9-cis-retinoic acid, a potent inducer of digit pattern duplications in the chick wing bud

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SUMMARY

The effects of retinoids are mediated by two types of receptors, the retinoic acid receptors (RARs) and the retinoid-X-receptors (RXRs). The physiological ligand of the RARs is all-trans-retinoic acid whereas RXRs have high affinity for 9-cis-retinoic acid, a naturally occurring retinoid isomer. RXRs are broadly expressed in embryonic and adult tissues, and they are capable of forming homodimers as well as heterodimers with RARs and other nuclear hormone receptors. The role of 9-cis-retinoic acid in regulating the activity of RXR homodimers and RXR-containing heterodimers is poorly understood in vivo. To begin to explore the function of 9-cis-retinoic acid in morphogenesis, we have examined the activity of this isomer in the chick wing. Using reverse transcriptase polymerase chain reaction analyses, we show that RXR isoform is expressed in stage 20 wing buds. Similar to all-trans-retinoic acid, the 9-cis-isomer induces pattern duplications when locally applied to chick wing buds, but the 9-cis isomer is about 25 times more potent than the all-trans form. Furthermore, applied all-trans-retinoic acid is converted to the 9-cis isomer in the wing bud. The ratio of 9-cis to all-trans-retinoic acid established in the tissue is approximately 1:25. This quantitative agreement between the degree of conversion and the 25-fold higher efficacy of the 9-cis isomer, raises the possibility that, at least in part, the effects of all-trans-retinoic acid on the wing pattern result from a conversion to the 9-cis isomer. Therefore, it is possible that, in this system, the actual active species is 9-cis-retinoic acid and that the all-trans form serves as a precursor.

Key words: 9-cis-retinoic acid, morphogenesis, limb development, pattern formation, retinoid receptor, chick wing

INTRODUCTION

Retinoids have diverse gene regulatory roles in vertebrate morphogenesis. Although the detailed mechanism of action of retinoids in development is not yet entirely understood, retinoids appear to play a role in establishing the limb pattern (Tickle et al., 1982; Summerbell, 1983; Dölle et al., 1989; Ruberte et al., 1990, 1992; Smith and Eichele, 1991; reviewed in Eichele, 1989; Tabin, 1991; Tickle, 1991; Bryant and Gardiner, 1992; Izpisúa-Belmonte and Duboule, 1992; Mendelsohn et al., 1992) and the pattern along the anteroposterior body axis (e.g. Durston et al., 1989; Sive et al., 1990; Kessel and Gruss, 1991; Morriss-Kay et al., 1991; Ruiz i Altaba and Jessell, 1991; Conlon and Rossant, 1992; Hogan et al., 1992; Kessel, 1992; Sundin and Eichele, 1992), possibly by regulating the expression of Hox genes (Izpisúa-Belmonte et al., 1991; Kessel and Gruss, 1991; Morriss-Kay et al., 1991; Conlon and Rossant, 1992; Kessel, 1992; Morgan et al., 1992; Sundin and Eichele, 1992; see also Boncinelli et al., 1991). At the biochemical level, retinoids exert their action through two types of receptors known as retinoic acid receptors (RARs) and retinoid-X-receptors (RXRs) (Gigueré et al., 1987; Petkovich et al., 1987; Benbrook et al., 1988; Brand et al., 1988; Hamada et al., 1989; Mangelsdorf et al., 1990; Noji et al., 1991; Rowe et al., 1991; Smith and Eichele; 1991; Mangelsdorf et al., 1992; reviewed in Chambon et al., 1991; Smith and Eichele; 1991, Mangelsdorf et al., 1992; reviewed in Chambon et al., 1991; Linney, 1992). RARs and RXRs are ligand-regulated transcription factors that modulate the expression of specific target genes by binding to cis-acting sequences known as retinoid-response elements (reviewed by Green and Chambon, 1988; Evans, 1988; Beato, 1989; Linney, 1992). Although all-trans-retinoic acid specifically binds and activates the members of the RAR subfamily, this isomer is known to have very low affinity for RXRs (Heyman et al., 1992; Allenby et al., 1993). Recent studies have established that 9-cis-retinoic acid is a ligand for RXRs (Heyman et al., 1992; Levin et al., 1992). Note, however, 9-cis-retinoic acid will also activate and bind to RARs (Heyman et al., 1992; Allenby et al., 1993) and hence is a bifunctional ligand. RARs and RXRs exist as homodimers and as RXR-RAR-heterodimers (Yu et al., 1991; Bugge et al., 1992; Durand et al., 1992; Kliwer et al., 1992a,b; Leid et al., 1992; Marks et al., 1992; Zhang et al., 1992a,b). Moreover, RXR is capable of forming heterodimers with other nuclear receptors such as thyroid hormone receptor, vitamin D receptor and peroxisome proliferator activator receptor (Yu et al., 1991; Kliwer et al., 1992a,b; Leid et al., 1992; Zhang et al., 1992a,b). Whether
RXRs form and operate as homodimers or heterodimers is thought to depend on several factors including the precise sequence of the retinoid-response element present in the target gene, the promoter context and the concentration and kind of receptors and ligands present in a particular cell (see e.g. Umesono et al., 1991; Durand et al., 1992; Zhang et al., 1992b).

In order to begin to explore the role of 9-cis-retinoic acid in morphogenesis, we have compared the activities of 9-cis-retinoic acid and all-trans-retinoic acid in the chick limb bud. Studies by Tickle and co-workers (1982, 1985), Summerbell (1983) and Thaller and Eichele (1990) have shown that the local application of the naturally occurring all-trans-retinoic acid or all-trans-3,4-didehydro-retinoic acid to the chick wing bud induces mirror-image duplications. Instead of the normal 234 pattern, these agents evoke the development of wings with additional digits. The formation of additional digits is dose-dependent. Low doses result in 2234 patterns and higher doses in complete mirror-image duplications (432234 pattern). Retinoid-induced duplications are virtually identical to those obtained after grafting a piece of posterior limb bud tissue (the zone of polarizing activity, ZPA) to the anterior margin of a host limb bud (Saunders and Gasseling, 1968; Tickle et al., 1975). This similarity has lead to the proposal that retinoic acid may be or is closely related to the postulated morphogen of the ZPA (Tickle et al., 1982, 1985; Summerbell 1983). However, it has also been argued that the action of all-trans-retinoic acid in the limb bud could well be indirect. For example, retinoic acid could induce a new ZPA which is then responsible for generating pattern duplications either by releasing a yet unknown morphogen or through some form of cell-cell interaction mechanism (Noguchi et al., 1991; Wanek et al., reviewed in Bryant and Gardiner, 1992). In this study, we report that a retinoid isomer, 9-cis-retinoic acid, a ligand of retinoid-X-receptor, is ~20 to 30 times more potent than all-trans-retinoic acid in inducing digit pattern duplications in the chick wing bud.

MATERIALS AND METHODS

Chemicals
All-trans-retinoic acid was purchased from Sigma, all-trans-[10,11-3H]retinoic acid (New England Nuclear) had a specific activity of 50.4 Ci/mmol. 9-cis-retinoic acid and 9-cis-[10,11-3H]retinoic acid were prepared by light isomerization from all-trans-retinoic acid or from all-trans-[10,11-3H]retinoic acid, respectively. Briefly, a quartz cuvette containing an ethanol solution of all-trans-retinoic acid (1 mg/ml for non-radioactive all-trans-retinoic acid, 5 µg/ml for [3H]all-trans-retinoic acid) in ethanol was placed between two fluorescent light boxes. After 40 minutes, the solvent was evaporated with nitrogen gas. The residue was redissolved in 30 µl ethanol and separated by HPLC using solvent system G (see below). Fractions containing 9-cis-retinoic acid were pooled, extracted with n-hexane/dichloromethane (1:1), the organic phase was evaporated with nitrogen gas and the residue redissolved in ethanol. To minimize isomerization and oxidation, all manipulations with retinoids were carried out under yellow light and in the presence of inert gas (nitrogen or helium).

Local treatment with retinoic acid
Fertile white Leghorn chicken eggs were purchased from Ideal Poultry (Cameron, Texas) and incubated at 37.5°C for 3.5 days to develop to stage 20 (Hamburger and Hamilton, 1951). 15 AG1-X2 ion-exchange beads (BioRad, Richmond, California) of 200-250 µm diameter were impregnated in 200 µl all-trans-retinoic acid or 9-cis-retinoic acid dissolved in DMSO. Retinoid concentrations used ranged from 0.02 µg/ml to 100 µg/ml. After washing in 200 µl Phenol Red containing phosphate-buffered saline (10 minutes, 2 changes), the beads were implanted into the right wing bud of stage 20 embryos at the anterior margin, below the apical ectodermal ridge (Tickle et al., 1985). After 7 days of incubation, wings were stained with Alcian Green and analyzed. Patterns were scored as described (Honig et al., 1981). A pattern with an additional digit 4 scored 100%. A wing with an additional digit 3 scored 66%, while a wing with an additional digit 2 scored 33%. A normal pattern obtained a score of 0%. Note, in this scoring scheme, only the anteriormost additional digit is considered.

In those experiments in which we determined tissue concentrations of all-trans-retinoic acid and 9-cis-retinoic acid established in the wing bud following local application, approximately 20 AG1-X2 beads were soaked either in tritiated all-trans-retinoic acid or in 9-cis-retinoic acid (specific activity of 50 Ci/mmol) dissolved in only 10 µl DMSO but at a concentration of 10 µg/ml. Beads were washed and implanted as described above. Wing buds were later removed and extracted as described below. In parallel experiments beads were also soaked in 10 µl non-radioactive all-trans-retinoic acid or 9-cis-retinoic acid (10 µg/ml). Beads were implanted and embryos were allowed to develop for 7 days when they were examined for pattern duplications.

Isolation of retinoid metabolites from wing buds
After either 10 or 22 hours of incubation, the beads releasing tritiated all-trans- or 9-cis-retinoic acid were removed, embryos were dissected out of the egg and rinsed in ice-cold stabilizing buffer (Eichele et al., 1985). All four limb buds were removed, rinsed in stabilizing buffer and separately collected into microfuge vials kept on dry ice. The time span between dissection and storage on dry ice was less than 2 minutes. To the frozen limb buds were added 200 µl of stabilizing buffer, 100 µl of a saturated Na2SO4 solution, 40 µl ethanol containing 2 mg/ml butylated hydroxytoluene (antioxidant) and 20 µl of a nonradioactive internal standard containing 100 ng of all-trans- and 100 ng of 9-cis-retinoic acid. The sample was sonicated and extracted three times with 800 µl ethylacetate/methyelacetate (8:1). The combined organic phases were evaporated to dryness at room temperature with nitrogen gas and the residue was dissolved in 20 µl methanol for subsequent HPLC analysis. We found that this procedure results in recoveries >90%.

Quantification of the metabolites by high-performance liquid chromatography
Samples were applied to a 30 cm C18 reversed phase column (Novapak, Waters) using a Waters UK6 injector. Mobile phases were delivered at 1 ml/min and absorbance of the effluent was measured at 350 nm. Peak areas were determined by integration. To determine radioactivity, 1 ml fractions were collected that were mixed with 5 ml of Instafluor scintillant (Packard) and counted. Mobile phases: mobile phase G consists of equal parts of solvent A (acetonitrile:methanol:2% aqueous acetic acid [6:2:2]) and solvent E (acetonitrile:methanol:2% aqueous acetic acid [5:5:1.5:3]); mobile phase K is acetonitrile:methanol:isopropanol: 1.2% acetic acid (30:25:15:30) (Motto et al., 1989).

RT-PCR analyses
Total RNA was extracted with guanidinium thiocyanate from stage 20 chick embryo wing buds and purified by CsCl gradient centrifugation (Chirgwin et al., 1979). RT-PCR cDNA synthesis:
RESULTS

9-cis-retinoic acid induces digit pattern duplications in a dose-dependent fashion

In order to examine whether 9-cis retinoic acid induces duplications of the wing digit pattern, AG-1 X2 ion-exchange beads were soaked in solutions of 9-cis retinoic acid that ranged from 0.02 µg/ml to 20 µg/ml. At concentrations of 0.02 µg/ml, wing patterns were normal (Fig. 1A). Wings with an additional digit 2 (2234 pattern) were most frequently obtained at concentrations ranging between 0.05 µg/ml and 0.1 µg/ml (Fig. 1B). Soaking concentrations around 0.5 µg/ml resulted in 32234 or 3234 patterns with extra digits 2 and 3 at the anterior wing margin (Fig. 1C). Patterns with an additional digit 2, 3, and 4 (432234, 43234, 4334 patterns) were found at soaking concentrations around 0.8 µg/ml and higher (Fig. 1D, E). Although digit pattern duplications are the most noticeable features resulting from 9-cis-retinoic acid application, we also observed mirror-symmetrical patterns of the feather rudiments in the forearm region. In a normal wing, feather rudiments are all pointing posteriorly whereas retinoid-treated wings exhibit an extra set of feather primordia, pointing away from the anterior wing margin (Fig. 1E). We conclude that 9-cis-retinoic acid

5 µg total RNA and 555 pmol of oligo(dT) primer (15-mer, Boeringer) were annealed by heating to 72°C for 10 minutes followed by chilling on ice. The sample was reverse transcribed at 37°C for 1.5 hours in 40 µl of 50 mM Tris-HCl (pH=8.3), 75 mM KCl, 3 mM MgCl₂, 0.5 mM each of dGTP, dTTP, dCTP, dATP (Pharmacia), 10 µM dithiothreitol, 40 U RNasin (Promega), 200 U of MoMuLV reverse transcriptase (BRL). After cDNA synthesis, the reaction mixture was heat-denatured (95°C for 10 minutes) and PCR-amplification was performed with 5 µl of the heat-treated sample. The primers used were RX5 (5′ ATG GATC-CCTCCGAGTGAGTCGCT 3′) and RX3 (5′ CGA ACGTTC-CAAAGAGGAGCAGAGGG 3′) deduced from the chicken RXRγ sequence (see Rowe et al., 1991). The resulting 339 bp PCR product corresponds to nt 1234 to nt 1556 (encoding part of the ligand binding domain and 3′ untranslated sequences, see Rowe et al., 1991) and is flanked by BamHI and HindIII restriction sites.

To provide an internal standard, 2.5 µl of the cDNA sample was subjected to PCR amplification using primers of the chicken β-actin gene ACT5 (5′ AAGAGGTATCCTGACCTGAAGTC 3′) and ACT3 (5′ ACCTGACCATCAGGGAGTTA 3′). Conditions used for PCR: 2.5 U of Taq polymerase (Promega), 40 cycles, denaturation for 1 minute at 94°C; reannealing for 1.5 minutes at 55°C; extension for 1.5 minutes at 72°C in 50 µl reaction volume. Amplified products were analyzed by electrophoresis on a 1.2% agarose gel and visualized by ethidium bromide staining.

Fig. 1. Effect of different doses of locally applied 9-cis-retinoic acid on the chick wing pattern. A bead soaked in 9-cis-retinoic acid was implanted at the anterior wing bud margin of a stage 20 embryo. Embryos were analyzed 7 days later. (A) Normal 234 pattern, the concentration of 9-cis-retinoic acid in the soaking solution was 0.02 µg/ml. (B) 2234 pattern, 0.05 µg/ml; (C) 32234 pattern, the soaking concentration was 0.4 µg/ml, note the absence of a radius in this particular specimen; (D) 43234 pattern; 2 µg/ml. (E) Dorsal aspect of a wing treated with 9-cis-retinoic acid (0.77 µg/ml). The wing pattern is 4334. Note the distinct feather primordia at the anterior wing margin (arrows). Normally this region has only short feather rudiments, but as a result of posteriorization of the anterior limb half by 9-cis-retinoic acid, the anterior feather rudiments now resemble those at the posterior wing margin. Abbreviations: h, humerus; r, radius; u, ulna; 2, 3, 4 digits.
induces additional digits in the chick wing in a way reminiscent of that reported for all-trans-retinoic acid and all-trans-3,4-didehydroretinoic acid (Tickle et al., 1982, 1985; Summerbell, 1983; Thaller and Eichele, 1990).

It has been found that high concentrations of all-trans-retinoic acid applied to the wing bud results in the partial or complete loss of the upper beak (reviewed in Wedden et al., 1988). Although we have not systematically studied this defect, we found that 9-cis-retinoic acid at concentrations of ≥ 5 µg/ml resulted in 100% of the cases in upper beak defects (data not shown) whereas all-trans-retinoic acid had no effect even at 100 µg/ml.

To compare the efficacy of 9-cis- and all-trans-retinoic acid quantitatively, dose-response curves were generated (Fig. 2). Although the curves have similar shapes, that of the 9-cis isomer is shifted towards lower concentrations indicating that this isomer is more potent than the all-trans form. We estimate an ED₅₀ of ~0.2 µg/ml for 9-cis-retinoic acid and an ED₅₀ of ~1 µg/ml for the all-trans isomer, respectively. The ED₅₀ for all-trans-retinoic acid reported here is very close to that found in an earlier study (Thaller and Eichele, 1990). In conclusion, based on a direct comparison of soaking concentrations, 9-cis-retinoic acid is approximately 5 times more active than all-trans-retinoic acid.

**Concentration of applied 9-cis- and all-trans-retinoic acid in the wing bud**

The comparison of potency of 9-cis- and all-trans-retinoic acid shown in Fig. 2 is based on retinoid concentrations in the soaking solution. However, it is possible that beads have different release kinetics for each isomer and/or that the two retinoids are metabolized at different rates by the tissue. This prompted us to measure directly the amount of each isomer present in the wing bud 10 and 22 hours after the bead was implanted at stage 20. These particular time points were chosen because they bracket the period required to specify pattern duplications (Eichele et al., 1985; G.E. unpublished data for 9-cis-retinoic acid). Beads were soaked in 10 µl of either [³H]9-cis-retinoic acid or [³H]all-trans-retinoic acid at a concentration of 10 µg/ml and implanted into wing buds of stage 20 embryos. Beads were removed 10 or 22 hours later, buds were cut off, extracted and fractionated over a reversed phase HPLC column. Figures 3 and 4 show chromatograms of the radioactive retinoid metabolites formed during each of the different treatments. In all cases, we found significant metabolism of the parent compound to polar species eluting in fractions 2 to 10. Nevertheless, at both time points, wing buds still contained radioactive parent compound that eluted with authentic internal standards.
standards (Figs 3, 4). Based on the known specific activity of the radiolabels used, the amounts of 9-cis-retinoic acid and all-trans-retinoic acid present in wing buds after 10 hours of treatment were determined as 1.45 and 5.5 pg, respectively (see Table 1). The corresponding figures for the 22 hours time point are 0.4 pg per bud (9-cis isomer) and 2.45 pg per bud (all-trans isomer). We conclude that at equal soaking concentrations, the amount of all-trans-retinoic acid in the bud tissue exceeds that of the 9-cis isomer by a factor of 4 to 6.

To apply radioactive 9-cis- and all-trans-retinoic acid in the above experiments, the soaking volume was reduced to 10 µl but the soaking concentration was augmented to 10 µg/ml. Since this protocol differs from that used for the dose-response analysis (Fig. 2), we redetermined the type of duplication patterns obtained using the alternative protocol. Consistent with the findings shown in Fig. 2, the 9-cis isomer was clearly more potent than all-trans-retinoic acid (Fig. 5).

In summary, these experiments show that, in order to evoke the same biological response, the concentrations of all-trans-retinoic acid in the soaking solution needs to be five times higher than that of 9-cis-retinoic acid (Fig. 2). Furthermore, our data also indicate that when beads are soaked in equal concentrations of all-trans or 9-cis-retinoic acid, the tissue concentration of 9-cis-retinoic acid will be 4 to 6 times lower than that of the all-trans isomer (Table 1). A numerical estimate of the difference in potency between the two compounds can be obtained by multiplying the 5-fold difference in responsiveness (Fig. 2) with the factor of 4-6 that reflects the difference in actual tissue concentrations (Table 1). Accordingly, it appears that the 9-cis isomer is approximately 20- to 30-fold more active than the all-trans form.

**Conversion of all-trans-retinoic acid to the 9-cis isomer**

When wing buds were treated with all-trans-retinoic acid, a small but reproducible peak of radioactivity at the elution position of authentic 9-cis-retinoic acid was seen (e.g. Fig. 4A). To identify this peak unambiguously, beads were soaked in a high concentration (100 µg/ml) of [3H]all-trans-retinoic acid and implanted at the anterior wing bud margin of stage 20 embryos. After 10 hours of treatment, beads were removed, the buds extracted and subjected to HPLC analysis. Chromatography on a C18 column developed with mobile phase K resulted in a chromatogram with several radioactivity peaks (Fig. 6A). One peak coeluted with the parent compound (fraction 41 and 42), another peak coeluted with authentic 13-cis-retinoic acid (fraction 32), there were several peaks representing polar metabolites (fraction 2-25), and a peak was found in fraction 37 that also contained internal standard 9-cis-retinoic acid. Fraction 37...
solution in which the beads were soaked and found that it is about 200:1 before and after soaking of the bead (data not shown). Hence, most of the 9-cis- of the 9-parent compound peak. As would be expected from the treatment was generated during the treatment and does not form during the soak or the extraction.

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Table 1. Amount of [3H] retinoid present in the wing bud following local application*

<table>
<thead>
<tr>
<th>Applied retinoid</th>
<th>Amount present 10 hours after treatment</th>
<th>Tissue concentration†</th>
<th>Amount present 22 hours after treatment</th>
<th>Tissue concentration†</th>
</tr>
</thead>
<tbody>
<tr>
<td>9-cis-retinoic acid</td>
<td>1.4 pg/bud</td>
<td>8 nM</td>
<td>0.4 pg/bud</td>
<td>1.5 nM</td>
</tr>
<tr>
<td>all-trans-retinoic acid</td>
<td>1.5 pg/bud</td>
<td>2.4 pg/bud</td>
<td>0.4 pg/bud</td>
<td>9 nM</td>
</tr>
<tr>
<td>5.5 pg/bud</td>
<td>30 nM</td>
<td>2.5 pg/bud</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.5 pg/bud</td>
<td>30 nM</td>
<td>2.5 pg/bud</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Beads were soaked in 10 µl of retinoid at a concentration of 10 µg ml⁻¹. Figures shown represent two independent measurements with 10 or 11 wing buds each.
†Wing bud volumes amount to 1.2 and 1.8 µl after 10 and 22 h. Estimates assume that most of the applied retinoid is in the half wing bud that obtained the implant (see Eichele and Thaller, 1987).

was extracted and rechromatographed on a second C₁₈ column eluted with mobile phase C (Fig. 6B). The major radioactivity peak coeluted with authentic 9-cis-retinoic acid. Based on peak area integration (Fig. 6A,B), we estimate that the ratio of all-trans-retinoic acid to 9-cis-retinoic acid is approximately 25:1. We also determined the ratio of all-trans-retinoic acid to 9-cis-retinoic acid in the solution in which the beads were soaked and found that it is about 200:1 before and after soaking of the bead (data not shown). Hence, most of the 9-cis-isomer present at the end of the treatment was generated during the treatment and does not form during the soak or the extraction.

Note, in extracts of wing buds exposed to 9-cis-retinoic acid, a radioactivity peak appears that coelutes with authentic all-trans-retinoic acid (Fig. 3A, B). At the 10 hour time point, the area of this peak corresponds to ~15% of the 9-cis peak, at 22 hours, it amounts to ~40% of the parent compound peak. As would be expected from the greater stability of the all-trans isomer, the conversion of 9-cis-retinoic acid to all-trans-isomer is more efficient than the reverse reaction (Fig. 4A,B; Rando and Chang, 1983).

Stage 20 wing buds express RXRγ

Although 9-cis retinoic acid binds and activates RARs, it is also a high affinity ligand for RXRs (Heyman et al., 1992; Allenby et al., 1993). So far, no expression data for RXRs in stage 20 limb buds have been reported, but Rowe et al. (1991) have detected low levels of RXRγ transcripts in stage 22 limb buds by northern blotting. To extend these studies to stage 20 wing buds, the time when 9-cis retinoic acid is locally applied, RNA was extracted from wing buds and subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Using specific primers for chicken RXRγ, cDNA was amplified by PCR and the appearance of a specific 339 bp reaction product was observed (Fig. 7, lane 1). In a parallel reaction in which the same reactants were used but reverse transcriptase was omitted, no PCR-product was detected (Fig. 7, lane 2). This provides evidence that the isolated RNA was not significantly contaminated with genomic DNA. In an additional control experiment, template DNA was subjected to PCR using chicken β-actin primers located in the first and second exon. If amplified from a cDNA template, the expected reaction product is 558 bp, whereas genomic DNA template would give rise to 1082 bp fragment. As can be seen in Fig. 7 (lane 3), the cDNA-based amplification results in an appropriate band. In contrast, the sample in which genomic DNA would have been amplified (the reverse transcriptase was omitted) has no detectable band, indicating that the sample was not contaminated with genomic DNA. We conclude that wing buds express RXRγ at the stage when they were treated with 9-cis-retinoic acid. Whether they also express other RXR isoforms remains to be determined.

Fig. 6. Locally applied [3H]all-trans-retinoic acid is converted to [3H]9-cis-retinoic acid. Beads were soaked in 10 µl of 100 µg/ml [3H]all-trans-retinoic acid and implanted at the anterior wing bud margin. 10 hours later the tissue was extracted. (A) Extract fractionated over a C₁₈ column eluted with mobile phase K at a flow-rate of 1 ml/minute. 1 minute fractions were collected and a 100 µl aliquot was counted. (B) Fraction 37, which coelutes with authentic 9-cis-retinoic acid internal standard, was extracted and reanalyzed on a second C₁₈ column developed with mobile phase C at a flow rate of 0.7 ml/minute. Note the distinct peak of radioactivity coeluting with authentic internal standard 9-cis-retinoic acid. For these chromatograms 23 treated wing buds were used.
Fig. 7. RT-PCR analysis demonstrating the presence of RXRγ mRNA in stage 20 chick wing buds. 5 μg total RNA isolated from chick wing buds were reverse transcribed and amplified by PCR. Lane 1, a specific PCR product of 339 bp (arrow head) resulting from amplification in the presence of primers specific for chicken RXRγ (Rowe et al., 1991). Lane 2, same reactants as lane 1 but reverse transcriptase was omitted. The absence of a band indicates that the RNA was not contaminated by genomic DNA. Lane 3, a specific PCR product of 558 bp (arrow) resulting from β-actin amplification in the presence of primers derived from neighboring exons of the chicken β-actin gene. Lane 4, same reactants as lane 3 except that reverse transcriptase was left out. No reaction product is detected. Markers: 1353, 1080, 870, 600, 310, 280 (270), 234, 194, 118, 72 bp.

DISCUSSION

The recent identification of retinoid-X-receptors (RXRs) and of 9-cis-retinoic acid as a RXR ligand has added another dimension to the physiology of retinoids (Heyman et al., 1992; Levin et al., 1992). Since RXRs are expressed in embryos, these receptors and their ligand are likely to have a role in embryonic development (Rowe et al., 1991; Blumberg et al., 1992; Mangelsdorf et al., 1992 and this report). What the function of the 9-cis-isomer might precisely be is currently unknown. The present study demonstrates that 9-cis-retinoic acid is 20-30 times more potent than the all-trans isomer in inducing pattern duplications in the chick wing bud. The higher potency of 9-cis-retinoic acid raises the possibility that this isomer represents a biologically highly active molecule and that the all-trans form serves as a biosynthetic precursor. Suggestive evidence for this hypothesis comes from our observation that the 25:1 concentration ratio of all-trans to 9-cis-retinoic acid established in the wing bud, following local application of the all-trans isomer, is in marked quantitative agreement with the 20 to 30 times greater potency of the 9-cis isomer.

How can one rationalize the higher potency of 9-cis-retinoic acid? 9-cis-retinoic acid is a bifunctional ligand capable of activating RARs and RXRs (Heyman et al., 1992; Allenby et al., 1993). In situ hybridization studies in the mouse and the chick showed that RARα, β and γ are expressed early in limb development (Dollé et al., 1989; Ruberte et al., 1990; Noji et al., 1991; Smith and Eichele, 1991; Schofield et al., 1992, reviewed in Mendelsohn et al., 1992) and the present investigation establishes that RXRγ is expressed in the chick wing bud by stage 20, the time when retinoid treatment is initiated (for expression of RXR in limb buds of older embryos, see Rowe et al., 1991). Given the presence of RARs and RXRs, the effect of applied 9-cis-retinoic acid on the wing pattern could be mediated by homodimers of RAR or RXR, RAR-RXR heterodimers, or heterodimers of RXR with other nuclear receptors.

Zhang et al. (1992b) showed that RXR homodimers bind with high affinity to the retinoic acid response element of the CRBP II and ApoAI genes and to TREp, a palindromic thyroid hormone response element. Furthermore, these investigators found that 9-cis-retinoic acid but not all-trans-retinoic acid effectively activates a reporter gene driven by TREp or RARE-ApoAI elements. Thus there apparently are natural retinoid response elements that interact specifically with RXR homodimers liganded to 9-cis-retinoic acid. Since all-trans-retinoic acid has very low affinity for RXRs (Heyman et al., 1992; Allenby et al., 1993), this would explain why all-trans-retinoic acid is less potent than 9-cis-retinoic acid. The fact that all-trans-retinoic acid is still capable of inducing duplications could be rationalized by our finding that the all-trans-isomer is converted, at least in part, to the 9-cis form.

It is also possible that both isomers operate entirely through RARs. 9-cis- and all-trans-retinoic acid bind to RARs with similar affinities (Allenby et al., 1993). The main argument against this mechanism is that in transactivation experiments using RAR-dependent reporter genes, 9-cis- and all-trans-retinoic acid are approximately equipotent (e.g. Heyman et al., 1992; Allenby et al., 1993) yet in the limb bud assay 9-cis-retinoic acid is clearly the more active isomer.

Another possibility is that 9-cis and all-trans-retinoic acid act through RXR-RAR heterodimers. There is compelling evidence that binding of RAR to retinoid response elements is greatly enhanced by RXR (Yu et al., 1991; Kliewer et al., 1992a, b; Leid et al., 1992; Zhang et al., 1992a; Marks et al., 1992; Bugge et al., 1992). The question of whether the activity of RAR-RXR heterodimers depends on the presence of ligand has recently been examined by Durand et al. (1992). These investigators found that the CRABP II promoter contains two cooperating response elements that bind RXR-RAR heterodimers more efficiently than either RAR or RXR alone. Furthermore, in P19 cells, the endogenous CRABP II gene is induced 3-8 times more efficiently by 9-cis-retinoic acid than by the all-trans isomer (Durand et al., 1992), and the strongest induction is seen by exposing cells simultaneously to both isomers. Not all retinoid-responsive genes are differentially activated by 9-cis and all-trans-retinoic acid. For example, the two isomers induce RARβ 2 mRNA expression with equal efficiency (Durand et al., 1992). It thus is possible that genes responding to applied 9-cis and all-trans-retinoic acid in the limb bud may have retinoid response elements capable of interacting with ligand-occupied RAR-RXR heterodimers. It is also possible that RARs are not involved in mediating the retinoid-induced digit pattern duplications. In this case, RXRs could form heterodimers with other, perhaps yet unknown nuclear receptors. The biological activity of all-trans-retinoic acid would then have to result from its isomerization to the 9-cis form (Fig. 6).

Another way of explaining the differential activity of 9-
cis- and all-trans-retinoic acid is that the two isomers exhibit different affinities for cellular retinoic acid binding proteins known to be expressed in the limb bud (Maden et al., 1988; Ruberte et al., 1992). CRABPs apparently do not bind 9-cis-retinoic acid (Allenby et al., 1993). At equal doses, the concentration of unbound 9-cis retinoic acid will, therefore, be greater than that of all-trans-retinoic acid. Accordingly, the concentration of free ligand available for binding to nuclear receptors may be greater in the case of the 9-cis isomer. However, it should be pointed out that it is presently not known whether retinooids bind to nuclear receptors in a free or a CRABP-bound form. Moreover, it remains to be determined whether or not limb bud cells contain binding proteins capable of specifically binding to 9-cis-retinoic acid.

In closing, we wish to point out that the different schemes discussed in the preceding section are not mutually exclusive. For example, 9-cis-retinoic acid could be more potent because it more effectively activates RAR-RXR heterodimers and, in addition, its free concentration in the cell could be greater because it cannot bind to CRABPs. At the mechanistic level the action of retinoids depends on a sequence of binding reactions. Retinoid ligands bind to receptors, receptors interact with each other and presumably also with other trans-acting factors. Receptors bind to a variety of DNA response elements located in different promoter contexts. These molecular interactions represent a multicomponent network. If the concentration of any one component is significantly changed (e.g. a high-affinity ligand is exogenously supplied), the concentrations of all remaining components readjust to comply with the law of mass action. Importantly, in such a network of reactions similar end points could be achieved by more than one path. Which one is chosen would depend on the concentration of each component in a particular cell.

This work was funded by a grant from the National Institutes of Health (HD 20209) and The McKnight Endowment Fund for Neuroscience. C.H. was supported by a fellowship from the German Academic Exchange Program (DAAD 312 402 756/2).

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(Accepted 13 April 1993)