see ref. 1); these cells can be induced to differentiate in monolayer culture into cells resembling extraembryonic endoderm, an epithelial cell of the mouse blastocyst, by treatment with at-RA over a 3- to 4-day period. We describe here the identification of a novel endogenous retinol metabolic pathway that is induced during the differentiation of the murine embryonic F9 stem cells. We identify all-*trans*-4-oxoretinol as a biologically active metabolite in this pathway and propose that it is a physiologically important retinoid signaling molecule.

MATERIALS AND METHODS

Cell Culture and Northern Analysis. F9 teratocarcinoma stem cells and CV-1 cells were cultured as described (9). For analysis of retinoid metabolism, F9 cells in monolayer culture were rinsed twice in phosphate-buffered saline (PBS) and then incubated in the presence of 50 nM 11,12-[^{3}H]retinol (specific activity, 50 μCi/nMol; 1 Ci = 37 GBq) for various periods of time. Northern analysis was performed as described (10).

Retinoid Extraction and HPLC Analysis. Retinoids were extracted from both cells and media immediately (11), and appropriate internal standards were added to the samples before extraction so that their elution profiles could be followed by absorbance. Each sample was then analyzed by HPLC (Waters) using a 5-μm analytical reversed-phase C18 column (Vydac, Hesperia, CA) at a flow rate of 1.5 ml/min, with a gradient of acetonitrile/ammonium acetate (15 mM, pH 6.5) from 40% to 67% acetonitrile in 35 min, followed by 100% acetonitrile for an additional 25 min. Detection was achieved by a photodiode array and an on-line scintillation counter (Packard model A-500).

To obtain retinoids for structure determination, a large number of cells (200 plates, each with 150-cm^2 surface area) were cultured and then treated with retinoic acid (RA) (1 μM) for 24 hr. The cells were washed and incubated in the presence of 10 μM retinol for 24 hr to maximize the production of the retinol derivatives. The media were then collected and the proteins were precipitated with 60% saturated ammonium sulfate for 6 hr. The retinoids were then extracted and purified

using gradients (water/acetonitrile or methanol buffer) and a series of preparative and analytical reversed-phase columns.

Retinoid Characterization and Synthesis. ^{1}H NMR spectra were recorded on a Varian model VXR-400 spectrometer in C^2HCl_3. The chemical shifts are given in parts per million ppm (δ reference peak = 7.24 ppm) and the coupling constants (J) in Hz. Assignments of the protons and the double-bond configurations were based on decoupling and nuclear Overhauser effect experiments. High-resolution electron impact mass spectroscopy (HR-EIMS) was measured on a JEOL DX-303 HF spectrometer (reference perfluorokerosine). Absorption spectra were recorded on a Perkin–Elmer Lambda 4B UV-vis spectrophotometer. CD spectra were measured on a Jasco (Easton, MD) model J-720 spectropolarimeter.

Retinoid a [all-*trans*-4-hydroxyretinol (all-*trans*-4-hydroxy-ROL)]. ^{1}H NMR: δ 1.03/1.06 [2s, 6H, 1-$(CH_3)_2$], 1.84 (s, 3H, 5-CH_3), 1.88 (s, 3H, 13-CH_3), 1.96 (s, 3H, 9-CH_3), 4.02 (m, 1H, 4-H), 4.33 (t, J 7.5, 2H, 15-H), 5.71 (t, J 7.2, 1H, 14-H), 6.10 (d, J 16, 1H, 8-H), 6.12 (d, J 11, 1H 10-H), 6.17 (d, J 16, 1H, 7-H), 6.31 (d, J 15, 1H, 12-H), 6.61 (dd, J 11, 15, 1H, 11-H). Retinoid **c** [all-*trans*-4-oxoretinol (all-*trans*-4-oxo-ROL)]. ^{1}H NMR: δ ppm 1.15 [s, 6H, 1-$(CH_3)_2$], 1.82 (t, J 7, 2H, 2-H_2), 1.82 (s, 3H, 5-CH_3), 1.84 (s, 3H, 13-CH_3), 1.95 (s, 3H, 9-CH_3), 2.48 (5, J 7, 2H, 3-H_2), 4.32 (t, J 2H, 15-H_2), 5.72 (t, J 14-H), 6.21 (d, J 15, 1H, 7-H), 6.22 d, J 10, 1H, 10-H), 6.31 (d J 15, 1H, 8-H), 6.35 d, J 15.2, 1H, 12-H), 6.59 (dd, J 15.2, 10, 1H, 11-H).

All-*trans*-4-oxo-ROL and racemic all-*trans*-4-hydroxy-ROL were prepared according to Surmatis (12) and Boehm *et al.* (13). 4-oxo-ROL was obtained as a mixture of isomers that were separated by HPLC (300 × 25 mm i.d. YMC-Pack 5 μm silica gel column (Waters), eluted with ethyl acetate/hexane 40:60 at 5 ml/min flow rate). The retention times of 13-*cis*-, all-*trans*-, and 9-*cis*-4-oxo–ROL were 40, 43, and 45 min, respectively. (4S)-all-*trans*-4-hydroxy-ROL was synthesized starting with (4S)-4-hydroxy-β-ionone obtained according to Haag *et al.* (14). Conversion to (4S)-4-hydroxyretinal (15), followed by sodium borohydride reduction and flash chromatography purification led to a mixture of (4S)-4-hydroxyretinol isomers that were further separated by HPLC (300 × 25 mm i.d. YMC-Pack 5 μM silica gel column, eluted with ethyl acetate/hexane 60:40 at 5 ml/min flow rate). The retention times of all-*trans*-, 13-*cis*-, and 9-*cis*-4-hydroxyretinol were 31, 33, and 35.5 min, respectively. (4R)-All-*trans*-4-hydroxyretinol was prepared similarly starting with (4R)-4-hydroxy-β-ionone (14).

Transfections, Nucleosol Preparations, and Binding Assays. Retinoids were assayed for their ability to bind the RARs and RXRs using nucleosol fractions from transfected COS-1 cells. COS-1 cells were transfected by electroporation with pSG5 expression vectors containing cDNAs for mouse RARs α, β, or γ or RXRs α, β, or γ (16). Aliquots of nucleosol or cytosol (17, 18) were incubated in nuclei lysis buffer (17) with tritiated ligands for 4 hr at 4°C. Retinoids were added in ethanolic solutions that did not exceed 2% of the total incubation volume. For competitive binding assays, the incubations were performed with increasing concentrations of unlabeled competing ligand and a fixed concentration of the radioligand (10 nM at-[3H]RA or 9-*cis*-[3H]RA). For all binding assays, bound radioactivity was separated as described (16). IC_{50}s values were calculated using a four parameter fit (19).

Assay of Transactivation in CV-1 Cells. Full-length CMX-hRARα (1 μg) or CMX-hRXRα (1 μg) (h, human) was cotransfected with the reporter tk-(TREp)$_2$-luc (5 μg) and the transfection control plasmid CMX-βGAL (4 μg) into CV-1 cells in 96-well plates using N-[1(2,3-dioleolyoxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) reagent (5 μg/ml) and medium plus 10% delipidated serum. Cells were washed with fresh medium after 18–24 hr and ligands (assayed for identity and concentration using HPLC) were added in triplicate. After 24–48 hr of further incubation, the cells were

lysed and assayed for luciferase and β-galactosidase activity as described (20). In other experiments, CMX-hRARα, -β, and -γ were cotransfected with the reporter tk-galp3-luc, ligands were added, and assays were performed as described above.

***Xenopus laevis* Embryos.** Fertilized *X. laevis* embryos (21) were cultured in 0.1× MBS (22) at 22°C and developmental stages were assigned according to Nieuwkoop and Faber (23). Varying concentrations of all-*trans*-retinol (at-ROL), at-RA (Sigma), or 4-oxo-ROL prepared in dimethyl sulfoxide were added to 0.1× MBS during the blastula stage (stage 8). Embryos were cultured in the same medium until harvesting, when control (dimethyl sulfoxide treatment) embryos reach the tadpole stage (stage 42).

RESULTS

Induction of Retinol Metabolites During F9 Cell Differentiation. F9 stem cells were cultured and induced to differentiate by treatment with exogenous 1×10^{-6} M at-RA for 72 hr. Subsequently, the cells were rinsed and incubated with 50 nM 11,12-[3H]retinol for 9 hr. Approximately 3×10^8 cells and their incubation media were collected, extracted, and analyzed on HPLC. HPLC radiochromatograms of cell extracts from both F9 stem cells and at-RA-treated F9 cells for 72 hr are shown (Fig. 1). Retinoids were identified by matching their retention times and their absorption spectra with those of standard compounds or by spectroscopic analysis. A major change in retinol metabolism occurs when F9 cells are induced to differentiate by at-RA (Fig. 1). The synthesis of several metabolites more polar than retinol (Fig. 1*B*, peaks a, b, and c) is induced by $\approx$15- to 20-fold in the differentiating cells and these metabolites are also found in the media from differentiating F9 cells (data not shown). This induction of retinol metabolism is seen as early as 12 hr after addition of exogenous RA and is maintained for as long as 4 days thereafter. The EC_{50} for this induction of retinol metabolism is approximately 1×10^{-7} M at-RA (data not shown). Approximately 10–15% of the exogenously added [3H]retinol is converted to these polar [3H]retinol metabolites by the differentiating F9 cells during an 18-hr period, resulting in an intracellular concentration of these metabolites of approximately 1×10^{-7} M; a portion of these metabolites remains in the cells while a greater portion is secreted into the cell culture medium. In contrast, less than 1% of the [3H]retinol is converted by the undifferentiated F9 stem cells over an 18-hr period. It is notable that the rates of synthesis of other retinol derivatives such as 14-hydroxy-4,14-*retro*-retinol are unchanged after at-RA treatment (Fig. 1).

Williams and Napoli (24) reported that retinol was converted to at-RA in F9 cells. In contrast to their results, we have not detected at-RA as an endogenous retinol metabolite in either the F9 stem cells or the at-RA-treated differentiated F9 cells (Fig. 1*B*). The level of detection of [3H]RA in this assay was calculated by using 50 nM exogenous [3H]retinol as a substrate; by determining the area under this intracellular [3H]retinol peak, we calculated that the level of detection of [3H]RA in this assay was about 1×10^{-9} M. The criteria used to identify at-RA were coelution with a known standard, at-RA, and UV spectral analysis of peaks in this region of the chromatogram (Fig. 1). By these criteria, no appreciable at-[3H]RA is generated, even in the differentiating F9 cells.

All-*trans*-4-hydroxy-ROL and all-*trans*-4-oxo-ROL Are the Induced Metabolites. To characterize the retinol metabolites induced in F9 cells after at-RA treatment, we purified to homogeneity peak **a** and peak **c**. From 20 liters of incubation media, 60 absorption units at 322 nm of retinoid **a** and 20 absorption units at 350 nm of retinoid **c** were isolated and characterized as all-*trans*-4-hydroxy-ROL and all-*trans*-4-oxo-ROL, respectively, based on absorption, CD, proton nuclear magnetic resonance (^{1}H NMR), and HR-EIMS analyses.

Retinoid **a** exhibits an absorption spectrum with λ_{max} at 322 nm (Fig. 2*B*). The HR-EIMS gave an observed value of 302.2234 (calculated for $C_{20}H_{30}O_2$ = 302.2246), suggesting that retinoid **a**
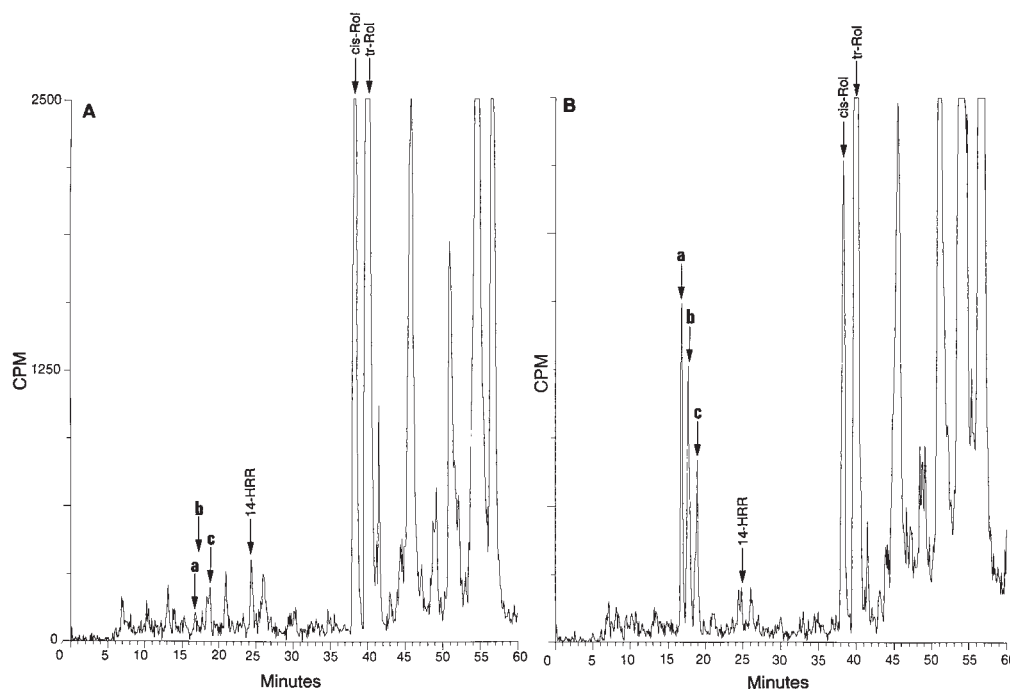
Fig. 1. Metabolism of retinol in F9 stem and RA-treated cells (72 hr). Cells were incubated in the presence of 50 nM [³H]retinol for 9 hr, followed by extraction and HPLC analysis of labeled retinoids from stem (*A*) and RA-treated cells (*B*). The abbreviations of some retinoid standards and their retention times (min) are: 4-hydroxy-retinoic acid (4-OH-RA), 8.4; 4-oxo-retinoic acid (4-oxo-RA), 8.6; retinoid **a**, 16.8; retinoid **b**, 17.7; retinoid **c**, 18.6; all-*trans*-retinoic acid (at-RA), 22.4; 14-hydroxy-4,14-*retro*-retinol (14-HRR), 24.4; 13-*cis*-retinol(s) (cis-Rol), 38.3; all-*trans*-retinol (at-Rol), 40; anhydroretinol (AR), 43.

has one oxygen atom more than its precursor retinol ($C_{20}H_{30}O$), which can be accounted for by an additional hydroxyl group. The [¹H] NMR spectrum (see *Materials and Methods*) establishes retinoid **a** as all-*trans*-4-hydroxy-ROL. Because the carbon at position 4 is an asymmetric center, retinoid **a** could be optically active. Indeed, its CD spectrum shows a positive Cotton effect at 328 nm ($\delta\varepsilon$ + 1.55), and a negative Cotton effect at 254 nm ($\delta\varepsilon$ − 0.5), indicating optical activity (Fig. 2*A*). To determine the absolute configuration at C-4, we synthesized (4*R*)-all-*trans*- and (4*S*)-all-*trans*-hydroxy-ROL, starting from (4*R*)- and (4*S*)-β-ionone, respectively (14). The signs of the CD spectrum of retinoid **a** (Fig. 2*A*) match that of synthetic (4*S*)-all-*trans*-4-hydroxy-ROL (data not shown). However, the $\delta\varepsilon$ values of retinoid **a** (+1.5 at 328 mn and −0.5 at 254 nm) are smaller than that of 98% optically pure (4*S*)-all-*trans* isomer (i.e., +2.68 at 328 nm and −0.87 at 254 nm). This suggests that the isolated 4-hydroxy-ROL is a mixture of enantiomers where the (4*S*)-all-*trans*-4-hydroxy-ROL predominates. Retinoid **b** (Fig. 1) could be 13-*cis*-4-hydroxy-ROL, based on its HPLC retention time and its UV–visible absorption spectrum. Retinoid **c** exhibits an absorption spectrum with λ_{max} at 276 nm and 350 nm (Fig. 2*B*). The HR-EIMS gave an observed value of 300.2088 (calculated for $C_{20}H_{28}O_2 = 300.2090$). These data, the red-shifted UV λ_{max} (350 nm as compared with 322 nm for retinol), and the band at 276 nm suggest a keto group at position 4. Indeed, the [¹H] NMR spectrum confirms that retinoid **c** is all-*trans*-4-oxo-ROL (Fig. 2*C*).

All-*trans*-ROL Binds Selectively to Subtypes of RARs. To determine whether all-*trans*-4-hydroxy-ROL and all-*trans*-4-oxo-ROL are ligands for RARs and RXRs, their abilities to compete for binding sites with at-RA and 9-*cis*-RA were examined under equilibrium conditions (25). The results indicate that all-*trans*-4-hydroxy-ROL is a relatively weak inhibitor of [³H]RA binding to all three subtypes of RARs as indicated by the IC_{50} values (Table 1). In contrast, all-*trans*-4-oxo-ROL is a good inhibitor of at-[³H]RA binding to RARα and RARβ and a weaker inhibitor (10-fold less) of at-[³H]RA binding to RARγ (Table 1). Thus, all-*trans*-4-oxo-ROL exhibits preferential binding to subtypes of RARs. The higher IC_{50} of 4-oxo-ROL for RARγ than for RARα and RARβ could reflect a faster dissociation rate, as was previously seen for 9-*cis*-RA and RARγ (26).

Neither all-*trans*-4-hydroxy-ROL nor all-*trans*-4-oxo-ROL inhibited the binding of 9-*cis*-[³H]RA to the RXRs (data not

shown). Retinol produced no apparent inhibition of at-[³H]RA binding to any of the RARs (Table 1) or 9-*cis*-[³H]RA binding to the RXRs (data not shown), even when used at concentrations of up to 50 μM. This result contrasts with the data previously reported by Repa *et al.* (27).

All-*trans*-4-oxo-ROL Activates RARs but not RXRs. We first tested the ability of 4-oxo-ROL to activate RAR or RXR. CV-1 cells were transiently transfected with full-length hRARα or hRXRα and the common reporter gene TREp (28). 4-oxo-ROL was able to activate hRARα but not hRXRα (Fig. 3 *A* and *B*). 4-oxo-ROL was very active at low (<10^{-8} M) doses and was considerably more potent than at-ROL at all doses tested. In this assay, 4-oxo-RA was slightly less active than at-RA (Fig. 3). At high retinoid doses, the acid retinoids were more active than 4-oxo-ROL. This result was unlikely to be caused by the conversion of 4-oxo-ROL to an acidic derivative because 4-oxo-ROL has a long half-life in these cells (see below).

To test the ability of 4-oxo-ROL to activate RAR subtypes differentially, we used chimeras between the RAR ligand binding domains and the yeast GAL4 DNA-binding domain (29), because this assay eliminates any background from the endogenous receptors in CV-1 cells. 4-oxo-ROL activated all three RARs at much lower concentrations than at-ROL (Fig. 4 *A–C*).

Effects of Various Retinoids on Gene Expression. We tested the effects of several retinol derivatives on F9 cells using the expression of Hoxa-1 (formerly Hox 1.6) and laminin B1 as markers (30–32). Northern blot analyses of F9 cells treated with a 1 μM concentration of one of four retinoids for different periods of time were performed. Hoxa-1 transcripts were induced 7-, 10-, 18-, and 22-fold by retinol, 4-hydroxy-ROL, 4-oxo-ROL, and at-RA, respectively, at 48 hr. Laminin B1 transcripts were increased by 6-, 7-, 9-, and 10-fold by retinol, 4-hydroxy-ROL, 4-oxo-ROL, and RA, respectively, at 72 hr after treatment. These assays show that all four retinoids, including retinol, can induce these two differentiation markers in F9 cells. 4-oxo-ROL is more biologically active than retinol in inducing F9 cell differentiation using the Hoxa-1 gene as a marker. Since retinol itself is converted to 4-oxo-ROL in the differentiating cells, the fact that retinol has some effect on gene expression is understandable.

4-oxo-ROL Exhibits a Long Half-Life in F9 Cells. 4-oxo-ROL has been shown in this study to elicit several biological responses. It was important to determine whether or not 4-hydroxy-ROL and 4-oxo-ROL were metabolized in F9 cells
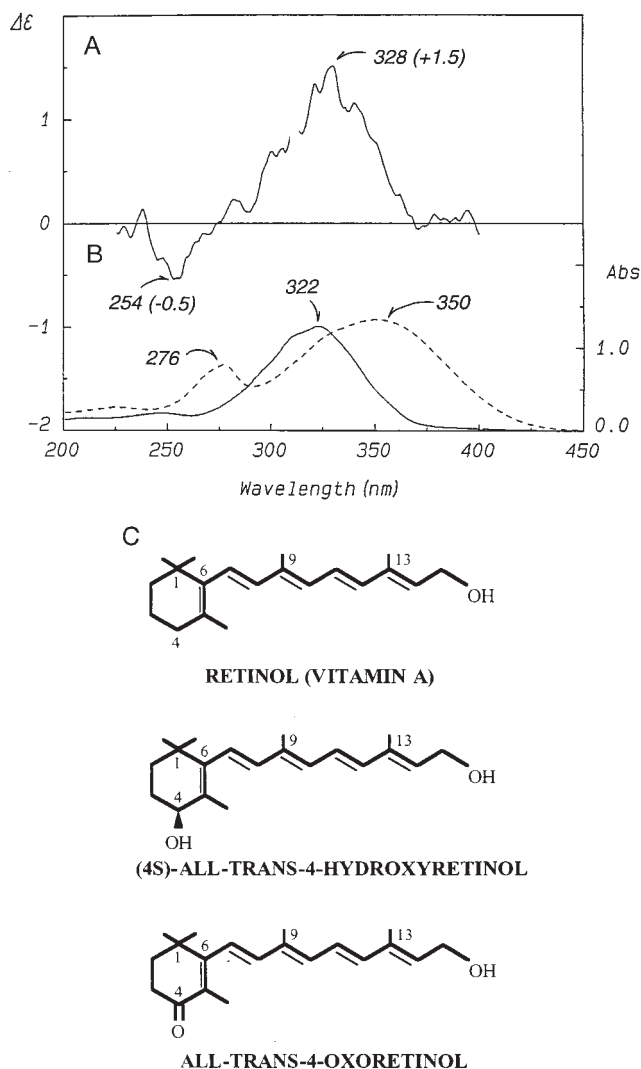
FIG. 2. (*A*) CD spectrum of retinoid **a** [(4*S*)-all-*trans*-4-hydroxy-ROL] in methanol. The ε values were estimated using a concentration derived from 52,000 $M^{-1}\cdot cm^{-1}$, the extinction coefficient value of at-ROL. (*B*) Absorption spectra of retinoid **a** (solid line) and retinoid **c** (all-*trans*-4-oxo-ROL (dotted line) in methanol. (*C*) Structures of at-ROL, (4*S*)-at-hydroxy-ROL, and at-4-oxo-ROL.

into other retinoids such as 4-hydroxy-RA or 4-oxo-RA, which were previously reported to be biologically active (33). For this purpose, all-*trans*-4-hydroxy-[³H]ROL and all-*trans*-4-oxo-[³H]ROL were purified from RA-treated F9 cells after the incubation of the cells with [³H]retinol for 18 hr.

Table 1. Comparison of IC_{50} values for binding of the different retinoids to RARs

Ligand	IC_{50},* nM		
	RARα	RARβ	RARγ
4-oxo-ROL	330 ± 54*	420 ± 110	3200 ± 520
4-hydroxy-ROL	5000 ± 1400	3800 ± 1100	3400 ± 1600
at-ROL	>50,000	>50,000	>50,000
at-RA†	5 ± 2	5 ± 0	4 ± 1

Nucleosol fractions isolated from COS-1 cells transfected with expression vectors for the indicated receptors were incubated with 5 nM of at-[³H]RA in the presence of various concentrations of unlabeled competing ligands as described (26).
*Results presented are the mean IC_{50} values (±SD) of at least three replicate experiments performed in duplicate.
†Data from Allenby *et al.* (26) provided for comparison purposes.

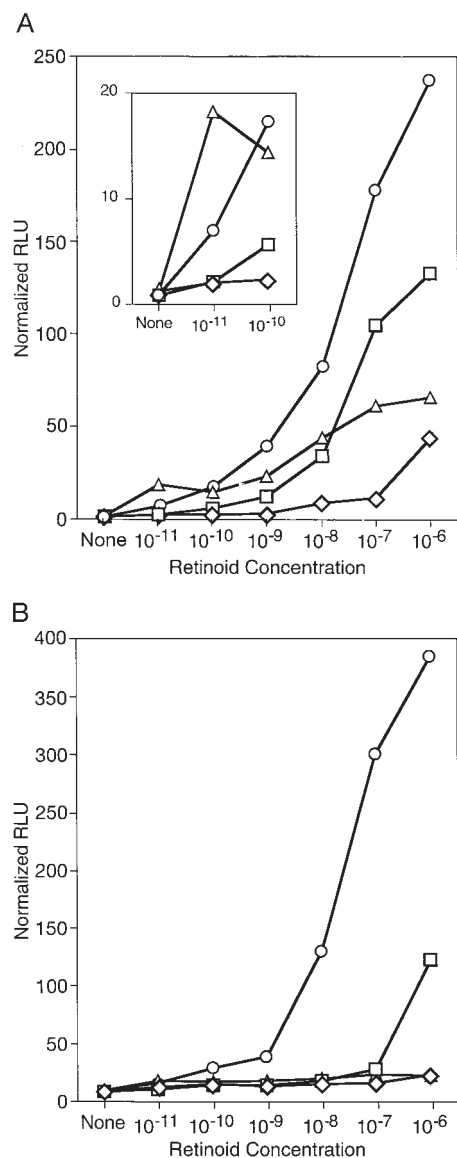FIG. 3. The ability of 4-oxo-ROL to activate hRARα (*A*) or hRXRα (*B*) in CV-1 cells was assessed using a common reporter gene containing the response element TREp. ○, 9-*cis*-RA; □, 4-oxo-at-RA; △, 4-oxo-ROL; ◇, at-ROL. Relative light units (RLU), A_{595}/min.

F9 stem cells or RA-treated F9 cells (72 hr) were cultured in the presence of 8 nM all-*trans*-4-hydroxy-[³H]ROL or 12 nM all-*trans*-4-oxo-[³H]ROL for different periods of time. Both F9 stem cells and RA-treated cells were able to convert all-*trans*-4-hydroxy-ROL to all-*trans*-4-oxo-ROL. No other major metabolites were detected in this assay (data not shown). The metabolism of [³H]4-oxo-ROL itself in F9 cells was very slow, resulting in a very long half-life (greater than 15 hr). 4-oxo-RA was not detected (limit of detection = 1×10^{-9} M) as an all-*trans*-4-oxo-ROL metabolite in a period up to 15 hr (data not shown).

4-oxo-ROL and 4-hydroxy-ROL Have the Ability to Cause Axial Truncations in the Developing *Xenopus* Embryo. *X. laevis* embryos were treated with various retinoids as described in the *Materials and Methods*. Dose-response curves for the different retinoids were established by measuring the degree of axial truncation according to the index of axis deficiency (34). In this assay, all-*trans*-4-oxo-ROL was much more potent than at-ROL in inducing axial truncation (Fig. 5). All-*trans*-ROL did not have any teratogenic activity when used at concentrations up to 1 μM (data not shown), whereas the ED_{50} for at-RA was about 100 nM and the ED_{50} for 4-oxo-ROL was ≈300–400 nM.
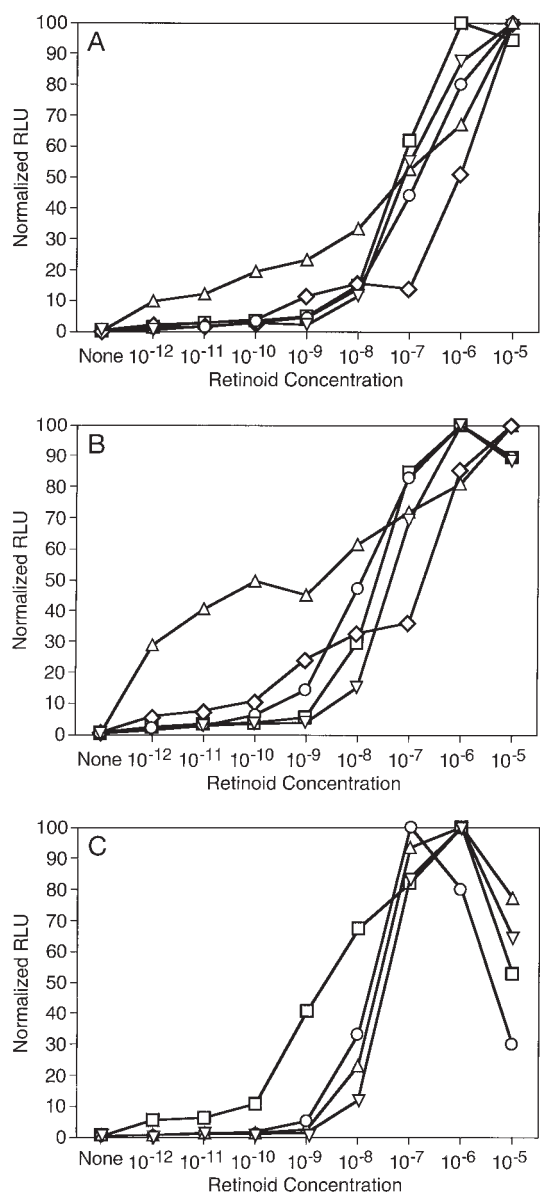
FIG. 4. The ability of 4-oxo-ROL to activate chimeric receptors containing the hRAR ligand binding domains fused to the GAL4 DNA binding domains in CV-1 cells was investigated. Data are shown for hRARα (*A*), hRARβ (*B*), and hRARγ (*C*). Results are plotted as relative light units (RLU) A_{595}/min as a percent of maximal induction. The data shown are derived from a single experiment but similar results were obtained from four other experiments. The induction of GAL-hRARγ by at-RA was anomalously low in this particular experiment. △, at-RA; ○, 4-oxo-at-RA; □, 4-oxo-ROL; ◇, at-ROL; ▽, 4-hydroxy-ROL.

DISCUSSION

Since the discoveries of the RAR and RXR nuclear receptors, each of which binds retinoids with terminal carbon 15 carboxylic acid groups, it has been assumed that most functions of retinoids are mediated at the molecular level by at-RA, or possibly by 9-*cis*-RA. However, current knowledge of retinol metabolism in various cell types is somewhat limited. The work presented here focuses on a novel metabolic pathway of retinol that can be positively regulated by at-RA and results in the production of 4-oxo-ROL, a biologically active retinoid in F9 cells. It is striking that this retinoid lacks the carboxylic acid moiety at carbon 15 but can transcriptionally activate the RARs. That 4-oxo-ROL is present intracellularly in differentiating F9 cells becomes even more relevant when one considers that we were not able to detect any at-RA or 9-*cis*-RA

FIG. 5. Exposure to exogenous 4-oxo-ROL induces dose-dependent progressive truncations along the anteroposterior axis in developing *X. laevis* embryos. The embryos were graded according to the index of axis deficiency by measuring the degree of axial truncation (34). *A* and *B* show representative embryos treated with varying concentrations of 4-oxo-ROL (*A*) or RA (*B*). In both panels, the final concentrations of retinoids are arranged from the top to the bottom: control (dimethyl sulfoxide), 1×10^{-9} M, 1×10^{-8} M, 1×10^{-7} M, and 1×10^{-6} M concentrations of either 4-oxo-ROL or at-RA.

production from retinol in either F9 stem cells or differentiating F9 cells (Fig. 1). Our results are in agreement with an

earlier study by Gubler and Sherman (35) who concluded that there is little, if any, metabolism of retinol to acidic retinoids in murine teratocarcinoma cells. Our current study shows that F9 cells convert retinol to 4-oxo-ROL, and that 4-oxo-ROL can induce F9 cell differentiation. The conversion of retinol to 4-oxo-ROL is not limited to F9 cells. In fact, 4-oxo-ROL was found to be a metabolite of retinol in several other cell lines such as RA-treated P19 teratocarcinoma cells and murine embryonic stem cells (data not shown).

4-hydroxy-ROL has been previously described as a metabolite of retinol (36, 37). Liver microsomes obtained from human subjects have the ability to convert retinol to 4-hydroxy-ROL but no 4-oxo-ROL was described (36, 37). The formation of 4-hydroxy-ROL was suggested to be catalyzed by the liver cytochrome P-450 IIC8 (37). Roberts *et al.* (38) showed that several cytochrome P-450 isoforms present in rat liver microsomes could catalyze the 4-hydroxylation of retinol. However, 4-hydroxy-ROL has generally been thought to be an inactive metabolite of retinol and the conversion of retinol to 4-oxo-ROL was not detected or discussed (36).

At-RA, 9-*cis*-RA, 4-oxo-RA, and didehydro-RA have been described as high affinity ligands for all three RARs (for review, see ref. 4). However, 4-oxo-ROL is the first physiological retinoid without a terminal carboxyl group that can activate transcription via the three different RARs (α, β, and γ). Additionally, at low concentrations, 4-oxo-ROL is more active than 4-oxo-RA (Fig. 3*A*) and didehydro-RA (data not shown). The binding of 4-oxo-ROL (Table 1) to the RARs appears to be less avid than one would expect given the transactivation data (Figs. 3 and 4). Because of differences in the chemical structures of 4-oxo-ROL versus at-RA, it is possible that the binding assays do not accurately reflect the affinity of 4-oxo-ROL for the RARs. For example, one difference between the binding data and the transactivation data is that the binding data are obtained using monomer receptors isolated under conditions of high salt. Moreover, the binding assays are done as competition assays using at-[^{3}H]RA; if 4-oxo-ROL binds to the RARs in a slightly different region of the ligand binding domain or when the RARs are in a different conformation (e.g., as heterodimers with RXR), 4-oxo-ROL may not displace at-[^{3}H]RA from its binding site in this assay. It was previously shown that the binding sites on the RARs for 9-*cis*-RA and at-RA are distinct but overlapping (39), indicating that retinoids may interact differently with the RARs.

A potentially important property of 4-oxo-ROL is its ability to transactivate RARs but not RXRs (Fig. 3). Another important characteristic of 4-oxo-ROL that differs from that of RA is its stability within cells. In F9 stem cells, at-[^{3}H]RA has a half-life of about 3.5–4 hr (24, 40). Culturing cells in the presence of at-RA significantly shortens this half-life, probably as a result of the induction of RA metabolic enzymes (refs. 24, 35, and 40; C.C.A and L.J.G., unpublished results). In contrast, the half-life of 4-oxo-ROL is greater than 15 hr. The long half-life of 4-oxo-ROL may be both biologically and clinically very significant, as its effects may be prolonged in some cell types relative to those of at-RA, especially in cells that metabolize at-RA.

Evidence is accumulating that at-RA and 9-*cis*-RA are not the only retinol metabolites responsible for the effects attributed to vitamin A. Our finding that 4-oxo-ROL, an abundant retinoid in some cells, binds and transcriptionally activates the three RARs indicates that it too may play an important morphogenic role. Additionally, our data suggest that at least part of the biological activity associated with at-ROL in various assays may result from the enzymatic conversion of at-ROL to 4-oxo-ROL. The characterization of the enzymes that convert retinol to active metabolites like 4-oxo-ROL and the identification of any additional biologically active metabolites of retinol are essential for our understanding of the sources and nature of retinoid signaling in animals.

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