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Relationship between retinoic acid and *sonic hedgehog*, two polarizing signals in the chick wing bud

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SUMMARY

Local application of all-*trans*-retinoic acid (RA) to the anterior margin of chick limb buds results in pattern duplications reminescent of those that develop after grafting cells from the zone of polarizing activity (ZPA). RA may act directly by conferring positional information to limb bud cells, or it may act indirectly by creating a polarizing region in the tissue distal to the RA source. Here we demonstrate that tissue distal to an RA-releasing bead acquires polarizing activity in a dose-dependent manner. Treatments with pharmacological (beads soaked in 330 μg/ml) and physiological (beads soaked in 10 μg/ml) doses of RA are equally capable of inducing digit pattern duplication. Additionally, both treatments induce *sonic hedgehog* (*shh;* also known as *vertebrate hedgehog-1, vhh-1*), a putative ZPA morphogen and *Hoxd-11*, a gene induced by the polarizing signal. However, tissue transplantation assays reveal that pharmacological, but not physiological, doses create a polarizing region. This differential response could be explained if physiological doses induced less *shh* than pharmacological doses. However, our in situ hybridization analyses demonstrate that both treatments result in similar amounts of mRNA encoding this candidate ZPA morphogen. We outline a model describing the apparently disparate effects of pharmcologic and physiological doses RA on limb bud tissue.

Key words: zone of polarizing activity, retinoic acid, limb development, *sonic hedgehog*, *Hoxd-11*, *vhh-1*

INTRODUCTION

Exogenously provided retinoids (vitamin A and its congeners) have marked effects on the body pattern of vertebrate embryos (reviewed by Brickell and Tickle, 1989; Eichele, 1989; Smith et al., 1989; Summerbell and Maden, 1990; Tabin, 1991; Bryant and Gardiner; 1992, Linney, 1992; Hofmann and Eichele, 1993). For example, all-*trans*-retinoic acid (RA) released from a bead implanted at the anterior margin of a chick wing bud will alter the normal *234* digit pattern to one that exhibits duplications (Tickle et al., 1982, 1985; Summerbell, 1983). This process is dose-dependent; increasing the RA dose leads to patterns with additional digits in a $2 \rightarrow 3 \rightarrow 4$ progression, yielding *2234*, *32234* and *432234* patterns. Such pattern duplications occur at concentrations of applied RA close to that found endogenously (Thaller and Eichele, 1987, 1990). RA mimics the zone of polarizing activity (ZPA, also known as the polarizing region; Saunders and Gasseling, 1968; Tickle et al., 1975), which evokes the development of additional digits in a cell number-dependent fashion (Tickle, 1981).

RA may act as a spatial signal that directly specifies cell identity, for example by inducing *HoxD* genes (Ipsizúa-Belmonte et al., 1991; Nohno et al., 1991). Alternatively, RA may act indirectly by initiating a cascade of signaling events in the tissue adjacent to an RA-releasing bead, ultimately resulting in the generation of a new polarizing region. Experiments in which tissue distal to an RA-releasing bead acquired polarizing activity support this theory (reviewed by Bryant and Gardiner, 1992). In this instance, RA could not be a polarizing signal since earlier studies suggest that grafts of the polarizing region do not induce polarizing activity (Smith, 1979). In the present study we show that, like a ZPA graft, physiological concentrations of RA do not induce polarizing activity in the tissue adjacent to an RA-releasing bead. However, in agreement with other studies (Summerbell and Harvey, 1983; Noji et al., 1991; Tickle 1991; Wanek et al., 1991; Tamura et al., 1993), high (pharmacological) doses induce polarizing activity.

Recently, *sonic hedgehog* (*shh*; also known as*vertebrate hedgehog-1, vhh-1*), a vertebrate homolog of the *Drosophila* segment polarity gene *hedgehog*, has been cloned. The site of expression of *shh* mRNA in the limb coincides with the polarizing region (Riddle et al., 1993; Roelink et al., 1994) making *shh* an ideal marker for the ZPA. Furthermore, overexpression of shh in the chick wing bud induces digit pattern duplications, raising the possibility that shh protein is the ZPA morphogen (Riddle et al., 1993). We found that physiological and pharmacological doses of RA induce *shh* expression at comparable levels. If shh is the ZPA morphogen, then transplanting *shh*-positive tissue taken from a region distal to the RA bead should induce digit duplication. In the case of pharmacological doses, this prediction holds true. However, when physiological doses of RA are used, the *shh*-positive graft did not affect the digit pattern. These results suggest that shh is only one of several signaling molecules that are required for polarizing activity.

MATERIALS AND METHODS

Local application of RA

AG1-X2 beads of 200-250 μm diameter were soaked in solutions of RA in dimethylsulfoxide (Eichele et al., 1984). Concentrations used ranged from 3 to 1000 μg/ml and were determined spectrophotometrically. Beads had formate as a counter ion and were kept at constant moisture by storing them in a closed jar containing a saturated solution of sodium chloride. Constant moisture results in a more uniform loading capacity of the ion-exchange resin. 100 μl RA solution was dispensed into 1.5 ml microcentrifuge tubes containing 20 beads each. Tubes were agitated on an Eppendorf tube shaker for 20 minutes. Thereafter, the RA solution was removed and beads first rinsed with 200 μl of phenol red-containing (5 mg/l) phosphate-buffered saline (PBS) and subsequently washed twice in 200 μl of this solution for 10 minutes each, again using the shaker. Beads were then implanted into Hamburger-Hamilton stage 20 embryos (Hamburger and Hamilton, 1951) underneath the apical ectodermal ridge (AER) as previously described (Tickle et al., 1985). Care was taken that the ridge was cleanly separated from underlying mesenchyme so that few mesenchymal cells were interspersed between the AER and the carrier. Eggs were sealed with tape and incubated for either 22-24 hours or 7 days at 37.5°C.

Tissue transplantations

Embryos treated with RA for 22-24 hours were removed from the egg, transferred into PBS, their treated wing buds were removed and placed in a freshly made 2% trypsin-PBS solution (Sigma T-8128). This and subsequent manipulations were carried out in a polystyrene Petri dish (3.5 cm diameter; Corning, Corning, NY) kept on a 2°C cold stage; all solutions were kept on ice. When the ectoderm started to detach after about 20-25 minutes, the buds were transferred into PBS and washed three times with approximately 3 ml of PBS. Thereafter, the ectoderm was peeled away with tungsten needles and forceps. Small prisms of tissue (about 200×100×100 μm) were cut from the region around the bead (see Fig. 2). One face of the prism was concave as a result of being in direct contact with the bead.

Grafting: The AER of stage 20 recipient embryos was lifted along the anterior margin using a sharp tungsten needle, tissue pieces were transferred from PBS into the egg using a 20 μl micropipette (Clay Adams, Becton Dickinson and Company, Parsippany, NJ) and manoeuvered underneath the loose ridge with a blunt tungsten needle. Embryos were inspected at 30 minutes and 24 hours to assure that the graft was properly placed and had not dislodged. Eggs were sealed with tape and incubated for a total of 7 days at 37.5°C. Primary host embryos were treated with RA for 22-24 hours for two reasons. First, RA has a maximal effect on patterning when wing buds are exposed for approximately 18-20 hours (Eichele et al., 1985). Second, the induction of polarizing activity by RA is first noted by approximately 15 hours after initiation of RA treatment (Wanek et al., 1991).

Analysis of wing patterns

Embryos were fixed in 3% trichloroacetic acid, stained in Alcian Green, differentiated in acidic ethanol and cleared with methyl salicylate. Patterns were numerically represented by assigning them a score (S). When the anteriormost additional digit was a digit *4*, S was 4. When the anteriormost digit was a *3* or a *2*, S was 3 and 2 respectively. A digit of equivocal identity had an S of 1. For each concentration, these scores were used to calculate the 'percentage respecification value' (PRV), where $PRV = \sum S \times 100/4 \times n$, and n is the number of wings included.

Application of exogenous RA and quantification by high-performance liquid chromatography

Experiments aimed at measuring the amount of applied RA in wing buds were conducted as described above under 'local application of RA'. Treatments with beads soaked in 100 μg/ml and higher were done with non-radioactive RA since, under these conditions, the amount of RA delivered into the bud is sufficient for UV detection at 350 nm. For the 10 μg/ml dose, tritiated RA (New England Nuclear, Boston, MA; specific activity of 50 Ci/mmol) was used.

Treated wing buds were collected from 15-20 embryos 15 hours after bead implantation at stage 20. Implanted beads were removed, the buds were cut off, washed in stabilizing buffer (Eichele et al., 1984) and collected into a microfuge vial kept on dry ice. The time-point of 15 hours was chosen for three reasons. First, it is halfway between the minimal time required to induce duplication and the time it takes to evoke a maximal response (Eichele et al., 1985). Second, the concentration of applied RA in the limb bud is approximately constant after 15 hours (Eichele et al., 1985). Third, polarizing activity is first detected in the treated bud at 15 hours (Wanek et al., 1991).

Tissue was extracted as previously described (e.g. Thaller et al., 1993). Tissue extracts were fractionated on a reverse-phase HPLC column (Microsorb C_{18}, Rainin, Woburn, MA) developed at 1 ml/min with either mobile phase A (acetonitrile:methanol:2% acetic acid = 60:20:20) or C (methanol:acetonitrile:1% acetic acid = 80:10:10). In the case of the 10 μg/ml tritiated RA treatments, the amount of RA was calculated based on the amount of radioactivity contained in the RA peak (measured by scintillation counting of 1 ml fractions that were collected) and using 20-nor RA (100 ng) as an internal standard to determine recoveries. In all other cases, the OD_{350} peak area was integrated to determine the amount of RA and recoveries were calculated using an added [^{3}H]RA-internal standard. In experiments where RA was detected by UV, a certain fraction of the absorbance was contributed by the endogenous retinoic acid; this value (7 pg/bud at stage 22/23; Thaller and Eichele, 1990) was subtracted from the total.

In situ hybridization

Embryo collection, sectioning and in situ hybridization were performed as previously described (Sundin et al., 1990). Subclones of *shh* and *Hoxd-11* in pBSII (nts 756-1566 and nts 884-1566 for *shh* [Riddle et al., 1993] and and nts 4-635 for *Hoxd-11* [Rogina et al., 1991]) were linearized with appropriate restriction enzymes to transcribe either sense or antisense ^{35}S-labeled riboprobes. The second *shh* probe was a truncated version of the first probe and did not contain sequence from the more conserved amino terminus; both *shh* probes produced identical hybridization patterns. Slides were exposed for 4 days. Photographs are double exposures; the red color represents the in situ hybridization signal, and the blue color shows the nuclei stained with Hoechst 33258 dye.

RESULTS

Experiments in which effects of RA on limb patterning are studied employ a wide range of soaking concentrations (e.g. 10 μg/ml by Thaller et al., [1993] to 1 mg/ml by Tamura et al., [1993]; Riddle et al., [1993]). It is an intrinsic property of receptor/ligand-based signal transduction systems that responses are dose-dependent. Whether or not tissue adjacent to a RA-releasing bead acquires polarizing activity may, therefore, be governed by the amount of RA applied to the wing bud. In a first series of experiments we wished to determine (i) how much RA was released into the wing buds as a function of the RA concentration in the soaking solution and (ii) how the amount of released RA related to the previously determined concentration of endogenous RA (physiological concentration). In a second set of experiments, we examined whether RA doses resulting in a physiological concentration of applied RA induced polarizing activity in tissue

adjacent to the bead. Polarizing activity was assessed by the classical digit pattern duplication assay (Saunders and Gasseling, 1968 as modified by Tickle, 1981). Lastly, we examined the effects of physiological and pharmacological RA doses on the induction of *sonic hedgehog*, a molecular marker associated with the ZPA and a putative ZPA morphogen (Riddle et al., 1993) and *Hoxd-11*, a gene induced by the polarizing signal (Ipsizúa-Belmonte et al., 1992).

Relationship between soaking concentration and the concentration of RA in the treated wing bud

We first determined the amount of RA released into a wing bud from a RA-impregnated bead. Briefly, beads were immersed in RA solutions ranging between 3 and 1000 μg/ml. Beads were washed and implanted into stage 20 wing buds (see Methods). The amount of RA in treated buds was determined 15 hours later when the embryos had progressed to stage 22/23. RA was quantified by high-performance liquid chromatography (Fig. 1A). The histogram in Fig. 1B illustrates the amount of RA present in the limb bud as a function of the soaking concentration. At 10 μg/ml, wing buds contained 6.5 pg of RA, which is virtually identical to the amount of endogenous RA (6 to 7 pg indicated by a horizontal solid arrow in Fig. 1B, and calculated from Thaller and Eichele, 1990). Therefore, we refer to soaking concentrations at or near 10 μg/ml as physiological concentrations. In contrast, presoaking beads in 100, 330 and 1000 μg/ml resulted in 137, 288 and 1362 pg of RA per bud, respectively. Soaking concentrations of 100 μg/ml and above result in levels of RA that are considerably above the endogenous RA level and therefore are referred to as pharmacological.

Pharmacological, but not physiological, doses of RA induce polarizing activity

We examined whether local application of RA to the chick wing bud evoked polarizing activity in tissue distal to the RA source. The presence of polarizing activity was determined by means of a digit pattern duplication assay (Fig. 2). RA-releasing beads were implanted at the anterior wing bud margin of stage 20 embryos. Some embryos (group 1) were incubated for 7 days to form a wing, while others (group 2) were incubated for 22-24 hours, after which time small prisms of mesenchyme were cut from the region surrounding the bead (Fig. 2). These prisms were grafted into stage 20 host embryos underneath the AER at the anterior wing bud margin opposite somite 16. Digit patterns of host embryos were examined after 7 days of incubation. Group 1 embryos exhibited pattern duplications in a dose-dependent fashion (Table 1 and Fig. 3, upper curve). At 1 mg/ml, RA induced *43234* duplications (Fig. 4A) or wings in which digits were either reduced in number (e.g. *4334*, *434*) or absent. Reducing the soaking concentration to 330, 100, 20 and 10 μg/ml frequently resulted in either *4334* or *43234* mirror-image patterns (Table 1, Fig. 4), i.e. wings with additional digit *3* and *4*. At 3 μg/ml, wings most frequently had either an additional digit *2* or *3*. Pattern duplication as a function of the RA dose is graphically illustrated in Fig. 3. Additional digits were assigned particular scores (see Methods) and these were plotted against the soaking concentration. In full agreement with our earlier studies (Thaller and Eichele, 1990; Thaller et al., 1993) soaking concentrations 10 μg/ml and above consistently yielded a maximal response.

Two types of responses were seen in group 2 embryos that received tissue from buds exposed to RA for 22-24 hours. When tissue from buds exposed to high doses of RA (either 1 mg/ml or 330 μg/ml) was grafted, the host bud usually developed additional digits *3* and *4*. In several instances, these digit patterns were symmetrical (Fig. 4C), and closely resembled those obtained following either a ZPA graft (Fig. 4D) or 10 μg/ml RA treatment (Fig. 4B). The ability of grafted, RA-exposed mesenchyme to induce additional digits dropped substantially when the soaking concentration was decreased to 100 μg/ml or 20 μg/ml. Out of a total of 16 wings, one wing had an additional digit *3*, 7 had an additional digit *2* (Fig. 4E)

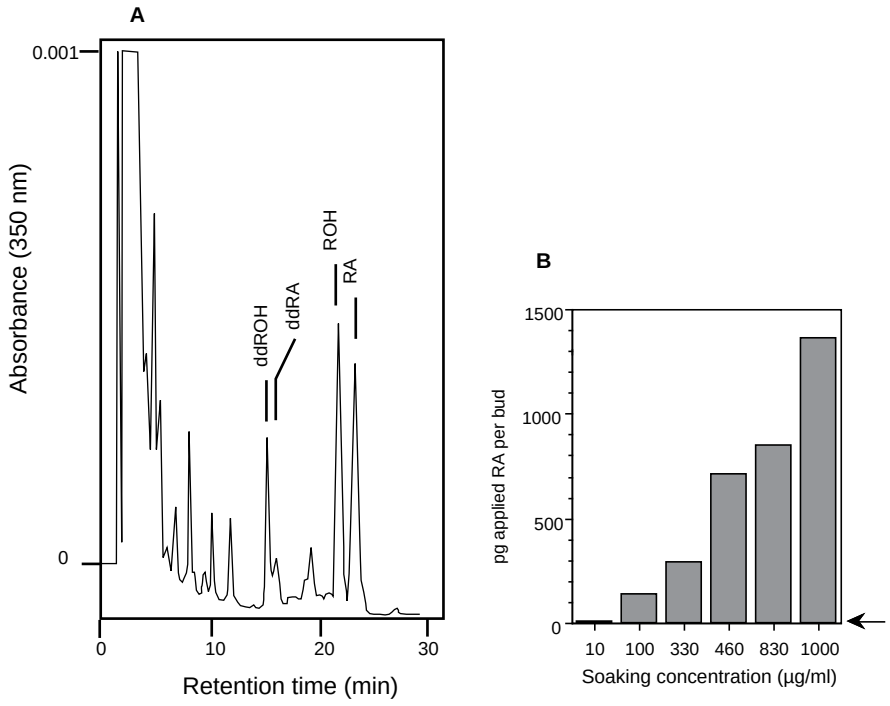

Fig. 1. Sample chromatogram (A) and amount of RA present in the wing bud as a function of the soaking concentration (B). (A) 20 beads were soaked in 100 μg/ml RA and implanted into stage 20 wing buds. 15 hours later, the treated bud was extracted and the extract analyzed on a reversed-phase C18 column eluted with solvent A at 1 ml/min. Note that the concentration of applied RA is very high, in the same range as that of endogenous retinol (ROH). In untreated buds RA is about 30 times less abundant than ROH (Thaller and Eichele, 1990). The elution position of two other endogenous retinoids, 3,4-didehydro retinol (ddROH) and 3,4-didehydro RA (ddRA) are indicated. (B) Amount of RA present in a wing bud following a 15-hour treatment with different doses of RA. The solid horizontal arrow represents the amount of endogenous RA at stage 22/23. The columns in the histogram correspond to the following amounts: 10 μg/ml, 6.7±1.2 pg; 100 μg/ml, 139±26 pg; 330 μg/ml, 288±38 pg; 460 μg/ml, 715±15 pg; 880 μg/ml, 846 pg; 1 mg/ml, 1362±13 pg.

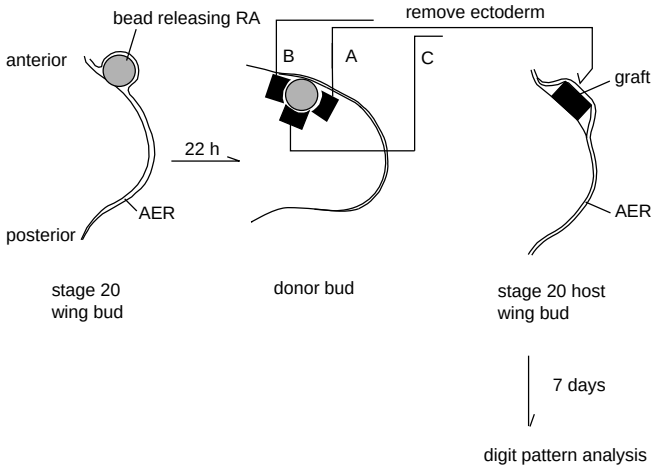

Fig. 2. Diagram of experimental manipulations carried out with wing buds. RA-releasing beads were implanted at the anterior wing bud margin of Hamburger-Hamilton stage 20 chick embryos. Embryos were treated in two different ways. Group 1 embryos were left to develop for 7 days. Group 2 embryos were exposed to an RA-releasing bead for 22-24 hours, then their wing buds were dissected away and incubated in 2% trypsin to remove the ectoderm. Prisms of tissue (A,B,C) surrounding the beads were cut out with a tungsten needle and grafted into a second, stage 20 host wing bud. The host embryo was left to develop for 7 days. AER, apical ectodermal ridge.

and 8 wings were normal (Table 1). At the lowest concentration of RA tested (10 μg/ml and 3 μg/ml), 16 out of 19 wings were normal, one wing had supernumerary digit *2* and two wings had an additional digit *2*-like element (Fig. 4F). The concentration-dependent loss of RA-exposed grafts to induce additional digits in a secondary host is reflected in the distinct decline of the dose response curve (Fig. 3, lower curve).

To determine whether RA induced polarizing activity all around the bead, similarly sized blocks of mesenchyme were taken from equidistant sites around the bead implant (regions B and C in Fig. 2). At a high dose (1 mg/ml) regions B and C were substantially less effective than region A (Table 1). Similarly, tissue taken from sites B and C of wing buds treated with low dose (20 μg/ml RA), were devoid of polarizing activity (Table 1). The high dose data confirm those reported by Tamura et al. (1993) who observed little activity in regions B and C. The finding that polarizing activity is confined to site A makes it less likely that polarizing activity results from a carryover of RA. If this were the case, one would expect that regions A, B and C are equally effective in inducing ZPA (excepting localized RA degradation in regions B and C). Taken together, our experiments show that high doses of RA endow tissue distal to the implanted bead with polarizing activity. However, when the dose of RA is lowered, tissue taken from a treated wing bud exhibits little or no polarizing activity irrespective of the site of origin of the transplant. Importantly, the lack of polarizing activity occurs at doses of RA capable of inducing full duplications when embryos are left to develop (Table 1 and Fig. 3).

Induction of *sonic hedgehog* and *Hoxd-11* by RA treatment

Transplantation of tissue that has acquired polarizing activity will produce mirror-image digit duplications in the host, an effect that is likely to require the coordinated action of many signaling molecules. To begin to examine the temporal and spatial order of molecular signals that constitute polarizing activity, we examined the expression of two genes, *sonic hedgehog* and *Hoxd-11*. *shh* is a candidate for the ZPA morphogen (Riddle et al., 1993), hence its expression should correlate directly with the acquisition of polarizing activity. *Hoxd-11* expression is induced as a result of polarizing activity, and hence represents a gene downstream of the polarizing signal (Ipsizúa-Belmonte et al., 1992). Embryos were treated with pharmacological (330 μg/ml) or physiological (10 μg/ml) doses of RA (see above), incubated for 22 hours, then examined for the expression of *shh* and *Hoxd-11* by means of in situ hybridization. A total of 3 or 4 specimens were examined in each case. Treatment with both 10 μg/ml and 330 μg/ml RA induced *shh* in the tissue immediately distal to the bead (Fig. 5A,B). The expression domains of *Hoxd-11* induced by treatment of embryos with 10 μg/ml and 330 μg/ml RA are

Table 1. Digit patterns resulting from RA treatment or tissue transplantation

Soaking concentration	Pattern if bead is left in place	*n*	Tissue block*	Pattern following tissue transplantation	*n*
1 mg/ml	no hand	2	A	44234	1
	4, 434, 34	4		43234	1
	4334	2		dd3234	1
	43234	2		3334	1
				3234	1
				234	1
			B	3234	1
				2234	1
				d234	1
				234	8
			C	2234	3
				234	10
330 μg/ml	4334	5	A	4334	1
	234	1		43234	4
				2234	2
				234	1
100 μg/ml	43234	3	A	2334	1
	4334	3		2234	2
				234	3
20 μg/ml	4334	3	A	2234	5
	43234	6		234	5
	d3234	1			
			B	2234	1
				234	6
			C	234	6
10 μg/ml	43234	9	A	d234	2
				234	9
6 μg/ml	43234	14			
	d3234	1			
	d2234	4			
	2234	3			
3 μg/ml	d3234	1	A	2234	1
	3234	2		234	7
	2234	4			
	234	1			
ZPA				4334	4
				43234	3

*see Fig. 2.

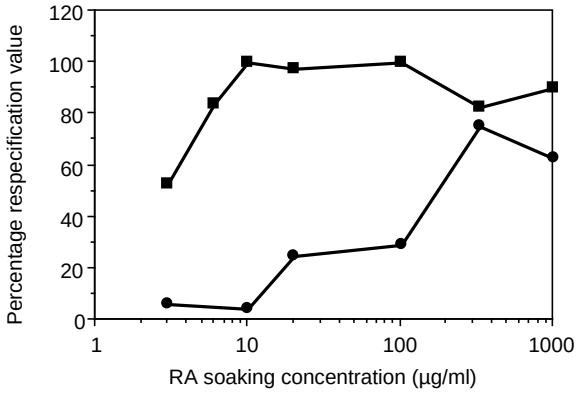

Fig. 3. Dose-response curves for embryos treated either with RA (upper curve) or with tissue taken from region A distal to the RA-releasing bead (lower curve). Concentrations specified on the abscissa were those used for soaking the bead that was implanted into the initial host.

illustrated in Fig. 5C and D. The relative size and intensity of the *shh* expression domains are similar for pharmacological and physiological treatments of RA. Based on these experiments we conclude that physiological and pharmacological doses of RA induce *shh* and *Hoxd-11*. Moreover, as judged by the silver grain density and the size of the expression domains of *shh* and *Hoxd-11* (Fig. 5) the degree of expression of both these genes is similar at pharmacological conditions, albeit only in the latter case can polarizing activity be demonstrated.

DISCUSSION

Local application of RA to the chick wing bud evokes mirror-image duplications of the limb pattern (Tickle et al., 1982, 1985; Summerbell, 1983). Furthermore, chick limb buds contain endogenous RA at a concentration very close to that required for inducing digit duplications (Thaller and Eichele, 1987, 1990), and RA is enriched in the region containing the polarizing activity (Thaller and Eichele, 1987). The mechanism by which pattern duplications are achieved is not well understood (reviewed in Brickell and Tickle, 1989; Eichele, 1989; Smith et al., 1989; Summerbell and Maden, 1990; Tabin, 1991; Bryant and Gardiner; 1992; Hofmann and Eichele, 1994). One possibility is that RA acts as a graded morphogen by specifying the anteroposterior limb pattern through induction of downstream genes that are directly

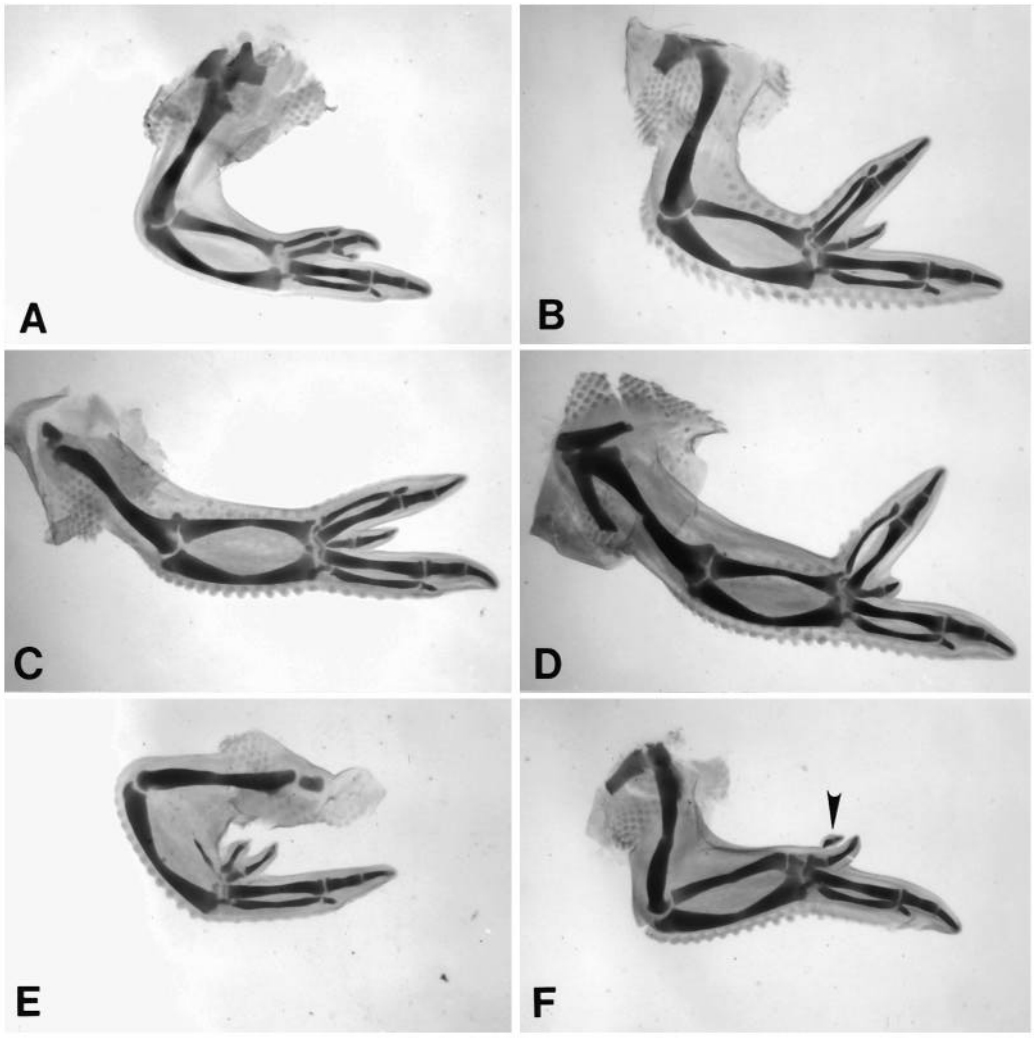

Fig. 4. Pattern duplications obtained after local application of RA (A,B), transplantation of tissue taken from a region next to the RA bead (C,E,F), or ZPA implantation (D). In A, the bead was soaked in 1 mg/ml RA and a *43234* pattern developed. Note, digit *2* is attached to the additional digit *3*. (B) A specimen with a *43234* pattern that resulted from application of 10 μg/ml RA. (C) A *43234* pattern that resulted from a graft of region A (see Fig. 2) taken from a bud exposed to 1 mg/ml for 22 hours. In D a *43234* pattern resulting from a ZPA graft is shown. (E) *2234* pattern that resulted from a graft of region A taken from a bud treated for 22 hours with a bead soaked in 100 μg/ml. Note the partial loss of the radius in this specimen. (F) A *234* pattern with a small anterior digit *2*-like element (arrow) resulted from a graft from region A taken from a bud treated for 22 hours with a bead soaked in 10 μg/ml.

involved in generating the pattern. Alternatively, RA may induce a new polarizing region in the tissue distal to the RA source. This ectopic polarizing region might either operate through an intercalation mechanism (Bryant and Gardiner, 1992) or it might synthesize and release the morphogen proper (Tabin, 1991).

The polarizing region is defined as signaling tissue capable of inducing pattern duplications when grafted into a host wing bud. By this operational definition, pharmacological doses of RA (330 μg/ml and above) induce polarizing activity in the tissue adjacent to the bead. This finding is consistent with earlier reports showing that beads soaked in 1 mg/ml RA induce ZPA (Wanek et al., 1991; Tamura et al., 1993). In the present study we have examined the dose-dependent acquis-

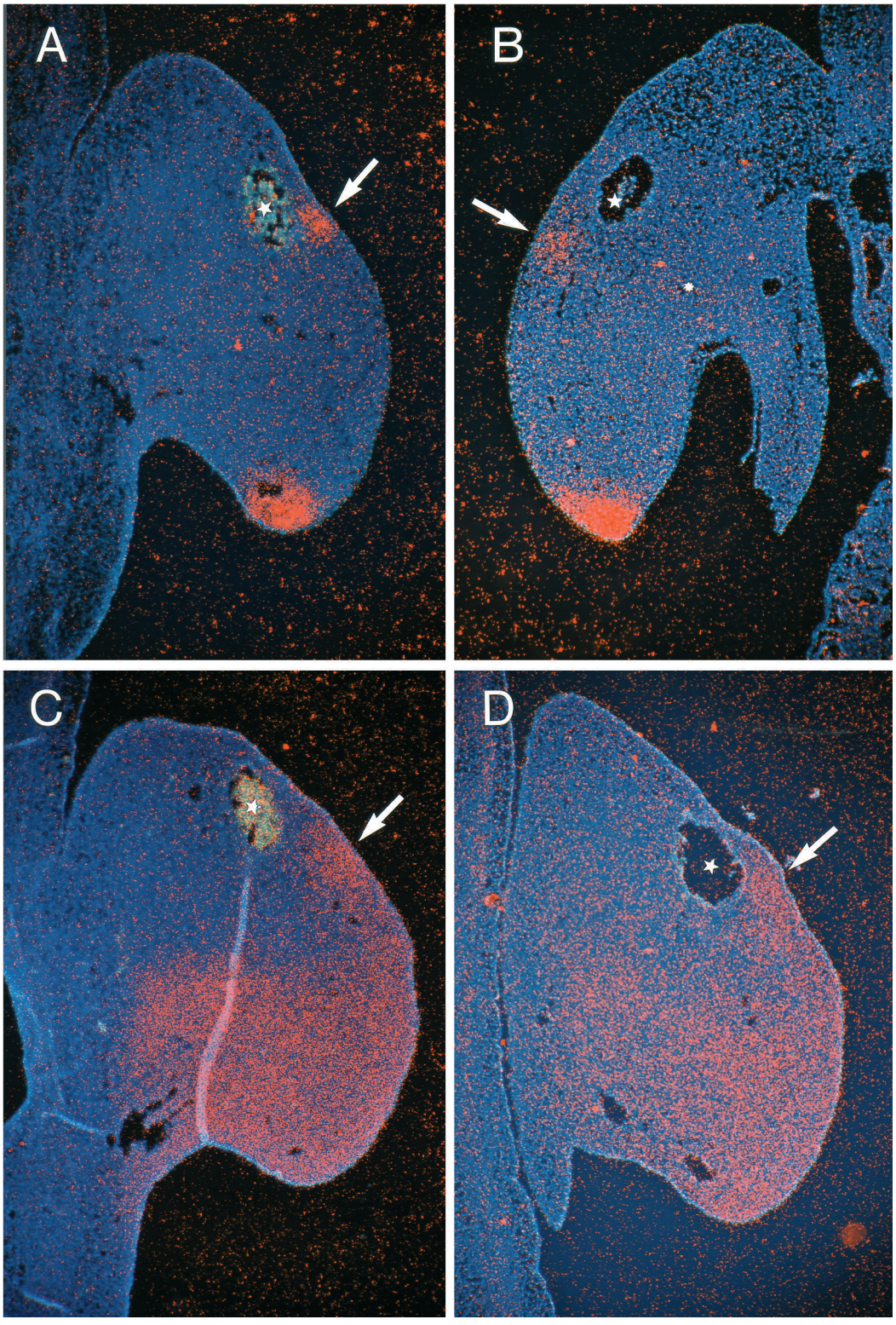

Fig. 5. Expression of *sonic hedgehog* and *Hoxd-11* in the chick embryo. Arrows indicate ectopic sites of expression and asterisks mark the site of bead implantation at the anterior wing margin. 7 μm sagittal sections were made from stage 23 embryos that received an RA bead at stage 20 and were treated with RA for 22-24 hours. Photographs show endogenous *shh* expression coinciding with the polarizing region, and induced expression of *shh* as a result of RA treatment. (A) *shh* expression induced by a bead soaked in 10 μg/ml RA. (B) Expression of *shh* induced by a bead soaked in 330 μg/ml RA. Note that the endogenous levels of *shh* mRNA are approximately equal in A and B, as are the induced levels, even though the amount of RA delivered to the tissues differed 33-fold. Levels of *Hoxd-11* mRNA induced by RA treatment with 10 μg/ml (C) are similar to that induced by 330 μg/ml RA (D).

tion of polarizing activity and we have directly measured the concentration of exogenous RA present in treated buds. Our data show that physiological doses of RA (approx. 10 μg/ml) evoke duplications but do not induce polarizing activity as determined by the functional assay of tissue transplantation (Table 1, Fig. 3). The concentration of endogenous RA at stage 22/23 (the stage attained by the embryo during the treatment) is about 6-7 pg per bud (calculated from Thaller and Eichele, 1990). The concentration of RA in the limb bud following treatment with beads soaked in 10 μg/ml is also approximately equal to 7 pg. In contrast, soaking concentrations of 330 or 1000 μg/ml, both effective in inducing polarizing activity, produce an RA concentration in the bud which is 45 to 210 times higher than the endogenous level.

Could the pattern duplications observed with grafts exposed to high concentrations of RA be due to carryover of RA with the transplant? This is unlikely for two reasons (see also Tickle, 1991; Wanek et al., 1991; Tamura et al., 1993). First, tissue from RA-treated limb buds fail to induce duplications when grafted 12 hours after bead implantation but do so when transplanted at 18 or 24 hours. However, the concentration of applied RA in the bud at 12 hours is higher than at the later time point (Eichele et al., 1985). Second, transplants taken from a site distal to the bead (Fig. 2A) induce duplications, yet tissues taken from equidistant sites proximal or posterior to the bead (Fig. 2B,C) are ineffective. Being equidistant from the source, and assuming no sitedependent difference in RA catabolism, all grafts should contain comparable amounts of RA and thus should be equipotent, yet they are not.

There are several explanations for the absence of polarizing activity when tissue has been exposed to physiological concentrations of RA. First, it is possible that a different signaling pathway is activiated by high doses of RA. However, two markers of polarizing activity, *shh* and *Hoxd-11*, were both expressed in response to either dosage. This does not exclude the possibility that unknown molecules are activated at different concentrations of RA. A second explanation is that the same sets of genes may be activated by high and low doses, but in the case of pharmacological doses, the level of the ZPA morphogen is above some minimum threshold required for polarizing activity. Although we are assessing levels of mRNA and not of protein, it is worth noting that a 33-fold increase in RA concentration has no discernable effect on *shh* or *Hoxd-11* expression levels as detected by in situ hybridization.

A third plausible explanation for the lack of digit duplication upon transplantation of *shh*-positive tissue is that the polarizing activity is initially present, but is lost during the course of grafting. This is unlikely because a high RA dose or a ZPA graft treated in the identical manner induces duplication (Table 1 and Fig. 4C,D). It should also be emphasized that as few as 100 ZPA cells are capable of evoking a full duplication (Tickle, 1981). Thus even a significant loss of the polarizing signal should not abolish the ability of the graft to induce additional digits. To increase the sensitivity of the transplantation assay, we grafted tissue underneath the intact apical ectodermal ridge. This procedure is several times more sensitive than the classical method of placing grafts into the limb bud mesenchyme (Tickle, 1981), yet we were unable to detect polarizing activity after physiological treatments.

It could be argued that tissue distal to a RA-releasing bead required a longer incubation period, and transplants taken later than 22 hours would have acquired the ability to induce digit duplication. Two observations argue against this. First, in a series of preliminary studies, we have transplanted grafts of mesenchyme taken 42 hours after bead implantation. Tissue blocks along the entire anterior limb bud margin were assayed, including blocks expressing *shh*. The resulting digit patterns were normal, with the rare exception of an additional digit *2*. Second, Fig. 5 clearly illustrates that *Hoxd-11*, a gene induced by ZPA grafts (Ipsizúa-Belmonte et al., 1992) or by *shh* (Riddle et al., 1993) is expressed by 22 hours, suggesting that the polarizing signal has begun to operate and that the time-point chosen is appropriate.

How do we envisage the mechanism of action of physiological versus pharmacological doses of RA? One hypothesis is that RA initiates a cascade of signaling molecules (*HoxD* gene products, shh, FGFs and presumably other molecules) that, when expressed together, bring about the formation of additional digits. Some of these factors may be expressed in adjacent domains that form boundary regions from which patterning is initiated (Ingham and Arias, 1992). At physiological doses, any given block of grafted tissue does not contain the full complement of signaling factors, and hence fails to provide polarizing activity. At pharmacological doses which are known to transiently reduce limb bud outgrowth (Lee and Tickle, 1985), the expression domains of the various signaling molecules are spatially compressed, and therefore overlap to a greater extent than under physiological conditions. As a result of this compression, a discrete group of cells express the full set of signaling molecules, and when transplanted, would act as polarizing region. This hypothesis has the merit of accomodating apparently different outcomes within a common molecular mechanism.

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