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Retinoic acid signaling is required during early chick limb development

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SUMMARY

In the chick limb bud, the zone of polarizing activity controls limb patterning along the anteroposterior and proximodistal axes. Since retinoic acid can induce ectopic polarizing activity, we examined whether this molecule plays a role in the establishment of the endogenous zone of polarizing activity. Grafts of wing bud mesenchyme treated with physiologic doses of retinoic acid had weak polarizing activity but inclusion of a retinoic acid-exposed apical ectodermal ridge or of prospective wing bud ectoderm evoked strong polarizing activity. Likewise, polarizing activity of prospective wing mesenchyme was markedly enhanced by co-grafting either a retinoic acid-exposed apical ectodermal ridge or ectoderm from the wing region. This equivalence of ectoderm-mesenchyme interactions required for the establishment of polarizing activity in retinoic acid-treated wing buds and in prospective wing tissue, suggests a role of retinoic acid in the establishment of the zone of polarizing activity. We found that prospective wing bud tissue is a high-point of retinoic acid synthesis. Furthermore, retinoid receptor-specific antagonists blocked limb morphogenesis and down-regulated a polarizing signal, *sonic hedgehog*. Limb agenesis was reversed when antagonist-exposed wing buds were treated with retinoic acid. Our results demonstrate a role of retinoic acid in the establishment of the endogenous zone of polarizing activity.

Key words: retinoic acid, retinoic acid antagonists, limb development, sonic hedgehog, ectoderm-mesenchyme interactions

INTRODUCTION

Much of the current research in limb morphogenesis has focused on events that occur when a distinct limb bud is present (Hamburger-Hamilton stages 18 and older, reviewed in Tickle and Eichele, 1994; Tabin, 1995). However, fate mapping and grafting data in the chick show that anteroposterior and dorsoventral limb axes are specified as early as stage 8 and stage 11, respectively, well before limb outgrowth begins (Chaube, 1959). Limb buds appear at distinct axial levels, possibly in response to signals from the lateral plate mesoderm that regulate cell proliferation (Murillo-Ferrol, 1965; Cohn et al., 1995). These signals may be members of the fibroblast growth factor family (FGFs; Niswander and Martin, 1993; Niswander et al., 1993; Mahmood et al., 1995), which can create an additional limb when applied to the interlimb flank (Cohn et al., 1995; Ohuchi et al., 1995).

The posterior limb bud mesenchyme, known as the zone of polarizing activity (ZPA), and the apical ectodermal ridge (AER) are two regions of the limb primordium that are essential for limb outgrowth and patterning. The ZPA specifies the pattern along the anteroposterior limb axis (Saunders and Gasseling, 1968; Tickle et al., 1975) and maintains the AER (Saunders, 1948; Laufer et al., 1994; Niswander et al., 1994). In turn, the AER maintains distal outgrowth and the ZPA. Polarizing activity is demonstrated by grafting a piece of the tissue in question to the anterior margin of a host wing bud, a manipulation that will result in a duplication of the digit pattern (e.g. Saunders and Gasseling, 1968; Tickle et al., 1975). Using this assay, it has been determined that cells with polarizing activity appear in the lateral plate within the future limb regions as early as stage 10/11 (Hornbruch and Wolpert, 1991). This is well before limb buds bulge out and prior to the expression of genes associated with polarizing activity such as *sonic hedgehog* (*shh*, Riddle et al., 1993), bone morphogenetic protein 2 (*bmp-2*, Francis et al., 1994) and *fgf-4* (Niswander et al., 1994; Laufer et al., 1994). We are interested in the nature of the signals that are responsible for establishing polarizing activity during these pre-limb-bud stages, with a focus on the role of retinoid signaling in this process.

Our approach to this problem is based on the observation that local application of RA to the anterior margin of a wing bud induces additional digits (Tickle et al., 1982, 1985; Summerbell, 1983) in a dose-dependent manner, a property also exhibited by polarizing region grafts (Tickle, 1981). When delivered ectopically, RA induces the expression of genes that are normally expressed in the posterior part of the limb bud including *Hoxd-11*, *Hoxd-12*, *Hoxd-13*, *fgf-4*, *bmp-2* and *shh* genes (Izpisúa-Belmonte et al., 1991; Nohno et al., 1991; Izpisúa-Belmonte and Duboule, 1992; Riddle et al., 1993; Francis et al., 1994; Helms et al., 1994). It has also been shown that polarizing activity is evoked in the tissue adjacent to beads releasing high doses of RA (Noji et al., 1991; Wanek et al., 1991; Tamura et al., 1993; Helms et al., 1994). These observations suggest that RA induces additional digits by first creating an ectopic ZPA. We reasoned that, if RA was capable

of inducing a new polarizing region at the anterior margin of stage 20 wing buds, then RA might have a similar function in the establishment of the native ZPA during the early stages of limb development. We explored this possibility in the present study.

MATERIALS AND METHODS

Local application of retinoids

All-*trans*-RA was applied as previously described (e.g. Helms et al., 1994). Soaking concentrations were 6 or 10 μg/ml. Retinoid antagonists LG754 (RXR selective) and LG629 (RAR selective) were disolved in dimethylsulfoxide. AG1-X2 beads of 200 μm diameter were soaked in this solutions for 60 minutes under constant shaking (Wedden et al., 1990). Thereafter they were briefly rinsed in phosphate-buffered saline (PBS) containing 10 mg/l Phenol Red. Stage 14 embryos (22 somites, Hamburger and Hamilton, 1951) were slightly stained with Neutral Red and two incisions were made at somite levels 15 and 20; beads were placed into these slits. Skeletal patterns were visualized as described (Helms et al., 1994).

Tissue transplantations

Embryos treated with RA for 22±1 hours at 37°C and wing buds were removed, incubated with 2% trypsin-PBS solution (Sigma T-8128, 2°C, 20 minutes) and washed with PBS containing 1% bovine serum albumin (BSA; see Helms et al., 1994 for details). Ectoderm and mesenchyme were separated and grafted underneath anterior the AER of stage 20 wing buds as described (Helms et al., 1994). Grafts of pre-limb-bud stage lateral plate tissues were prepared the same way except that trypsin digestion was halted after 10 minutes.

In situ hybridization

Embryo collection, sectioning and in situ hybridization were performed as previously described (Sundin et al., 1990). Subclones of *shh*, *bmp-2* (Francis et al., 1994) and *Hoxd-11* in pBSII (Helms et al., 1994) were linearized with appropriate restriction enzymes to transcribe either sense or antisense ^{35}S-labeled riboprobes. Slides were exposed for 4 to 6 days. Images illustrating the expression pattern and tissue were created as described previously (Helms et al., 1994).

For whole-mount in situ hybridization, embryos were dehydrated and rehydrated through an methanol/0.9% sodium chloride series, digested with proteinase K (Sigma) 10 μg/ml for 30 minutes at room temperature, then incubated with digoxigenin-labeled RNA probe at 60°C overnight. Embryos were washed stringently (0.2× SSC, 0.1% CHAPS for 2 hours at 60°C), blocked with heat-inactivated lamb serum and incubated with anti-digoxigenin Fab fragments. Embryos were washed with BSA-PBS containing 0.1% Triton-X 100 and a color precipitate was produced as described previously (Wilkinson, 1993).

High-performance liquid chromatography

Blocks of lateral plate tissue consisting of ectoderm, somitic and splanchnic mesoderm were dissected from stage 14 embryos and incubated (5% CO_2 for 3 hours at 37°C) in 50 μl of serum-free F12 medium containing insulin (5 μg/ml), transferrin (5 μg/ml), sodium selenite (5 ng/ml) and 36 μM all-*trans*-retinal. Thereafter, 200 μl of stabilizing buffer (Eichele et al., 1984), 50 μl of saturated sodium sulphate and 60 pg of [^{3}H]all-*trans*-RA (New England Nuclear, 1861.1 Gbq/mmol) were added to serve as internal standard. Samples were sonicated and extracted as described (Thaller and Eichele, 1987). Extracts were first fractionated on a C_{18} HPLC column (Microsorb, Rainin) developed at 1 ml/minute with methanol/acetonitrile/0.1 M ammonium acetate pH 6.8 (64:18:18). 1 ml fractions were collected and 100 μl aliquots were counted in a scintillation counter. Fractions coeluting with internal standard [^{3}H]all-*trans*-RA were pooled, extracted and rechromatographed on the same HPLC column using methanol/acetonitrile/10% acetic acid (80:1:1) at 0.8 ml/minute. Fractions were counted to determine the elution position and recovery of the internal standard.

RESULTS

Retinoic acid induces factors in the apical ectodermal ridge that are required for the maintainence of *shh* and *bmp-2*, and for polarizing activity

Grafts of mesenchyme from wing buds exposed to low doses of RA have no polarizing activity although the grafts express *shh* at the time of transplantation (20-22 h after initating RA exposure; Helms et al., 1994). We now show that the absence of polarizing activity in these grafts is due to the lack of signals being supplied by a RA-treated AER (hereafter referred to as AER^{RA}). To demonstrate this, three types of grafts were placed underneath an (untreated) anterior AER of a stage 20 host wing bud: (1) grafts of RA-treated mesenchyme only (mesenchyme^{RA}), (2) grafts of RA-treated mesenchyme, its overlying ectoderm and its associated AER and (3) grafts containing mesenchyme^{RA} and ectoderm, but without the AER. Grafts of mesenchyme^{RA} had little polarizing activity, giving rise to an occasional small digit resembling digit **2** (Table 1, first column). This was in marked contrast with grafts of mesenchyme^{RA} containing its ectoderm and AER^{RA}, that often resulted in full duplications (Table 1, second column). To determine if these signals resided in the AER^{RA} or in the ectodermal component of the graft, we dissected away the AER but left the ectoderm intact. These ridge-less grafts had almost no effect on the digit pattern (Table 1, third column), thus indicating that the AER^{RA} was the primary source of signal(s) required for mesenchyme^{RA} to exhibit polarizing activity. As controls, we determined that the AER^{RA} itself did not exhibit polarizing activity since grafting it alone had no effect on the digit pattern (Table 1, fourth column). Likewise, enzymatic digestion, which was required to isolate AER^{RA} and mesenchyme^{RA}, had no adverse effect on polarizing activity. This was determined by subjecting to RA-treated wing buds to trypsin digestion, then recombining mesenchyme^{RA} and AER^{RA} and grafting them to the anterior wing bud margin of a host embryo (Table 1, fifth column).

A posterior AER is similar to AER^{RA} in that both appear to be required for the maintenance of polarizing activity in the underlying mesenchyme (Todt and Fallon, 1987; Vogel and Tickle, 1993; Niswander et al., 1993). Therefore, we expected that posterior AER could substitute for an AER^{RA} in a polarizing activity assay. We combined posterior AERs from untreated stage 20 embryos with mesenchyme^{RA} and placed them underneath an anterior AER of a host. The patterns that developed were mostly normal (Table 1, last column). Thus we conclude that posterior AER was not functionally equivalent to an AER^{RA} in this assay.

Embryos that had received grafts composed of either mesenchyme^{RA}, or mesenchyme^{RA} and AER^{RA} were examined for the expression of *shh* and *bmp-2* at 9, 13 and 18 hours after transplantation (Fig. 1). Grafts that consisted of mesenchyme^{RA} expressed *shh* at the time of transplantation and continued to do so in their new environment at 9 h (Fig. 1A).

Table 1. Digit-inducing capacity of various types of grafts*

Type 1 graft mesenchyme^{RA}†	Type 2 graft mesenchyme^{RA} AER^{RA} ectoderm^{RA}	Type 3 graft mesenchyme^{RA} ectoderm^{RA}	AER^{RA}	Mesenchyme^{RA} AER^{RA} ectoderm^{RA} trypsinized	Mesenchyme^{RA} posterior AER
d**234** (3)‡	**44334** (1)	**23234** (1)	**234** (6)	**4334** (3)	**23234** (1)
234 (6)	**4334** (3)	**3234** (1)		**43234** (4)	**2334** (1)
	432234 (1)	**2334** (1)		**32234** (4)	**2234** (3)
	32234 (2)	d**234** (3)		**3234** (2)	d**234** (1)
	3234 (1)	**234** (6)		**2234** (6)	**234** (13)
	2234 (3)			**234** (2)	
	d**234** (1)				
	234 (1)				

*Grafts originated from a site distal to the RA bead (region 'A' as defined by Helms et al., 1994).
†Tissue treated with RA is marked by 'RA'. For RA treatment, beads soaked in 10 μg/ml RA, were left in situ for 22±1 hours.
‡Number of cases.

However, transcripts were not detected 13 hours after grafting (Fig. 1B). Similarly, *bmp-2* expression was lost in the graft (data not shown). In contrast, grafts consisting of mesenchyme^{RA} and AER^{RA} expressed *shh* and *bmp-2* for a minimum of 18 hours (Fig. 1C-H). In addition, *bmp-2* was strongly induced in the host's AER next to the graft (Fig. 1G,H), exactly as we observed when a ZPA was transplanted (data not shown). From these experiments, we conclude that an AER exposed to RA contains signals that are required for the maintenance of *shh* and *bmp-2* in the mesenchyme^{RA} (Fig. 1) and for polarizing activity (Table 1). Neither stage 20 posterior AER nor the host's anterior AER can substitute for an AER^{RA}. This contrasts with ZPA grafts taken from a stage 20 wing bud and placed underneath a host's AER. These grafts continue to express *shh* and *bmp-2* and evoke complete digit pattern duplications with (n=5) or without (n=7) their overlying ectoderm and AER.

Ectodermal signals are required for early flank mesenchyme to exhibit polarizing activity

We have shown that there are at least two significant discrepancies between mesenchyme that has been treated with RA and a ZPA graft. The first difference is that mesenchyme^{RA} requires an AER^{RA} to exhibit polarizing activity, whereas a ZPA graft does not require signals from the posterior AER to exhibit polarizing activity. The other difference is that the expression of *shh* and *bmp-2* is maintained in a ZPA graft without benefit of its overlying AER/ectoderm whereas RA-treated tissues will lose the expression of both genes in the absence of an AER^{RA}. One possible explanation for these discrepancies is that RA exposure reprograms the mesenchyme and the AER to a developmental state earlier than a stage 20 ZPA. To test this idea, we assayed for polarizing activity in tissues from earlier embryos. These grafts were composed of either mesenchyme only, or mesenchyme and its overlying ectoderm. First we grafted posterior mesenchyme from stage

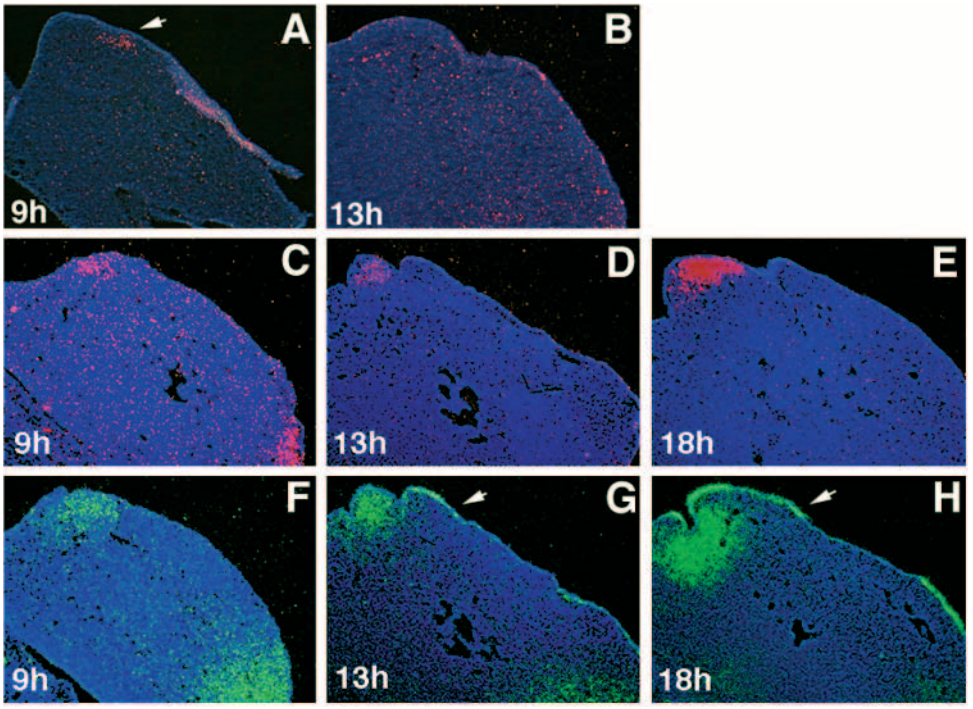

Fig. 1. An AER^{RA} is required for the continued expression of *shh* and *bmp-2* in mesenchyme^{RA} transplanted into a host wing bud. Grafts taken from a donor embryo treated with 10 μg/ml RA were transplanted under the anterior AER of a host embryo and allowed to develop. The embryos were examined by in situ hybridization for the expression of *shh* (red) and *bmp-2* (green). (A,B) Grafts of mesenchyme^{RA} only expressed *shh* by 9 hours (A, arrow), but this expression was no longer observed by 13 hours after transplantation (B). (C-H) Grafts consisting of mesenchyme^{RA} and AER^{RA} maintain the expression of *shh* (C-E) and *bmp-2* (F-H) in the transplanted tissue. In addition, *bmp-2* is strongly induced in the host's AER next to the graft (G,H arrows). For all time points, adjacent sections were hybridized with either *shh* or *bmp-2* cRNAs; since the expression of *shh* delimits the graft and the expression domain of *bmp-2* is greater than that of *shh*, we conclude that an induction of *bmp-2* in the host tissues had occurred.

17/18 embryos whose limb buds had begun to form. Such grafts were almost as effective in evoking duplications (**43234**, *n*=6; **3234**, *n*=2; **2234** or **234**, *n*=2) as posterior mesenchyme taken from stage 20 embryos (**43234**, *n*=7). These results demonstrate that full duplications can be achieved without including the posterior AER from a stage 17/18 embryo in the graft, just as we see with a stage 20 ZPA graft. Therefore, it appears that, by stage 17 and later, polarizing activity is limited to the mesenchyme. This ability of posterior mesenchyme to act as a ZPA closely correlates with the onset of *shh* and *fgf-4* expression.

Next we assayed polarizing activity in grafts from the flanks of stage 14/15 embryos (Fig. 2). Note, neither *shh* nor *fgf-4* transcripts are detected at this early stage of limb development. Since the limb buds had not begun to protrude from the body wall, grafts were taken from the posterior half of the presumptive wing field opposite somites 18-22 (Hornbruch and Wolpert, 1991). We found that grafting prospective wing mesenchyme (somatopleure) together with its overlying ectoderm (a discernible AER had yet to form) induced complete duplications with additional digits **3** and **4** much more frequently than grafts of mesenchyme alone (Fig. 2; Table 2). Grafts of flank ectoderm had no effect on the digit pattern (Table 2). These results demonstrate that, on or around stage 14, ectodermal signals are required for prospective wing mesenchyme to exhibit polarizing activity when grafted to a stage 20 wing bud. These ectodermal factors are not supplied, or not supplied in sufficient amounts, by a host's anterior AER. Therefore, with regard to a requirement for an ectodermal signal, prospective wing mesenchyme and mesenchyme treated with RA are functionally equivalent in this polarizing activity assay. These findings support the hypothesis that RA treatment reprograms anterior limb bud tissues to a state similar to prospective wing mesenchyme.

An RA-treated AER is functionally interchangeable with early flank ectoderm

In the previous sections, we demonstrated that prospective wing ectoderm and an AER^{RA} provided similar signals that were required for polarizing activity. This led us to hypothesize that treating a stage 20 anterior limb bud with a low dose of RA created a state resembling that of stage 14 prospective wing bud. To test this hypothesis further, we swapped prospective wing-bud tissues and RA-treated tissues (Fig. 3A,B) and assayed for polarizing activity. First we grafted recombinants of mesenchyme from stage 14 embryos and an AER^{RA} (Fig. 3A). In a second series of experiments, we combined mesenchyme^{RA} with stage 14 ectoderm (Fig. 3B). Recall that, by itself, stage 14/15 mesenchyme was a weak inducer of duplications (Table 2, first column). However, when prospective wing mesenchyme was combined with an AER^{RA} the result was a complete duplication of the digit pattern in 4 out of 7 cases (Fig. 3A; Table 3). Polarizing activity was not due to mesenchymal cells attached to the AER^{RA} since such a ridge graft alone did not cause duplications (Table 1, fourth column). Combinations of mesenchyme^{RA} and prospective wing ectoderm were also effective in evoking full pattern duplications (Fig. 3B; Table 3). Collectively these data demonstrate that AER^{RA} and prospective wing ectoderm can functionally substitute for each other in a polarizing activity assay. These results underscore our conclusion that both types of mesenchyme depend on ectodermal signals for their subsequent acquisition of polarizing activity. While these experiments do not provide information about the molecular nature of the ectodermal signals, they are consistent with the idea that both types of ectoderm provide the same types of signals.

RA is locally synthesized in the presumptive wing region

Thus far we have shown that AER^{RA} and early flank ectoderm were necessary and sufficient to establish polarizing activity in mesenchyme^{RA} and presumptive wing bud mesenchyme, respectively. Moreover, these early flank tissues and RA-exposed tissues were interchangeable in a polarizing activity assay. While these experiments illustrate that ectopic RA

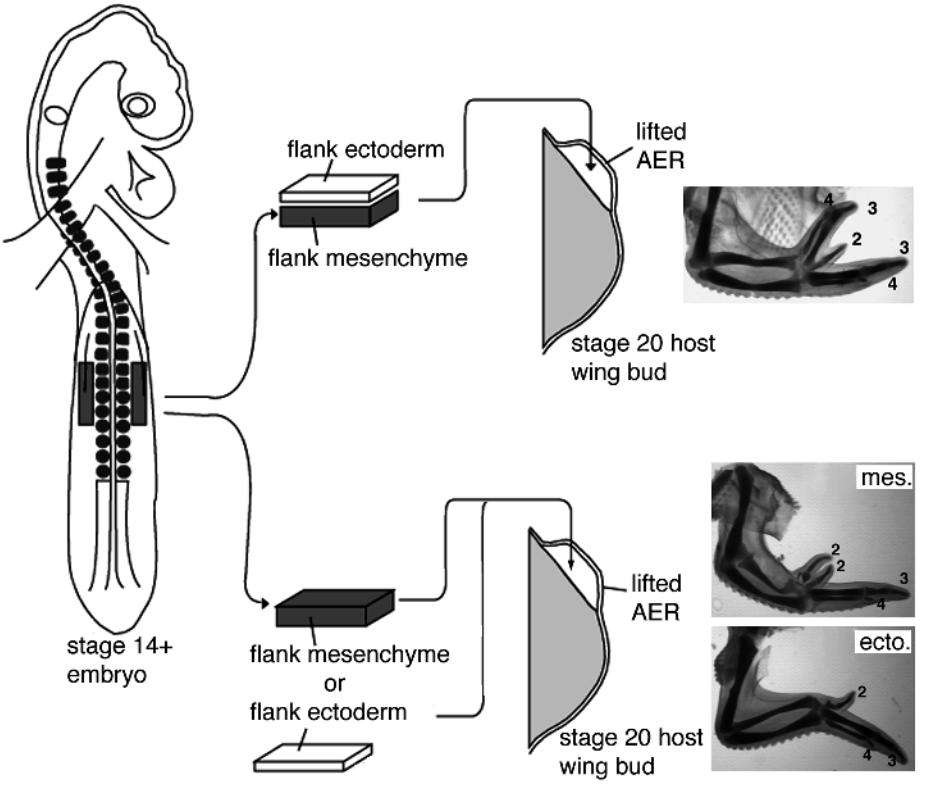

Fig. 2. Both early flank ectoderm and mesenchyme are required for polarizing activity. Ectoderm and mesenchyme taken from the wing-forming region of a stage 14 embryo (shaded rectangle) were grafted, either together (top) or separately (bottom), underneath the anterior margin of a stage 20 wing bud. Grafts consisting of ectoderm and mesenchyme frequently resulted in full digit pattern duplications, whereas grafts consisting of mesenchyme only were much less effective. Grafts of ectoderm never induced additional digits.

Table 2. Digit pattern duplications induced by stage 14 mesenchyme and ectoderm

Digit pattern	Mesenchyme only	Ectoderm only	Mesenchyme and ectoderm
234	5*	8	4
d**234**			
2234			
dd**234**	15	0	8
d**2234**			
23234			
3234			
32234	7	0	0
d**3234**			
333234			
43234			
4334	1	0	8

*Number of cases.

treatment mimics processes that occur during the establishment of a polarizing region, they do not directly demonstrate a role of RA in this process. To provide further evidence that RA is involved in the establishment of the ZPA, we showed that tissue from the wing region was capable of synthesizing RA. Specifically, we assayed for the ability of lateral plate tissues to synthesize RA from its precursor (retinal). Stage 14 lateral plate tissue was divided into two parts, one representing presumptive wing bud tissue and the other consisting of tissue located posterior to the wing bud region but not including the hindlimb territory (Fig. 4A). The tissues were incubated in 36 μM retinal dissolved in a fully defined medium and the metabolites that formed were identified by high performance liquid chromatography (HPLC). Fractionation of incubates on two sequential reverse phase columns resulted in a peak that co-eluted with authentic [^{3}H]RA (Fig. 4B,C). Tissue from the presumptive wing bud synthesized RA at a rate of 5.83±1.7 pg RA/μg DNA/3 hours while interlimb flank tissue exhibited a rate of 1.7±1.7 pg RA/μg DNA/3 hours, 3.4 times less than the presumptive wing bud. Rates of RA synthesis were also determined for tissue from stage 20 wing buds and were 0.5 pg RA/μg DNA/3 hours, which is almost 12 times less than the rate found in the presumptive wing region.

Antagonists of retinoic acid receptors and retinoid-X-receptors produce abnormal limbs and down-regulate the expression of *sonic hedgehog*

Thus far our experiments suggest that, when applied to stage 20 anterior wing bud cells, RA initiated ectoderm-mesenchyme interactions that mimic those observed during pre-wing-bud stages and showed that prospective wing tissues synthesize RA. Next we disrupted the endogenous retinoid signaling pathway by applying the RAR and RXR pan-specific antagonists LG629 and LG754 (R. Heyman, personal communication). Beads soaked in an mixture of these antagonists (500 μg/ml each) were implanted at the anterior (somite 15) and posterior (somite 20) boundaries of the presumptive wing field of a stage 14 (22 somite) embryo (Fig. 5A). Wing buds formed at the same rate as the contralateral buds and by stage 20 a distinct AER was visible (data not shown). By stage 25, the untreated wing bud (Fig. 5B) displayed a distinct handplate whereas the treated wing bud was usually lacking, or had a misshaped, handplate (Fig. 5C). By day 10, the skeletal pattern of the wings revealed a spectrum of defects (Fig. 5D-F). In 90% of the cases, a humerus had formed (n=45) although, in most instances, it was thinner. In 10% of the wings, the humerus was distally truncated or completely absent (Fig. 5F). A partial or full radius was present in 87% of the specimens while only 26% of the wings had an ulna. Often the

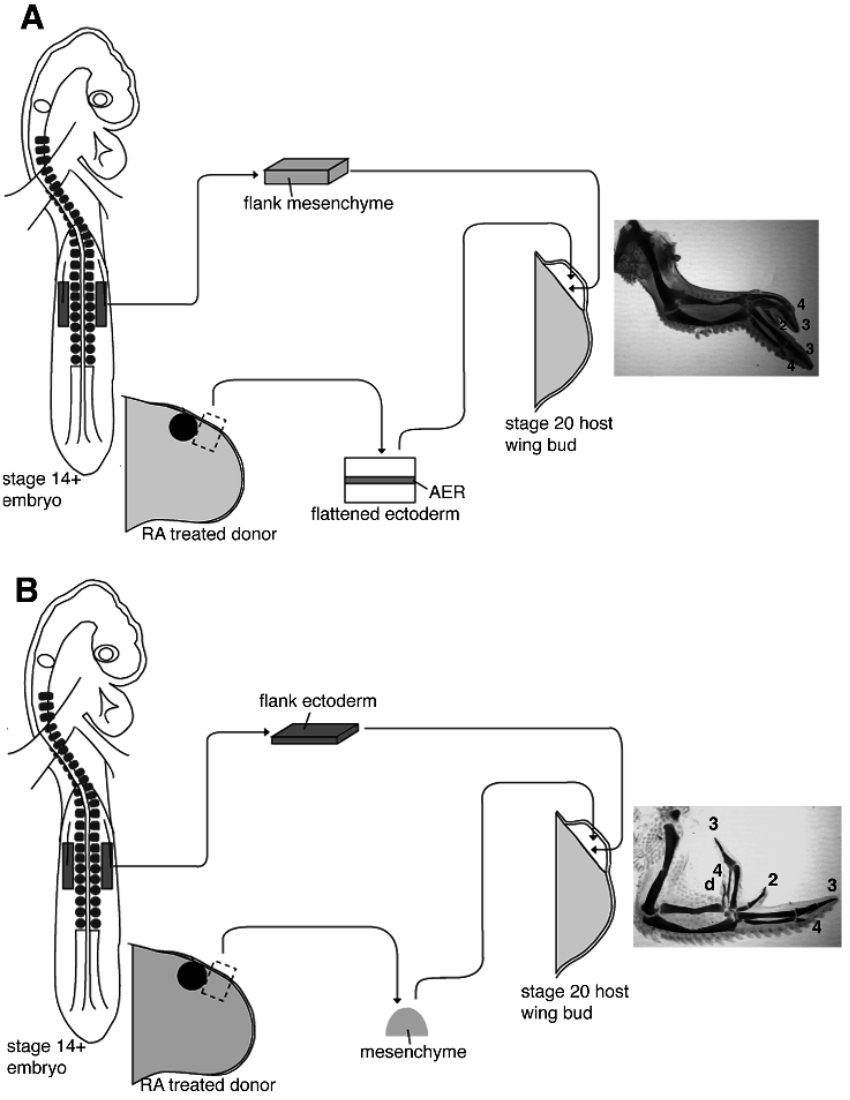

Fig. 3. RA-treated tissues can substitute for early flank tissues in a polarizing assay. (A) Stage 14 flank mesenchyme was combined with an AER^{RA} and placed underneath the AER of a stage 20 host wing bud. This combination frequently resulted in duplications of digits **3** and **4**. (B) Identical results were obtained when mesenchyme^{RA} was combined with stage 14 flank ectoderm.

Table 3. Digit pattern duplications resulting from tissue swapping between stage 14 presumptive wing bud tissues and tissues from RA-treated wing buds

Digit pattern	Stage 14 flank mesenchyme + AER^{RA}	Stage 14 flank ectoderm + $mesenchyme^{RA}$
234	0*	3
2234	1	3
3234	0	3
d**3234**	2	4
333234	0	1
43234	3	3
4334	1	0

*Number of cases.

handplate was completely absent (74%, Fig. 5E,F). If digits were present, they were more frequently the anterior digits than the posterior digits (digit **2**, 26%; digit **3**, 17%; digit **4**, 9%; see Fig. 5D). In general, treatment with RAR and RXR antagonists resulted in a loss of structures in a posterior-to-anterior progression. In those eleven cases when a hand plate was partially present, the digits were either digit **2** or **3** but never a digit **4**. Likewise, in 39 of the 40 wings which had only one forearm element, this element was a radius.

If RAR and RXR antagonists blocked retinoid signaling in a selective manner, then application of ectopic RA should restore a pattern. However, if LG629 and LG754 caused irreversible tissue damage, no such rescue should occur. To distinguish between these possibilities, the presumptive wing bud region was treated with a combination of the antagonists then allowed to develop to stage 20, at which time a bead soaked in a physiologic dose of RA (20 μg/ml; Helms et al., 1994) was implanted underneath the AER at the anterior wing bud margin. By stage 25, it was clear that treatment with RA restored the handplate (Fig. 5G) as compared with antagonist-treated limbs (Fig. 5C). We found that out of a total of 24 wings, 16 had a handplate with one or more identifiable digits. Six showed a **43** pattern where **4** was situated anterior to **3** (Fig. 5H,I), an unambiguous reversal of anteroposterior polarity. Such a reversal in polarity demonstrates that the digits did not arise because the antagonists had been ineffective and failed to truncate the limb. Rather, the reversed digit pattern was specified from the anteriorly implanted RA source which acted as a new signaling center.

To investigate whether the morphological data that we obtained with the retinoid antagonists had a molecular correlate, we examined LG629- and LG754-treated embryos for the expression of *shh*. RAR and RXR antagonists were applied to the presumptive wing bud region of stage 14 embryos and the embryos were analyzed by whole-mount in situ hybridization at approximately stage 20. As with the skeletal patterns, there was a range of effects of the antagonists on *shh* expression. In 13 out of 26 embryos, the expression of *shh* in the treated limb bud was undetectable or greatly diminished as compared to the contralateral limb bud (Fig. 6A,B). In the remaining cases, *shh* expression appeared to be equivalent to that seen on the untreated contralateral limb (Fig. 6C) as would be expected in those cases where the truncations of the limbs were minimal (for example, see Fig. 5D).

To determine whether LG629 and LG754 have an effect on the wing pattern once wing buds have formed, beads soaked

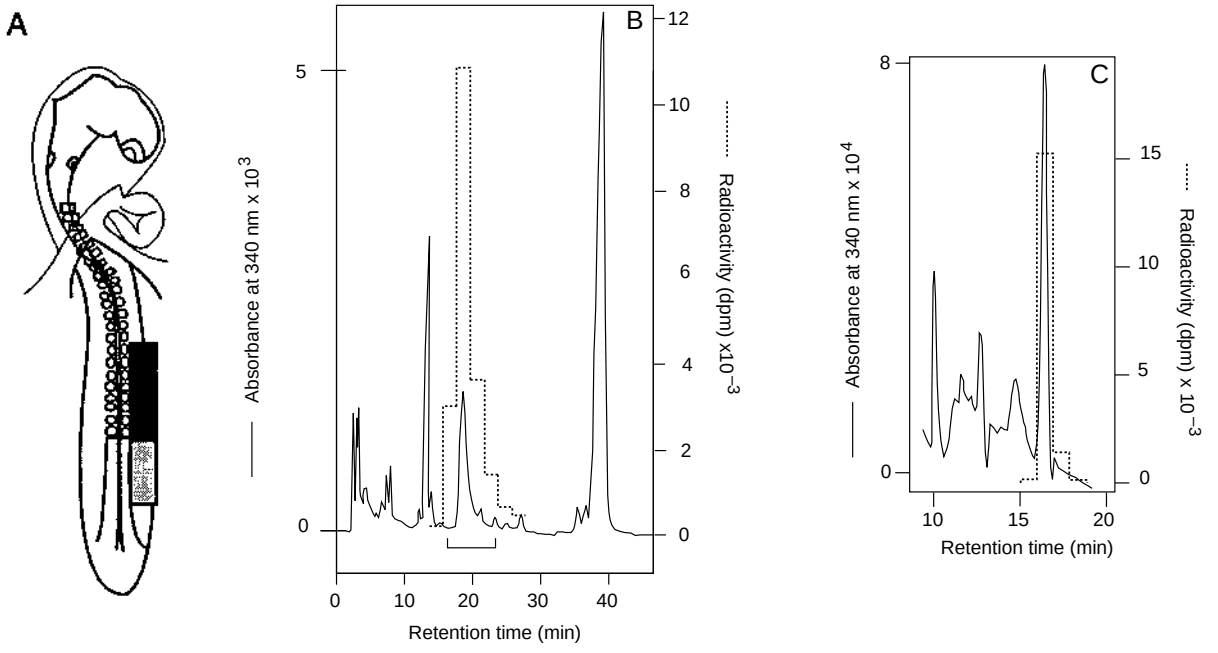

Fig. 4. Sample chromatograms illustrating the identification of RA generated from all-*trans*-retinal by tissue from the wing-forming region. (A) Tissue regions in a stage 14 embryo examined for RA production are indicated by black (prospective wing region) and gray (interlimb region). (B) UV absorption (solid line) of the metabolites generated from all-*trans*-retinal. Fractions 18-23 containing tracer [^{3}H]RA (dotted line) were pooled and rechromatographed. The resulting chromatogram (C) exhibited a distinct UV peak (solid line) that co-eluted with the internal standard [^{3}H]RA (dotted line).

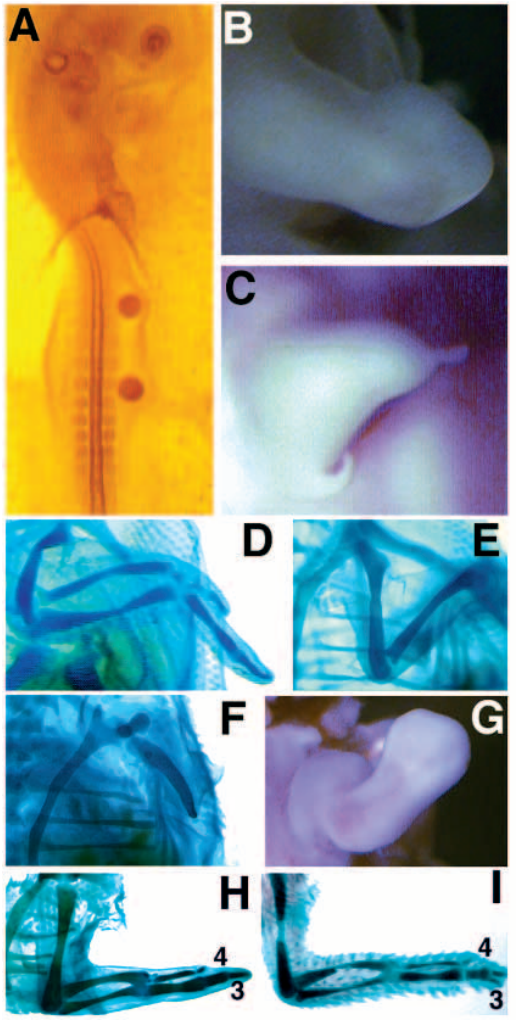

Fig. 5. Local application of a combination of RAR and RXR antagonists to the wing-forming region of stage 14 embryos resulted in the loss of wing skeletal elements. (A) AG1-X2 ion exchange beads of 200 μm diameter soaked with LG629 and LG754 were placed opposite somites 15 and 20 of a 22- to 24-somite chick embryo. To visualize the anatomical details, embryos were slightly stained with Neutral Red, a vital stain. (B) Untreated wing rudiment at stage 25. (C) An antagonist-treated wing rudiment at stage 25; note lack of distal tissue such as the handplate. (D-F) Skeletal patterns resulting from antagonist-treated wing buds. (D) Wing consisting of a humerus, a radius and an ulna, and digits **2** and **3**. (E) Wing consisting of a humerus and a radius. (F) Wing consisting of a distally truncated humerus. Control experiments included implantation of beads soaked in DMSO, the solvent for LG629 and LG754, as well as implantation of untreated beads. In most cases (n=15) the wings were normal with the exception of two wings, which lacked a radius, and one wing, which lacked a radius and a digit **2**. (G) Example of a wing primordium treated first with retinoid antagonists at stage 14, then treated with 20 μg/ml RA at stage 20 and allowed to develop to stage 25. Note the restoration of distal structures and a handplate that resembles the untreated wing bud seen in B. (H,I) Wings that developed after antagonist treatment followed by rescue with RA. (H) Wing consisting of a normal humerus, a radius, an ulna and digits **4** and **3**. Note, digit **4** is most anterior. (I) Skeletal pattern consists of a humerus, an ulna, a radius and digits **4** and **3**; digit **3** is split distally. The reversal of the digit pattern polarity proves that the handplate pattern was specified by the RA bead at the anterior bud margin.

in these antagonists (2.5 mg/ml each) were implanted underneath the posterior AER at the ZPA of stage 20 and 21 embryos. 14 wings that developed were normal and 2 had a partially truncated digit **4**. This suggests that blocking retinoid signaling in the ZPA by that time has little effect on the pattern.

Limb truncations can result from the application of very high doses of RA (Tickle et al., 1985). Since treatment of stage 14 embryos with LG629 and LG754 caused partial limb truncations (Fig. 5E,F), the possibility existed that these RAR and RXR antagonists could be acting as agonists in this system and causing the loss of skeletal elements in a similar manner as high doses of RA. The following experiment argues against this. We applied beads soaked in 500 μg/ml LG629 and LG754 to the anterior margin of stage 20 wing buds and assayed for pattern duplications. Neither LG629, LG754 (n=5, each) nor the antagonists in combination (n=20), induced additional digits. In addition, regardless of the dose used at stage 14 (from 10 μg/ml to 500 μg/ml), these antagonists never evoked any digit duplications and only caused a range of limb truncations (illustrated in Fig. 5D-F). Collectively these studies show that

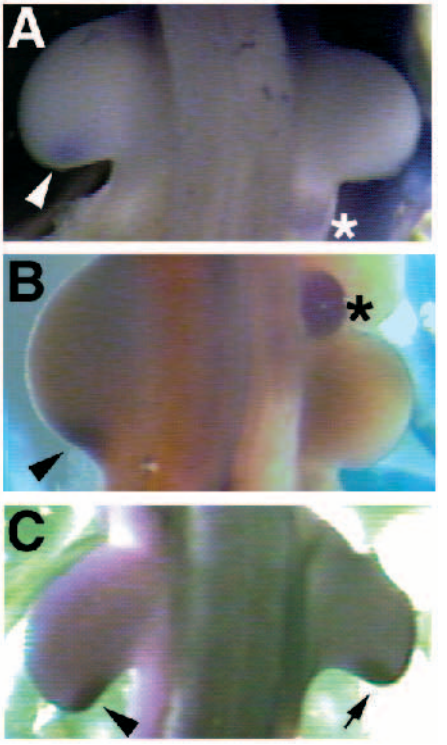

Fig. 6. Expression of *shh* following treatment with RAR and RXR antagonists. Stage 14 embryos treated with LG629 and LG754 developed to stage 20-22, then subjected to whole-mount in situ hybridization. The treated limb bud is on the right side and the control (untreated) limb bud is on the left. Where visible, the antagonist-releasing beads are indicated with an asterisk. (A) Expression of *shh* was evident in the untreated wing bud (white arrowhead), but was undetectable in the treated wing bud. (B) *shh* expression was detected in the untreated limb bud (black arrowhead) but was not visible on the treated side. The posterior bead is not visible from this view. In this case, the anterior bead resulted in a loss of anterior tissue. (C) Although both antagonist beads were present (not visible from this aspect), *shh* transcripts were detected in both wing buds (arrow, arrowhead).

retinoid antagonists can down-regulate or abolish the expression of *shh* and cause a loss of proper proximodistal and anteroposterior patterning. Since *shh* is normally expressed at stage 17 and later, and antagonists can interfere with the expression of this gene, we infer that retinoid signaling blocked by local application of antagonists takes place prior to stage 17 (see Discussion).

DISCUSSION

This study indicates a role of retinoids in early limb development, at the time prior to or concomitant with limb bud formation. This conclusion is based on three observations. (1) Blocking the retinoid signaling pathway with RAR and RXR antagonists at Hamburger-Hamilton stage 14 prior to limb bud formation results in down-regulation or loss of *shh* expression in the ZPA and in a partial or near-complete loss of wing structures. (2) Presumptive wing bud tissue from the same stage is a high-point of RA synthesis. (3) Exogenous RA induces factors in limb bud mesenchyme and ectoderm that, in a polarizing activity assay, substitute for stage 14 ectoderm or mesenchyme of the presumptive wing region.

RA regulates ectodermal-mesenchymal interactions underlying outgrowth and patterning of the limb

Grafts of posterior mesenchyme (ZPA) placed underneath the anterior AER of a stage 20 host wing bud maintain *shh* expression and exhibit polarizing activity as revealed by a digit induction assay. In contrast, grafts consisting of *shh*-positive mesenchyme^{RA} placed into an identical environment cannot maintain the expression of this gene and do not possess polarizing activity unless an RA-treated AER (AER^{RA}) is co-grafted. Similarly, grafts of presumptive wing mesenchyme have weak polarizing activity unless the overlying flank ectoderm is co-grafted. Importantly, presumptive wing tissues and RA-treated wing bud tissues can be interchanged in this polarizing activity assay. Three main points emerge from these observations. First, the presumptive wing bud ectoderm (or an AER^{RA}) provides signals that are required for polarizing activity to be established in the mesenchymal component of the graft. Second, ectodermal signals present in the presumptive wing ectoderm and those induced by RA in the AER^{RA} are functionally analogous. This functional homology of RA-treated tissue and presumptive wing tissue in conjunction with our retinoid antagonist experiments (see below), suggest a role for RA in early limb development. Third, the ectodermal signals contributing to the formation of polarizing activity in the pre-wing-bud mesoderm or in mesenchyme^{RA} and those required to maintain polarizing activity in a ZPA graft from a stage 20 bud seem to be different. From this follows that there are two kinds of ectodermal signals, a set required during the establishment of the ZPA ('early factors') and a set to maintain the ZPA ('late factors').

Two candidates for early acting factors are FGF-4 and FGF-8. The host AER expresses *fgf-8*, and *fgf-8* mRNA is found in the limb ectoderm as early as stage 16 (Crossley and Martin, 1995; Mahmood et al., 1995; Crossley et al., 1996). It has recently been shown that FGF-8 funtions as a limb-inducing signal and is involved in the establishment (Crossley et al., 1996) and maintenance of *shh* expression (Crossley et al., 1996; but see also Mahmood et al., 1995). In light of this it is surprising that, in our experiments, a *fgf-8*-positive host AER cannot support the establishment of polarizing activity in grafts of presumptive wing mesoderm or in mesenchyme^{RA} grafts. The reason for this discrepancy remains to be investigated.

FGF-4, the other candidate factor, is induced by RA in the anterior AER (Niswander et al., 1994), it is expressed in the posterior AER overlying the ZPA (Laufer et al., 1994; Niswander et al., 1994) and, in conjunction with high doses of RA, locally applied FGF-4 can create digits in a ridge-less wing bud (Niswander et al., 1993, 1994). Invoking FGF-4 as an early signal would also be consistent with our finding that the *fgf-4*-positive AER^{RA} will maintain polarizing activity in mesenchyme^{RA}. However, we found that *fgf-4*-positive posterior AER cannot substitute for an AER^{RA}. One cause for this failure might be that *fgf-4* expression is down-regulated after grafting and, as a result of this loss of expression, the postulated SHH-FGF-4 feed-back loop would no longer operate (reviewed e.g. in Tabin, 1995). However, it is not obvious why such a down-regulation of *fgf-4* expression should occur in a grafted posterior AER but not in a grafted AER^{RA}. Furthermore, pre-limb-bud flank ectoderm taken from stage 14 embryos evokes polarizing activity in the mesenchymal component of the graft despite the fact that *fgf-4* is not expressed in the ectoderm (*fgf-4* transcripts are first seen at stage 18, Laufer et al., 1994). Furthermore, mouse embryos homozygous for the *limb deformity* (*ld*) mutation in which *fgf-4* mRNA is not detectable in the AER, form distal structures such as digits. The limb buds of $ld^{-/-}$ embryos begin to express *shh* at the same time as wild-type embryos (Chan et al., 1995; Haramis et al., 1995). Apparently, here too, FGF-4 is dispensible for much of limb development, or can be substituted by another factor. Taken together, our experiments do not reveal a consistent correlation between the presence of *fgf-4* or *fgf-8* transcripts in ectoderm (or AER) and the establishment of polarizing activity in grafts of presumptive wing bud mesenchyme and mesenchyme^{RA}.

Once a wing bud has formed (stage 17/18 and beyond), the role of the ectoderm/AER appears to be one of maintenance. The AER and ectoderm are now required for the continued expression of *shh* and for the maintenance of the ZPA and progress zone (see Tabin, 1995 for a review). FGF-4 and Wnt-7A are implicated in this later function, since their ectopic application is sufficient to maintain *shh* expression in the absence of an AER and ectoderm (Laufer et al., 1994; Niswander et al., 1994; Parr and McMahon, 1995; Yang and Niswander, 1995). Consistent with FGF-4 acting as a maintenance factor is the finding that in *ld* mice *shh* expression does not persist as long as it does in wild-type buds (Chan et al., 1995; Haramis et al., 1995).

Comparison of limbs from retinoid receptor mutants and antagonist-treated embryos

Vertebrates have three RARs and three RXRs (RARα,β,γ; RXRα,β,γ) and they may be functionally redundant (for discussion, see e.g. Lohnes et al., 1994). In fact, null mutations in individual RAR genes do not result in limb malformations (Li et al., 1993; Lufkin et al., 1993; Lohnes et al., 1993, 1994; Mendelsohn et al., 1994; Luo et al., 1995). Likewise, null mutants in RXRα have normal limbs (Kastner et al., 1994; Sucov et al., 1994); null mutants of RXRβ and RXRγ have not

yet been reported. Consistent with the idea of functional redundancy, double mutants of RARα and RARγ have forelimb defects such as agenesis of the scapula, shortening of the humerus, loss of the radius, malformation of carpal bones, size reduction or loss of digit **4** and the formation of an additional anterior digit accompanied by a transformation of the second digit (Lohnes et al., 1994). Superficially, the wings developing after antagonist treatment (Fig. 5D-F) have some resemblence to those seen in RARα/γ$^{-/-}$ mice. However, there are two major differences. First, antagonist exposed limbs exhibit much more severe defects, in 74% of all cases, the handplate was missing and many limbs had just a humerus and a forearm element. At the molecular level, a main difference is that, in double mutants of RARα and RARγ, the expression of *shh* appears unaffected (Lohnes et al., 1994), while antagonist treatment either reduces or totally abolishes the expression of this gene in half of the cases. One possible interpretation of this discrepancy is that these particular double mutants reveal only a subset of functions retinoids have in limb development.

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