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Reciprocal Changes in *Hox D13* and RAR-β2 Expression in Response to Retinoic Acid in Chick Limb Buds

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The finding that retinoic acid (RA) has major effects on pattern formation in developing chick limbs has led to the conclusion that RA plays a central role in the normal development of the limb. In addition, it has been demonstrated that treatment with exogenous RA leads to changes in expression of homeobox-containing *Hox* complex genes within the developing limb. This has been used to argue that, since RA activates 5' *Hox D* complex genes in the same temporal and spatial sequence as in normal development, *Hox* genes might be regulated by RA during normal limb development. In this study, we further examine the temporal and spatial changes in expression of *Hox D13* and RAR-β2 transcripts in wing buds in response to local application of RA *in vivo*. We confirm reports that RAR-β2 expression is induced early and locally by RA. However, we find that the effect of RA on *Hox D13* gene expression at the distal end of wing buds at stage 25/26 is downregulation of transcript expression. Furthermore, we find that activation of ectopic *Hox D13* expression in response to implantation of RA beads along the anterior margin at stage 20/21 is indirect. Finally, the effects of RA exposure on *Hox D13* expression appear to directly correlate with effects on the pattern of distal skeletal elements. © 1994 Academic Press, Inc.

INTRODUCTION

The homeobox-containing *Hox* complex genes are considered to play a central role in the specification of regional identity along the anterior–posterior (AP) axis of the body during development. Direct evidence for such a role has been obtained in numerous organisms, including *Drosophila* and mice (McGinnis and Krumlauf, 1992; Hunt and Krumlauf, 1992). In vertebrates, there are four separate *Hox* gene clusters, located on different chromosomes. Among the striking features of these genes is the colinearity that exists between a gene's position within a cluster and its temporal and spatial pattern of expression along the AP axis of the embryo. The

Hox genes are activated in a 3' to 5' order, and the anterior borders of their expression domains form an anterior to posterior sequence along the body axis.

In addition to having a role in pattern formation along the main body axis, members of all four *Hox* complexes, primarily those at the 5' ends, are expressed in developing limbs. As in the body axis, there is a colinearity between position of a gene in a complex and its temporal and spatial expression in the limb bud. Genes located at the 3' end of a cluster are expressed earlier and with more proximal boundaries than the 5' genes. Expression patterns of the *Hox A* genes have been interpreted to suggest a role in proximal–distal (PrDi) limb patterning (Yokouchi *et al.*, 1991; Haack and Gruss, 1993). The *Hox D* genes are expressed in a nested series along the PrDi axis of the limb, but primarily toward the posterior side of the limb bud (Dollé *et al.*, 1989a; Izpisúa-Belmonte *et al.*, 1991; Nohno *et al.*, 1991; Yokouchi *et al.*, 1991). Evidence for a functional role of *Hox D* genes in limb pattern formation has also been presented by Morgan and colleagues (1992), who reported that overexpression of *Hox D11* in chick hindlimb buds leads in some cases to a transformation of digit 1 to a digit 2. In addition, Dollé *et al.* (1993) recently demonstrated that targeted disruption of the *Hox D13* gene in mice induces alterations of skeletal elements in both the vertebral column and in limbs.

It is probable that an understanding of pattern formation will involve an understanding of how expression of the *Hox* genes is controlled. One agent that is known to influence *Hox* gene expression is retinoic acid (RA). In a series of studies, Boncinelli and colleagues have shown that in human embryonal carcinoma (EC) cells, RA induces both differentiation and *Hox* gene expression (Mavilio *et al.*, 1988; Boncinelli *et al.*, 1991) Furthermore, they have shown that there is a colinearity between position along the complex and RA responsiveness, with the most 3' genes giving an immediate response at low RA concentrations, intermediate genes responding only at higher doses and after longer exposures to RA, and the most 5' genes either not responding to RA at all or

being inhibited (Stornaiuolo *et al.*, 1990; Simeone *et al.*, 1990, 1991; Mavilio, 1993).

The direct effects of RA are believed to be mediated by retinoic acid receptors (RARs) (see Linney, 1992; Rowe and Brickell, 1993, for review), which activate gene expression by binding to retinoic acid response elements (RAREs) in the regulatory regions of target genes (de The *et al.*, 1990; Hoffmann *et al.*, 1990; Sucov *et al.*, 1990). A number of RA-responsive genes have been identified, including RAR-β2 (de The *et al.*, 1989). In the case of *Hox* gene induction by RA, it is known that at least some of the 3' genes are directly responsive to RA (LaRosa and Gudas, 1988; Simeone *et al.*, 1991; Conlon and Rossant, 1992) and that *Hox A1* and *Hox D4* have RAREs located in their 3' regulatory domains (Langston and Gudas, 1992; Pöpperl and Featherstone, 1993). Other RA-responsive genes within a complex appear to be responding to products from other *Hox* genes (Arcioni *et al.*, 1992; Mavilio, 1993).

RA is well known for its dramatic effects on pattern formation in the developing chick limb. Local application of exogenous RA along the anterior margin of chick wing buds at stages 18 to 21 mimics the effects of grafts of posterior (zone of polarizing activity, ZPA) tissue by inducing supernumerary digits in a dose-dependent manner (Tickle *et al.*, 1982; Summerbell, 1983). Subsequent studies have provided evidence that RA exerts this morphogenetic effect by locally converting anterior limb bud cells into cells with ZPA properties (Wanek *et al.*, 1991; Noji *et al.*, 1991; Hayamizu and Bryant, 1992; Tamura *et al.*, 1993).

It is not yet clear whether RA plays a role in normal limb pattern formation (see Discussion). However, the fact that RA can initiate a cascade of events that leads to the formation of an extra limb means that it can be used as a tool to dissect the process of limb pattern formation. Since it is known that *Hox* genes are involved in pattern formation, a study of changes in their expression patterns in response to RA is expected to lead to insights into how the pattern of the limb is formed. Previous studies have shown that implantation of RA beads at the anterior limb bud margin leads to expansion of the proximal-anterior expression domain of *Hox C6* (Oliver *et al.*, 1990). It has been suggested that the formation of extra shoulder girdle structures is related to the expansion of the *Hox C6* domain in response to RA. Induction of *Hox C6* expression is also observed after RA treatment of teratocarcinoma cells *in vitro* (Simeone *et al.*, 1991).

In other studies, it has been reported that RA beads implanted along the anterior margin of wing buds lead to ectopic induction of 5' *Hox D* genes (Izpisúa-Belmonte *et al.*, 1991; Nohno *et al.*, 1991). The temporal and spatial aspects of ectopic *Hox D* gene expression mimic their expression in the posterior region of normal limbs, with

Hox D13 appearing later and nested within the *Hox D11* domain. Izpisúa-Belmonte *et al.* (1991) reported ectopic *Hox D11* expression 20 hr after RA bead implantation. Both studies reported ectopic domains of *Hox D13* expression after 24 to 48 hr. Correlations between the normal patterns of *Hox D* gene expression and the induced anterior expression after RA treatment support the idea that RA might play a role in the normal development of the limb pattern. However, as discussed above, when *Hox* expression has been examined in other contexts, the 5' genes are not responsive to RA, or are inhibited by it.

To investigate this apparent paradox further, we decided to examine the RA responsiveness of cells that under normal circumstances show strong *Hox D13* expression. By stage 25/26, the domain of *Hox D13* expression is well-defined and large enough for a bead implanted in the posterior-distal tip to be completely surrounded by *Hox D13*-expressing cells (Izpisúa-Belmonte *et al.*, 1991; Nohno *et al.*, 1991). Since we know that RAR-β2 contains a RARE element in its promoter (de The *et al.*, 1990; Hoffmann *et al.*, 1990; Sucov *et al.*, 1990) and has been shown to be activated in limb cells exposed to duplication-inducing doses of RA (Noji *et al.*, 1991), we used RAR-β2 expression to indicate those cells that respond directly to the presence of RA by altering gene expression. We found that where cells upregulated RAR-β2, *Hox D13* was downregulated and *vice versa*. We also found that downregulation of *Hox D13* correlates with loss of digit 4. Finally, in order to place the observed changes in expression patterns into the context of previous studies, we also examined the dose- and interval-dependent nature of the response to RA beads implanted anteriorly in wing buds at stage 20/21.

From this evidence, we conclude that RA downregulates the expression of *Hox D13* in limbs, consistent with earlier findings of Boncinelli and others on the effects of RA on 5' *Hox* genes in teratocarcinoma cells *in vitro*. Furthermore, we propose that activation of 5' *Hox D* genes in response to RA treatment, previously reported as a consequence of RA treatment of anterior cells, occurs as a downstream effect subsequent to the reinitiation of the limb formation cascade and that it does not occur until exogenuous RA has been removed.

MATERIALS AND METHODS

Preparation of Chick Embryos

Fertilized White Leghorn chicken eggs (K&R Enterprises, Westminster, CA) were incubated at 38°C. On Day 4 of incubation, the eggs were prepared by withdrawing some of the albumen and creating a window in the shell overlying the embryo. Embryos were staged according to the criteria of Hamburger and Hamilton (1951).

Implantation of RA-Containing Beads

Ion-exchange beads (Dowex AG1-X2, 250-300 μm diameter, Bio-Rad) were soaked in solutions containing all-trans retinoic acid (Sigma) dissolved in dimethyl sulfoxide (DMSO) at concentrations ranging from 0.01 to 1.0 mg/ml as described by Wanek *et al.* (1991). Control beads were soaked in DMSO alone. Beads were implanted into chick wing buds at stage 20/21 along the anterior margin immediately subjacent to the AER, or at stage 25/26 posteriorly along the distal margin of the wing bud. Following experimental manipulation, eggs were resealed and incubated at 38°C. At various time points following bead implantation, embryos were fixed and processed for whole-mount *in situ* hybridization analysis or skeletal analysis.

Preparation of Digoxigenin-Labeled Riboprobes

Antisense RNA probes specific for chick *Hox D13* were transcribed as previously described (Hayamizu *et al.*, 1994) from cloned DNA provided by D. Duboule (Izpisúa-Belmonte *et al.*, 1991). RAR-β2-specific probes were transcribed from DNA provided by B. Blumberg and K. Umesono.

Whole-Mount in Situ Hybridization

Detection of RAR-β2 and *Hox D13* transcripts in whole-mount preparations of developing chick limbs was performed as previously described (Hayamizu *et al.*, 1994). Following the alkaline phosphatase-mediated color reaction, limbs were analyzed either dehydrated in methanol or after clearing in methyl salicylate. Tissue samples were photographed under either incident or transmitted light using a Wild M8 Stereomicroscope with a Wild Photoautomat MPS50 and Kodak Ectachrome 160 Tungsten film.

Skeletal Analysis

On Day 11 of incubation, wings were dissected out and fixed in alcoholic Bouin's solution. Left wings were used as controls for assessing the effects on the treated right wings. The pattern of cartilage structures in the limbs were determined in whole-mount preparations of limbs stained with Victoria blue (Bryant and Iten, 1974) or Alcian blue (Wanek *et al.*, 1989) and cleared in methyl salicylate. Tissue analysis and photography were performed as described above.

RESULTS

Distal RA Beads at Stage 25/26: RAR-β2 Expression

In this study, *in situ* hybridization techniques were used to analyze RAR-β2 and *Hox D13* transcript expression in whole-mount preparations of developing chick wing buds. In normal limb buds at stages 25/26, RAR-β2 expression is restricted to the proximal part of the bud (Fig. 1a). Implantation into the distal tip of control beads soaked in solutions of DMSO without RA have no detectable effect on the expression of RAR-β2 transcripts (data not shown). However, in response to implanted RA-containing beads, RAR-β2 expression is locally induced in mesenchymal cells surrounding the bead within 6 hr (Fig. 1b; Table 1).

Furthermore, the extent of RAR-β2 induction is dependent on the RA dose. That is, at 12 hr, beads soaked in higher concentrations of RA (1.0 mg/ml; Fig. 1c) result in more extensive areas of RAR-β2 induction than lower RA doses (0.1 mg/ml; Fig. 1d). In addition, persistence over time of the induced RAR-β2 expression is also RA dose-dependent. With 0.1 mg/ml RA beads, distal RAR-β2 expression is no longer detected at 24 hr in the distal part of the limb bud (Table 1). In contrast, 24 hr after implantation of a 1.0 mg/ml RA bead, RAR-β2 expression is still found near the bead (Fig. 1e).

Distal RA Beads at Stage 25/26: Hox D13 Expression

The normal expression of *Hox D13* at this stage is restricted to a well-defined domain at the distal tip of the wing bud (Fig. 1f; see also Izpisúa-Belmont *et al.*, 1991; Nohno *et al.*, 1991; Yokouchi *et al.*, 1991). Following implantation of control beads (data not shown) and 6 hr after distal RA bead implantation, the *Hox D13* expression domain is unaffected, regardless of the dose of RA (Fig. 1g; Table 1). However, after 12 hr the expression of *Hox D13* transcripts is reduced in the region surrounding the implanted bead (Figs. 1h and 1i).

Downregulation of *Hox D13* expression appears to be correlated with the upregulation of RAR-β2 (compare Figs. 1a through 1j; Table 1). As seen with RAR-β2, the extent of the RA effect on *Hox D13* expression is dose-dependent (see Table 1). After implantation of a high-dose (1.0 mg/ml) RA bead, expression of *Hox D13* in the entire distal tip of the wing bud is greatly reduced at 12 hr (Fig. 1h). With lower RA doses, the area of *Hox D13* expression affected by RA is progressively reduced (Fig. 1i).

Furthermore, persistence of the RA effect on *Hox D13* expression is also dose-dependent. At 24 hr after implantation of a 1.0-mg/ml RA bead, *Hox D13* expression near the bead remains reduced (Fig. 1j). This is reciprocal to the effect of RA on RAR-β2 which continues to be expressed at the same dose and time point (Fig. 1e). Neither *Hox D13* nor RAR-β2 expression are affected at 24 hr with a 0.1-mg/ml bead. At even lower doses (0.01 mg/ml), *Hox D13* is no longer affected at 16 hr (Table 1).

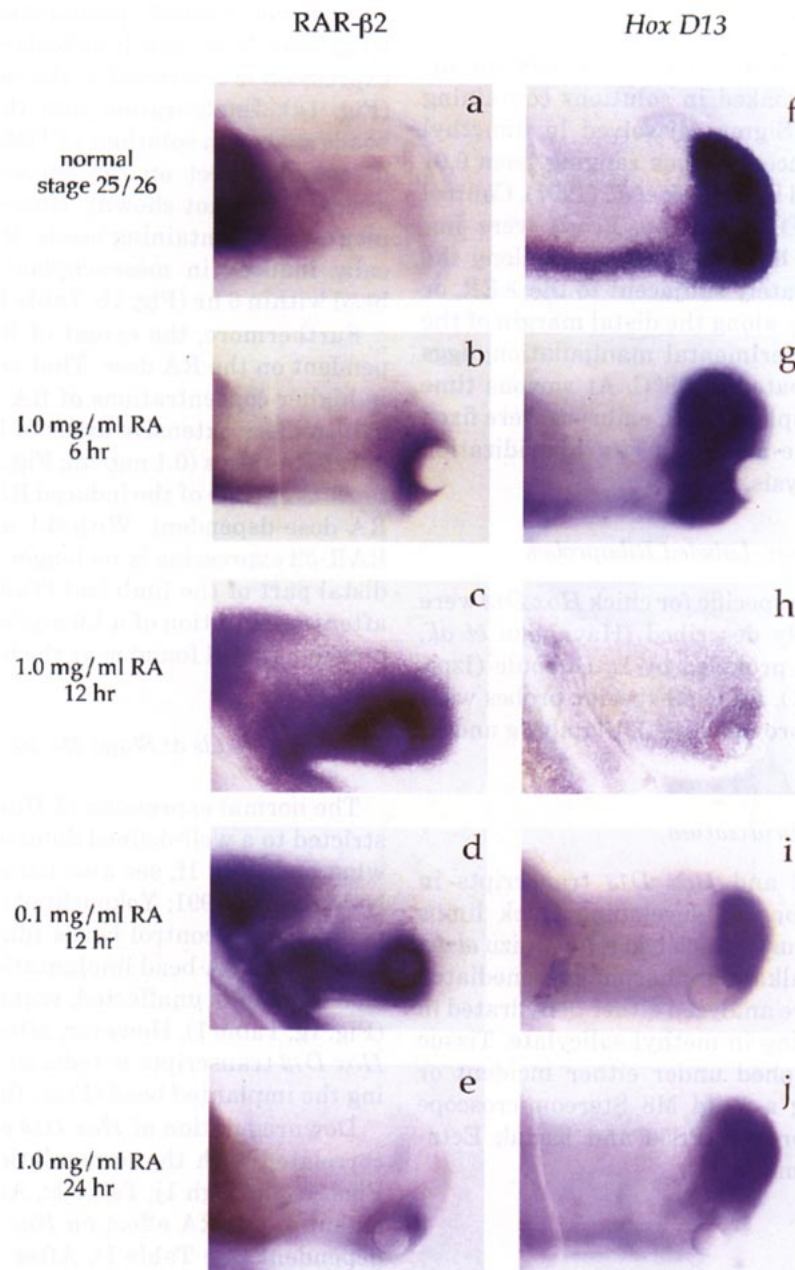Finally, the effect of exogenous RA on *Hox D13* down-

RAR-β2   Hox D13

FIG. 1. Whole-mount *in situ* hybridization analysis of RAR-β2 and *Hox D13* gene expression in response to RA-containing beads implanted distally at stage 25/26. (a–e): RAR-β2 and (f–j): *Hox D13* expression in normal wing buds (a, f), 6 hr following distal implantation of 1.0 mg/ml RA beads (b, g), 12 hr after 1.0 mg/ml (c, h) and 0.1 mg/ml (d, i) RA bead implants, and 24 hr after implantation of 1.0 mg/ml RA beads (e, j).

regulation does not appear to be mediated by the AER, since cells expressing *Hox D13* transcripts persist between the AER and the region next to the bead in which *Hox D13* expression is downregulated (data not shown).

Distal RA Beads at Stage 25/26: Skeletal Pattern

To evaluate the effect of distal RA treatment on limb skeletal pattern, embryos from each experimental group were allowed incubate further until Day 11. Limbs were fixed and processed for skeletal analysis. Representative stained and cleared preparations are shown in Figs. 2a and 2b.

As shown in Table 2, the effect of exogenous RA on distal skeletal elements ranged from no effect with lower-dose RA beads (digit pattern 234; Fig. 2a) to absence of digit 4 (digit pattern 23; Fig. 2b) seen with higher doses of RA.
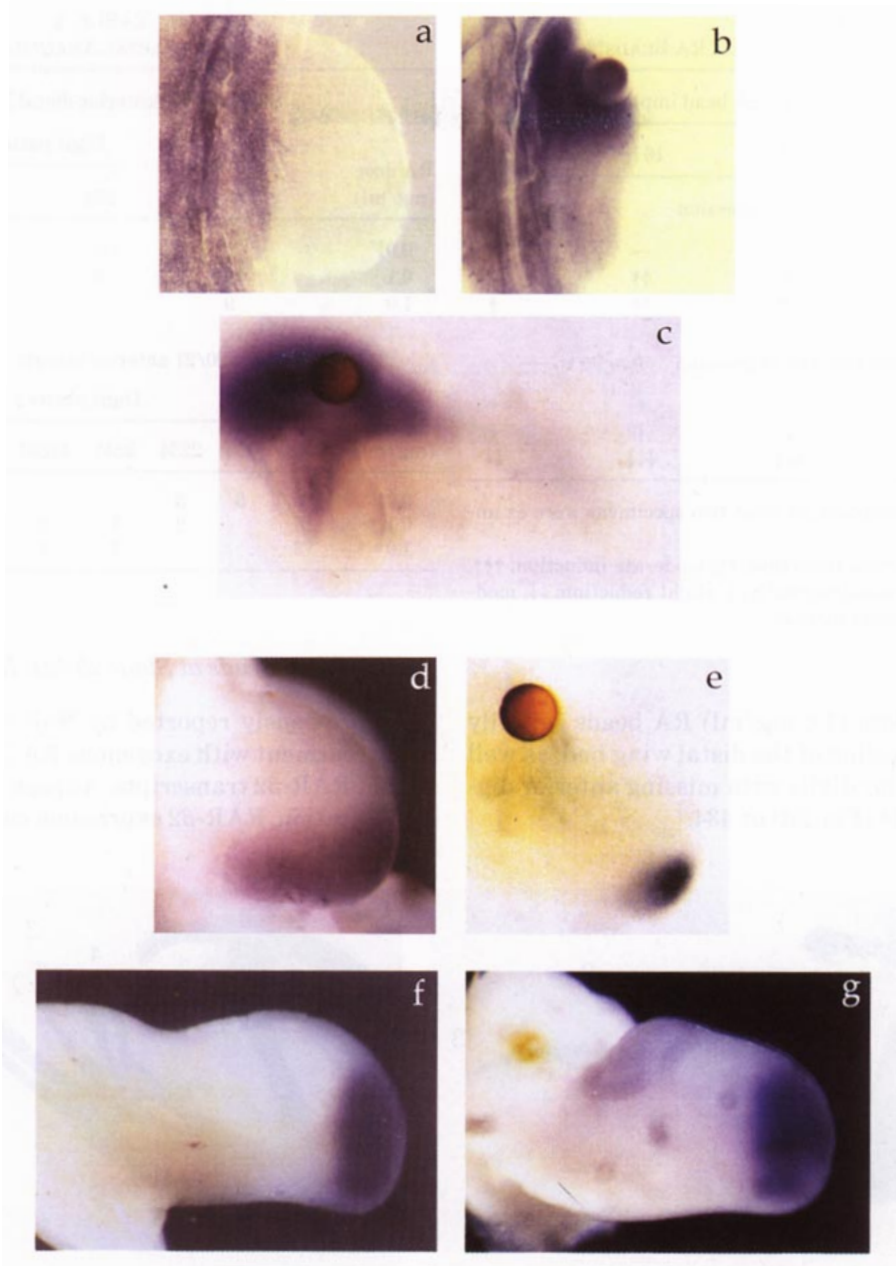
FIG. 3. Expression of RAR-β2 and *Hox D13* transcripts in normal wing buds and in response to RA beads implanted anteriorly at stage 20/21. (a–c): RAR-β2 expression in untreated wing bud at stage 21/22 (a), 6 hr after a 0.1 mg/ml RA bead implant (b), and 48 hr following implantation of a 1.0 mg/ml RA bead (limb bud now at stage 25) (c). (d–g): *Hox D13* expression in untreated wing buds at stage 23/24 (d) and stage 25 (f), 24 hr after a 1.0 mg/ml RA bead (limb bud now at stage 23/24) (e), and 48 hr following implantation of a 0.05 mg/ml RA bead (limb bud now at stage 25) (g).

Anterior RA Beads at Stage 20/21: Skeletal Pattern

In order to evaluate these results in the context of published data, we also examined the dose-dependent nature of the response to RA beads implanted anteriorly in wing buds at stage 20/21. As has been reported previously, RA beads implanted anteriorly at stage 20/21 induce extra skeletal elements. In the experiments re-ported here, the extent of the RA effect is dose-dependent (Table 2) within a range of RA concentrations comparable to those used in other studies.

Specifically, low-dose (0.01 mg/ml) RA beads result in either normal wings (digit pattern 234) or wings with an extra digit 2 (digit patter 2234). Implantation of beads soaked in 0.1 mg/ml RA result in a widening of the distal bud and extra digits; digit pattern 2234, 3234, 43234 (Fig.

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TABLE 1

STAGE 25/26 POSTERIOR DISTAL RA BEADS[a,b]

RA dose (mg/ml)	Time after RA bead implantation			
	6 hr	12 hr	16 hr	24 hr
Distal RAR-$\beta2$ expression				
0.01	—/↑	—	—	—
0.1	↑	↑	↑↑	—
1.0	↑	↑↑↑	↑↑	↑
Distal Hox D13 expression				
0.01	+	+/↓	+	+
0.1	+	↓	↓	+
1.0	+	↓↓↓	↓↓↓	↓↓

[a] At every dose and time point, at least two specimens were examined.

[b] —, Not expressed; ↑, weak induction; ↑↑, moderate induction; ↑↑↑, strong induction; +, expressed normally; ↓, slight reduction; ↓↓, moderate reduction; ↓↓↓, strong reduction.

TABLE 2

SKELETAL ANALYSIS

Stage 25/26 posterior distal RA beads

RA dose (mg/ml)		Digit pattern		
	Total	234	23	% Affected
0.01	8	8		0
0.1	10	3	7	70
1.0	9		9	100

Stage 20/21 anterior margin RA beads

RA dose (mg/ml)	Total	Digit pattern						% Affected
		234	2234	3234	43234	4334	434	
0.01	6	3	3					50
0.1	16		2	5	3	6		100
1.0	13			2	1	8	2	100

2c), or 4334. High-dose (1.0 mg/ml) RA beads initially lead to a slight narrowing of the distal wing bud, as well as duplicated posterior digits with missing anterior digits; digit pattern 4334 (Fig. 2d) or 434.

Anterior RA Beads at Stage 20/21: RAR-$\beta2$ Expression

As previously reported by Noji et al. (1991), we find that treatment with exogenous RA induces local expression of RAR-$\beta2$ transcripts. As seen with distal RA bead implantation, RAR-$\beta2$ expression can be detected in me-
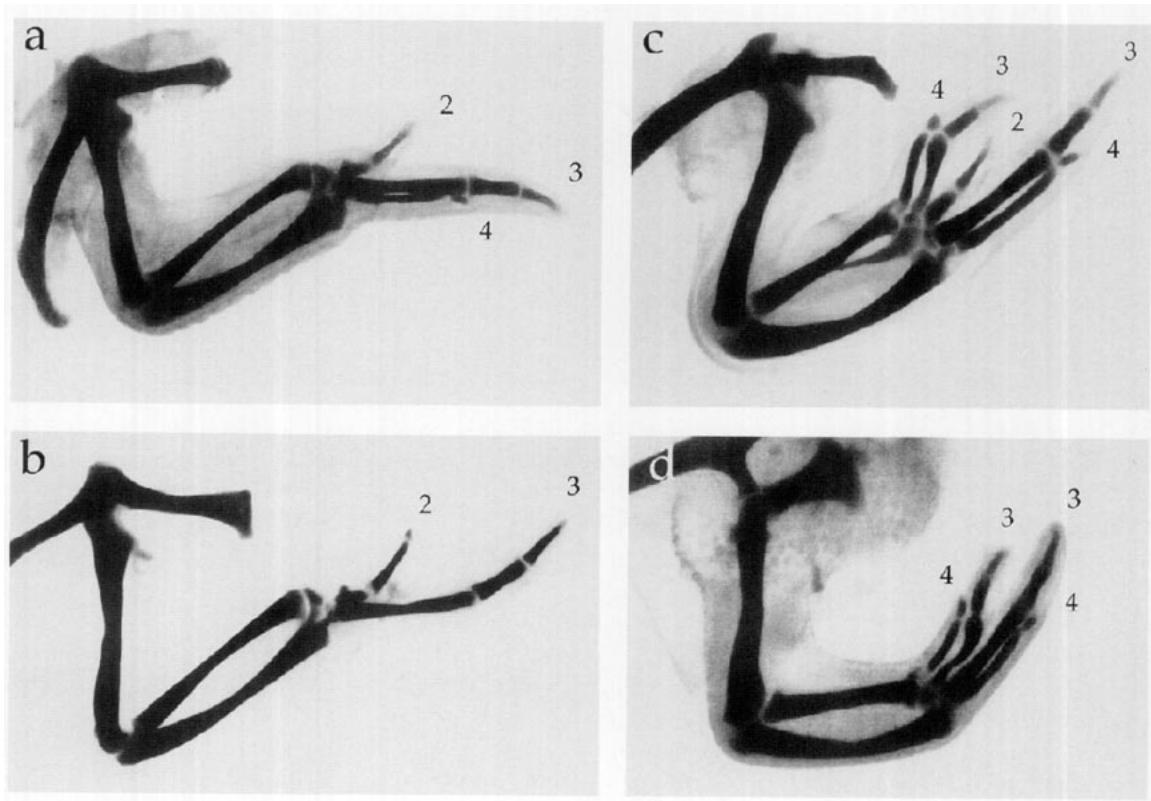

FIG. 2. Victoria blue-stained whole-mount skeletal preparations of wing buds following implantation of RA beads. (a) Normal wing bud; pattern of digits is 234. (b) Wing from implantation of a 1.0-mg/ml RA bead distally at stage 25/26; digit pattern is 23. (c, d) Wings resulting from implantation of 0.1 mg/ml (c) and 1.0 mg/ml (d) RA beads along the anterior margin at stage 20/21. Digit patterns 43234 (c) and 4334 (d).

TABLE 3
STAGE 20/21 ANTERIOR MARGIN RA BEADS[a,b]

RA dose (mg/ml)	Time after RA bead implantation			
	24 hr	36 hr	48 hr	60 hr
Anterior Hox D13 expression				
0.01	—	—	—	
0.05	—	—	—/Ectopic	—[c]
0.1	—	—	—/Ectopic	Ectopic
1.0	—	—/Ectopic	Ectopic	Ectopic
Posterior Hox D13 expression				
0.01	+	+	+	
0.05	+	+	+	+[c]
0.1	Reduced	+/Reduced	+	+
1.0	Reduced	+/Reduced	Reduced	+

[a] Except as noted below, at every dose and time point, at least two specimens were examined.

[b] —, Not expressed; +, expressed normally.

[c] Only one case was examined at this dose and time.

sodermal cells near the RA-containing bead within 6 hr (Fig. 3b). The extent and persistence of induced RAR-β2 expression is correlated with RA dose. That is, higher doses (1.0 and 0.1 mg/ml) of RA result in more dramatic responses than lower dose (0.01 mg/ml) RA beads (data not shown). Furthermore, following implantation of 1.0 mg/ml RA beads, RAR-β2 transcripts are still apparent at 36 and 48 hr (Fig. 3c). In contrast, RAR-β2 expression is not detectable 12 hr after 0.1 mg/ml RA bead implants (data not shown). In all cases, the induced RAR-β2 expression is restricted to the proximal part of the limb bud. No RAR-β2 expression is detected distally.

Anterior RA Beads at Stage 20/21: Hox D13 Expression

As reported in other studies, we also find that RA beads implanted anteriorly at doses which lead to digit pattern duplication also result in ectopic anterior domains of *Hox D13* expression. In the present study, anterior *Hox D13* expression is seen after implantation of anterior RA beads at stage 20/21 (Fig. 3g; compare with Fig. 3f). Also as reported, the ectopic domains do not appear next to the bead, but rather distally along the anterior margin of the limb bud, in mesodermal cells adjacent to the AER.

The appearance of ectopic anterior *Hox D13* expression is RA dose- and exposure time-dependent. With very-low-dose (0.01 mg/ml) RA beads, anterior expression of *Hox D13* is not detected at any time point (n = 6). However, 5 of 12 limbs implanted with RA beads at doses from 0.05 to 1.0 mg/ml show distinct anterior *Hox D13* domains at 36 and 48 hr (Table 3). At 60 hr, ectopic

Hox D13 expression was seen in all cases with RA bead implants at doses of 0.1 and 1.0 mg/ml (n = 3).

Izpisúa-Belmont et al. (1991) reported the appearance of an anterior *Hox D13* expression domain in some cases at 24 hr after implanting 0.1 mg/ml RA beads. In other cases, they observed ectopic *Hox D13* expression at 48 and 40 hr in cases where 0.1 and 1.0 mg/ml RA beads, respectively, were removed after 24 hr of treatment. In this study, subsequent to implantation of RA beads at doses ranging from 0.01 to 1.0 mg/ml, ectopic *Hox D13* expression is not detected at 24 hr in any case (n = 8). Anterior *Hox D13* expression is first seen at 36 hr with a 1.0-mg/ml RA bead (Table 3). At 48 and 60 hr, 7 of 10 cases in which 0.05- to 1.0-mg/ml RA beads were implanted along the anterior margin at stage 20/21 show ectopic expression of *Hox D13* transcripts.

High-dose (1.0 mg/ml and, in some cases, 0.1 mg/ml) RA beads give rise to duplicated wings with missing anterior digits. These limbs also exhibit a narrowing of the distal wing bud at 24 to 48 hr after bead implantation and, as shown in Table 3, a concommitant reduction in the extent of normal *Hox D13* expression domain (Fig. 3e) in comparison with the normal posterior-distal *Hox D13* expression domain (Fig. 3d). The persistence of this effect also appears to be RA dose-dependent; distal-posterior *Hox D13* expression returns to normal at 48 hr after implantation of 0.1 mg/ml RA beads but remains reduced with 1.0 mg/ml RA beads until 60 hr.

DISCUSSION

We have compared temporal and spatial changes in the expression of *Hox D13* and RAR-β2 transcripts in distal as well as anterior limb bud cells after local exposure to exogenous RA. We find that both anterior and distal cells, neither of which normally express RAR-β2, respond to exogenous RA by upregulation of RAR-β2 expression. This finding is similar to that reported previously for anterior limb bud cells by Noji et al. (1991). RAR-β2 gene induction is an expected consequence of exogenous RA treatment because the RAR-β2 gene contains a RARE in its promoter (de The et al., 1990; Hoffmann et al., 1990; Sucov et al., 1990). Activation of RAR-β2 expression has similar characteristics in both anterior cells of wing buds at stage 20/21 and distal cells of stage 25/26 limb buds. Under either set of conditions, RAR-β2 expression is induced within 6 hr of implantation of the RA-containing bead. The spatial extent of the RA-mediated effect, as well as the persistence of RAR-β2 expression with time, are dose-dependent.

In contrast to the effects of RA treatment of RAR-β2 expression, we find that expression of *Hox D13* in distal limb bud cells is downregulated in response to RA. In the distal portion of stage 25/26 wing buds, downregulation of *Hox D13* expression is first detected at 12 hr fol-

lowing the onset of RA exposure, and persistence of this effect as well as the extent of the affected area are dose-dependent. The effect of RA is seen in cells surrounding the bead, which also respond to the RA treatment by upregulating RAR-β2. In fact, RA effects on expression of RAR-β2 and Hox D13 in distal cells appear to be reciprocal. That is, while RAR-β2 is upregulated in cells next to the bead, Hox D13 is downregulated in this area. When RA levels decline and RAR-β2 can no longer be detected next to the bead, Hox D13 levels are no longer downregulated. The consequence of prolonged downregulation of Hox D13 expression, especially at the higher doses of RA, is the deletion of digit 4.

The results led us to reevaluate the observed effects of local exposure to exogenous RA on Hox D13 transcripts in anterior limb bud cells. In anterior limb bud cells near the RA bead, which normally express neither RAR-β2 nor Hox D13 transcripts, RAR-β2 expression is upregulated, and this local effect persists for up to 48 hr at the higher RA doses. On the other hand, Hox D13 expression is never induced in cells in the immediate vicinity of the bead, and ectopic anterior expression only occurs 1 to 2 days later at some distance from the RA source. An earlier response, at higher RA doses which also lead to a greater area of RAR-β2 response, is a reduction in the normal posterior domain of Hox D13 expression. As seen with RA beads implanted distally at stage 25/26, expression of Hox D13 transcripts appears to be downregulated in cells in which RAR-β2 expression is induced by RA. These findings are consistent with earlier studies in vitro which have shown that RA inhibits the expression of the most 5' located members of the Hox complexes (Stornaiuolo et al., 1990; Simeone et al., 1990, 1991; Mavilio, 1993).

Given these results, how then does RA activate the 5' members of the Hox D complex in the anterior of the chick limb bud? In order to reconcile these findings, it is necessary to consider the role of RA in limb development. First of all, despite the dramatic effects of RA on patterning in the developing limb, the issue of whether RA plays a role in normal limb pattern formation remains unresolved (Tabin, 1991; Bryant and Gardiner, 1992). Information about the distribution and functional activity of endogenous retinoids is relevant to this issue.

Biochemical analyses of tissue extracts indicates the presence of several retinoids, including RA, in the developing Xenopus embryo (Durston et al., 1989), in mouse embryos and limb buds (Satre and Kochhar, 1989), and in chick limb buds (Thaller and Eichele, 1987). Experiments using retinol or retinal as a substrate have shown that the ability to synthesize RA is a property of cells in the chick limb bud (Thaller and Eichele, 1988), in Hensen's node of both chick (Chen et al., 1992) and mouse (Hogan et al., 1992) embryos, in the floor plate of the neural tube (Wagner et al., 1990), and in the developing

mouse retina (McCaffery et al., 1992). In addition, analyses of tissue extracts using an in vitro reporter assay indicate that retinoic acid is enriched in Hensen's node (Chen et al., 1992), in the floorplate (Wagner et al., 1992), and in the retina (McCaffery et al., 1992). Finally, using an antibody that recognizes retinol and retinal as well as RA, Tamura et al. (1990) described the localization of these molecules in developing limb buds. From these studies it is clear that some cells of the embryo, including limb bud cells, have the ability to synthesize RA and other biologically active retinoids and that such retinoids are present at detectable levels at appropriate developmental stages.

A different type of study addresses the question of where in the embryo RA may function to regulate gene expression. Such studies make use of the fact that the RA effects are thought to be mediated by receptors (RARs) which activate gene expression by binding to response elements (RAREs) in regulatory regions of target genes (de The et al., 1990; Hoffmann et al., 1990; Sucov et al., 1990). Transgenic mice containing reporter gene contructs driven by promoters containing RAREs have been studied to determine where endogenous retinoids activate gene expression in normal development. In several such studies (Mendelsohn et al., 1991; Reynolds et al., 1991; Rossant et al., 1991; Balkan et al., 1992), reporter genes were activated in the trunk region and in the most proximal portion of limb buds. Reporter expression was not detected in the head or in the tail, nor was it detected in distal regions of limb buds.

Expression of endogenous RA-responsive genes such as RAR-β2, which contains a RARE in its promoter region, can also be used to deduce the presence of biologically active retinoids in development. In mouse embryos, RAR-β transcripts have been observed in early limb bud mesenchyme, but only in the most proximal regions (Dollé et al., 1989b; 1990, 1991; Ruberte et al., 1991). In the chick, RAR-β2 transcripts are expressed in proximal regions of both wing and leg buds from stages 19 to 27 (Noji et al., 1991; Smith and Eichele, 1991; Schofield et al., 1992, and this study), but not in distal regions, including posterior (ZPA) tissue. Together with the reporter studies, these data show that the distribution of biologically active retinoids may be more restricted than either the ability of cells to synthesize retinoids or the actual presence of such compounds in the embryo would suggest. It is likely that the availability and function of various binding proteins (such as the CRABPs) and receptor molecules modulate the ability of RA to affect gene expression.

Since RAR-β2 is not expressed in the distal tip of limb bud, we conclude that biologically active retinoids are not functionally available in the region of the developing limb where pattern formation is taking place, and therefore that RA is not involved in pattern formation during

normal limb outgrowth. Further support for this idea comes from studies of RA-deficient mice. In embryos in which RA deficiency-related malformations are present elsewhere, RAR-β2 promoter activity in limb buds is abolished, but *Hox D13* expression is not affected and the limbs develop normally (Wood, Ward, and Morriss-Kay, personal communication). In the present study, we have shown that RA has reciprocal effects on RAR-β2 and *Hox D13* expression, suggesting that *Hox D13* is only expressed in places where RA is absent.

In conclusion, we propose that activation of 5′ *Hox D* genes in response to RA treatment, previously reported as a consequence of RA treatment of anterior cells, occurs as a downstream effect subsequent to the reinitiation of the limb formation cascade and that it does not occur until exogenous RA has been removed. The evidence supports the idea that RA converts cells adjacent to the bead to a more proximal and as well as posterior positional identity (Tamura *et al.*, 1993). In the developing chick limb, interactions between anterior limb bud cells and respecified posterior cells results in proliferation of the anterior cells. Subsequent distal outgrowth along the anterior of the limb bud would eventually generate cells with a distal identity that: (a) are no longer exposed to functional levels of RA, evidenced by the lack of expression of RA-responsive genes such as RAR-β2 (b) express 5′ *Hox D* complex gene transcripts, such as *Hox D13*; and (c) go on to form the most distal portion of the limbs, the digits.

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