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## Regeneration of *HoxD* Expression Domains during Pattern Regulation in Chick Wing Buds

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The expression domains of genes located at the 5' end of the *HoxD* (formerly *Hox-4*) complex appear to correlate with pattern along both the proximal-distal (PrDi) and the anterior-posterior (AP) axes of the developing limb bud, and it has been suggested that the *HoxD* gene products are involved in the specification of positional information during limb development. The apical ectodermal ridge is required for limb outgrowth and is thought to influence mesodermal cells at the distal end of the limb bud in a region within which patterning events occur. In this paper, we examine the expression of 5' *HoxD* genes during PrDi pattern regulation in chick wing buds. In limbs undergoing pattern regulation, we demonstrate that the domains of *HoxD11* and *HoxD13* gene expression are "regenerated" within 24 hr of removal of the distal mesenchyme. In contrast, in limbs which will not form distal structures, *HoxD13* expression becomes reduced. © 1994 Academic Press, Inc.

### INTRODUCTION

The expression domains of genes located at the 5' ends of the *HoxA* and *HoxD* gene complexes (formerly *Hox-1* and *Hox-4*, respectively; Scott, 1992, 1993) appear to correlate with regions that will give rise to distinct skeletal elements along the proximal-distal (PrDi) and anterior-posterior (AP) axes of developing limb buds in both the mouse (Dollé *et al.*, 1989) and the chick (Izpisua-Belmonte *et al.*, 1991b; Mackem and Mahon, 1991; Noji *et al.*, 1991; Yokouchi *et al.*, 1991; Morgan *et al.*, 1992). Specifically, the expression domains of the 5' *HoxA* genes appear to identify different PrDi segments of the limb (Yokouchi *et al.*, 1991), while the *HoxD* genes are expressed in domains that correlate with pattern along both PrDi and AP axes (Dollé *et al.*, 1989; Izpisua-

Belmonte *et al.*, 1991b; Mackem and Mahon, 1991; Noji *et al.*, 1991; Yokouchi *et al.*, 1991). Furthermore, correlations between *Hox* gene expression domains and regions of the limb bud that will give rise to specific skeletal structures are apparent prior to any overt sign of differentiation, and they persist into the period in which differentiation of the skeletal elements is occurring. These observations support the idea that the *Hox* gene expression domains represent an early indication of "determination" in terms of positional identity of cells in the limb bud.

Distal outgrowth of the developing limb bud is dependent upon interactions between mesodermal cells and the overlying ectoderm. The apical ectodermal ridge (AER) is a transient ectodermal specialization that is thought to influence a region of mesoderm at the distal end of the limb bud (the progress zone) within which pattern specification occurs (Summerbell *et al.*, 1973; Summerbell, 1974). Studies on developing chick limbs demonstrate that the AER is essential for outgrowth of the chick limb bud (Saunders, 1948), although early limb bud formation occurs without the AER in the *limbless* chick mutant (Carrington and Fallon, 1988) and also in the normal mouse (Wanek *et al.*, 1989). The influence of the AER has been demonstrated by experiments in which the AER is removed at various stages during chick limb development, resulting in limbs that are truncated at different PrDi levels (Saunders, 1948; Summerbell, 1974; Rowe and Fallon, 1982). These results are similar to those observed following amputation of the distal tip of the limb bud (Saunders, 1948). Consequently it appears that, in the chick, the outgrowth-promoting ability of the limb ectoderm has become restricted to the AER and that the AER is not reformed *in vivo* after its removal.

Further evidence for the influence of the AER on limb outgrowth is its ability to induce extra limb pattern. AERs have been shown to induce supernumerary limb

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outgrowth following transplantation to different regions of the chick limb bud (Zwilling, 1956; Saunders *et al.*, 1976; Carrington and Fallon, 1986). In the *eutrophia* mutant, formation of ectopic AERs correlates with formation of extra structures (Goetinck, 1964). When isolated leg bud mesoderm is grafted subjacent to the AER of a host wing bud in which the distal mesoderm has been excised (Gasseling and Saunders, 1961), or when proximal leg bud tissue is grafted under an AER (Saunders *et al.*, 1957, 1959), distal leg structures develop. AERs can also induce limb outgrowth following transplantation onto the proximal stumps of amputated limb buds (Zwilling, 1956; Rubin and Saunders, 1972). Finally, previous reports have provided limited documentation that chick limb buds can reform and develop normally following removal of a portion of the mesodermal component of the limb bud, provided that the AER is left intact (Hansborough, 1954; Saunders *et al.*, 1957; Hampé, 1959, 1960; Barasa, 1964).

In this study, we have used nonradioactive *in situ* hybridization methods on whole-mount preparations of chick embryos to analyze the expression patterns of *HoxD* genes in wing buds during normal limb development, after amputation and AER removal, and while limbs are undergoing PrDi pattern regulation. Pattern regulation following removal of distal mesenchyme ("excavation") was examined in wing buds at stages 20/21 and 23/24. Excavated limb buds with an intact AER reform a normal morphology within 48 hr, and all regulate to form normal distal skeletal elements from proximal mesenchyme cells. As controls, wing buds were amputated, or the AER alone was removed, leaving the underlying mesoderm intact. Neither of the two control groups reformed the distal part of the wing bud skeletal pattern. At various time points after experimental intervention at stage 23/24, the patterns of expression of *HoxD11* and *HoxD13* (formerly *Hox-4.6* and *Hox-4.8*) transcripts were examined. We chose to study expression after excavation at this stage because the excavated region is greater than and contains the entire *HoxD13* domain. The distal domains of *HoxD11* and *HoxD13* expression are "regenerated" during PrDi pattern regulation. In contrast, when distal pattern elements are not formed, *HoxD13* expression becomes reduced. Thus, we present evidence that regulation of the distal limb pattern involves reestablishment of the expression of *HoxD13*.

## MATERIALS AND METHODS

### *Preparation of Embryos*

Fertilized White Leghorn chicken eggs (K&R Enterprises, Westminster, CA) were incubated at 38°C. On the fourth day of incubation, the eggs were prepared by

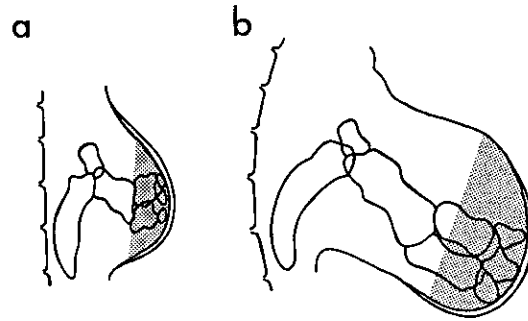


FIG. 1. Diagram showing fate maps of stage 21 (a) and stage 24 (b) wing buds (adapted from Stark and Searls, 1973), shaded to indicate the regions removed in the excavations and amputations. (In all figures, the anterior sides of the limb buds or limbs are oriented toward top of figure.)

withdrawing some of the albumen and creating a window in the shell overlying the embryo. Chick embryos were staged according to the criteria of Hamburger and Hamilton (1951).

### *Experimental Manipulations*

Prior to operations, dorsal surfaces of limb buds were lightly stained with 1% Nile blue sulfate. Electrolytically sharpened tungsten needles were used to make incisions along the distal rim of wing buds at stages 20/21 and 23/24 at the base of the AER. Distal mesoderm was then removed by making an AP incision through the limb bud without severing the isolated AER (Figs. 1 and 2a). The plane of excision mapped to the prospective distal humerus for stage 20/21 and to the prospective radius and ulna for stage 23/24 wing buds (Stark and Searls, 1973).

In order to demonstrate that the results we obtained were due to regeneration of the limb from proximal limb bud cells, rather than from residual distal cells that remain attached to the AER, an additional set of experiments was performed. Excavated stage 21/22 wing buds were used as graft sites for tissue from stage 24/25 leg buds (Fig. 2e). Axial alignment of the donor tissue was achieved by marking the dorsal surface of the leg tissue with Nile blue sulfate or with carbon particles prior to grafting.

In addition, to control for the results reported for excavations, the distal end of stage 23/24 wing buds was amputated at the same plane of incision as was used for the excavation experiments (see Fig. 1). In a second set of controls, the AER alone was removed from limb buds at stage 23/24, leaving the underlying mesoderm intact. Eggs were returned for further incubation at 38°C without rotation. At various time points following experimental intervention, selected embryos were dissected

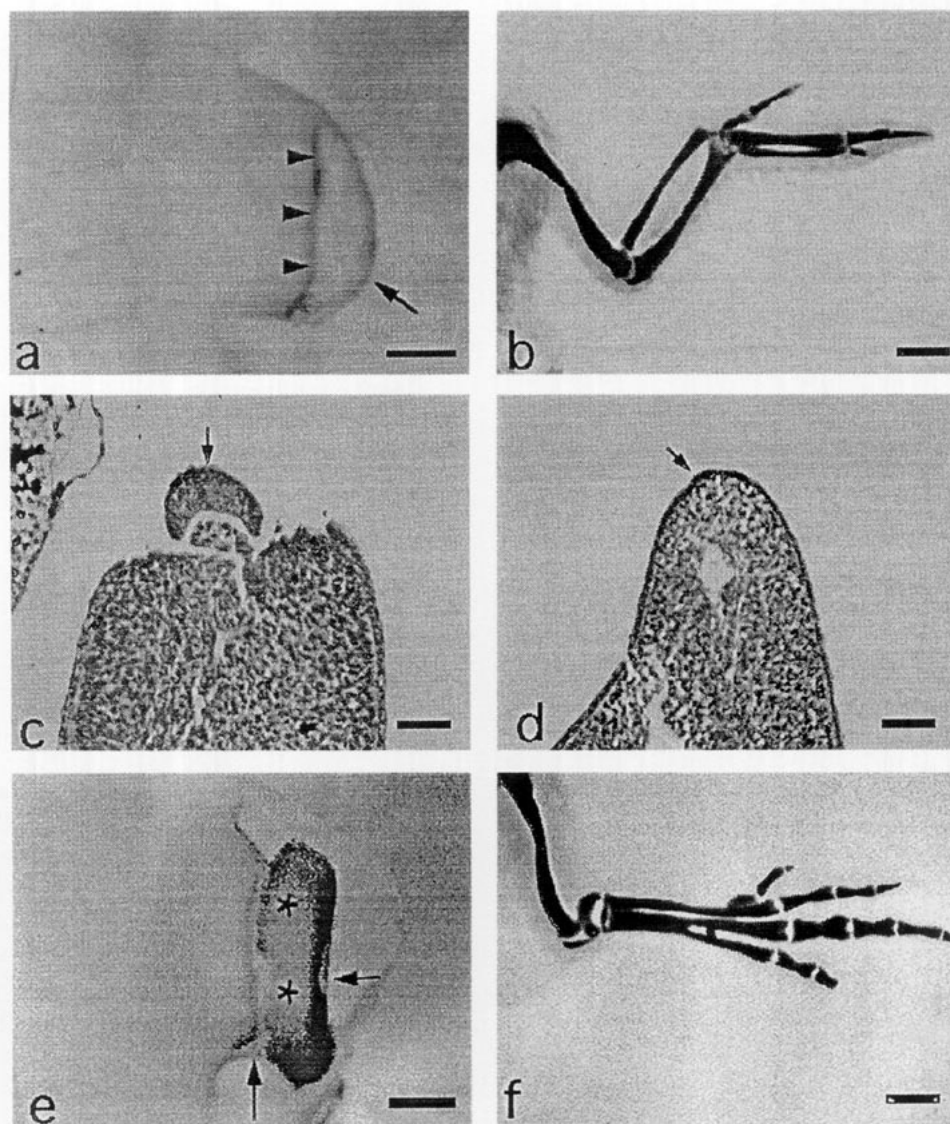


FIG. 2. (a) Wing bud immediately after removal of distal mesenchyme at stage 23/24. Arrowheads mark the cut edge of the proximal stump. Bar, 250  $\mu$ m. (b) Alcian blue-stained whole-mount skeletal preparation of wing fixed 6 days after excavation. PrDi pattern regulation is complete and a normal limb has formed. Bar, 1 mm. (c) Histological section of wing bud fixed 7 hr after excavation shows retraction of the AER onto the remaining mesenchyme. Bar, 40  $\mu$ m. (d) Histological section of wing bud after 48 hr. Wing bud appears normal in morphology and has developed a DV flattening. Bar, 40  $\mu$ m. (e) Proximal tissue from stage 24/25 leg buds (indicated by asterisks) grafted into the excavated distal site of a stage 21/22 wing bud. Bar, 250  $\mu$ m. (f) Skeletal preparation of wing fixed 6 days after grafting proximal leg bud tissue. Bar, 1 mm. In all figures, arrows indicate the AERs. Orientation of limb buds in (a), (b), (e) and (f) is as in the legend to Fig. 1.

out, rinsed in PBS, and fixed for whole-mount *in situ* hybridization as described above.

#### Skeletal Analysis

On Day 11 of incubation, embryos were dissected out and rinsed in normal saline, and torsos with wings were excised. Wings were fixed in alcoholic Bouin's solution for at least 2 days, then rinsed, and stored in 70% eth-

anol. The limbs were then stained with Victoria blue (Bryant and Iten, 1974), dehydrated in ethanol, and cleared in methyl salicylate. Whole-mount preparations of limbs were examined, and the pattern of cartilage structures was determined.

In host wings receiving grafts of leg bud tissue, it was possible to determine whether the resulting limb structures originated from proximal (grafted leg) or distal (host wing) cells, since skeletal elements of the leg are readily distinguishable from wing structures.

### *Preparation of Labeled RNA Probes*

Antisense RNA probes specific for chick *HoxD11* and *HoxD13* genes were transcribed from cloned DNA provided by D. Duboule. These templates were first described by Izpisua-Belmonte *et al.* (1991b) and subsequently used in several published studies on *HoxD* gene expression in developing limbs (Izpisua-Belmonte *et al.*, 1992a, b, c). Linearized DNA templates were used for transcription reactions with digoxigenin-11-UTP and either T3 or T7 RNA polymerase. Labeled RNA was precipitated and then resuspended in hybridization solution (50% formamide, 5× SSC, 1 mg/ml yeast RNA, 100 µg/ml heparin, 1× Denhart's, 0.1% Tween 20, 0.1% Chaps, 5 mM EDTA).

### *Whole-mount in Situ Hybridization*

The protocol for *in situ* hybridizations on whole mounts of chick embryos was adapted from published protocols developed for *Xenopus* embryos (Hemmati-Brivanlou *et al.*, 1990; Harland, 1991; Cho *et al.*, 1991) with minor modifications. Embryos or isolated limb buds were fixed in MEMFA (0.1 M Mops, pH 7.4, 2 mM EGTA, 1 mM MgSO<sub>4</sub>, 3.7% formaldehyde) at room temperature for 1 to 2 hr. Fixed tissues were stored in methanol (MeOH) at -20°C.

Embryos were rehydrated stepwise to PTw (PBS with 0.1% Tween-20). In some cases, tissue was incubated in 10 µg/ml proteinase K at room temperature for 15–30 min. Tissue was washed in 0.1 M triethanolamine (pH 7.8), acetic anhydride was added, and then tissue was washed in PTw, refixed in 4% paraformaldehyde, re-washed in PTw, and prehybridized in hybridization solution without probe at 60°C in a shaking water bath. Tissue was then incubated in hybridization solution containing digoxigenin-labeled RNA probe (1 µg/ml) at 60°C overnight. After hybridization, the tissue was transferred stepwise to 2× SSC/0.3% Chaps and washed at 37°C. Nonhybridized RNA was digested with RNase A (20 µg/ml) and RNase T1 (10 units/ml) at 37°C for 30 min. Tissue was washed in 2× SSC/0.3% Chaps at room temperature, with 0.2% SSC/0.3% Chaps at 60°C, and then transferred stepwise to TNT (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween-20).

Immunohistochemical detection of labeled RNA hybrids was performed using reagents from the Genius 3 kit (Boehringer Mannheim). After blocking (2 mg/ml blocking reagent in TNT with 20% heat-inactivated sheep serum) at 4°C, tissue was incubated with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (1:5000 dilution) at 4°C overnight. Tissue was washed in TNT containing 1 mM levamisole and then in a solution of 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 50 mM MgCl<sub>2</sub>. Nitro blue tetrazolium (NBT) and

5-bromo-4-chloro-3-indoyl phosphate (BCIP) were added. The reaction was allowed to proceed at room temperature for 1 to 6 hr and was then stopped by transferring to MeOH. Tissue was analyzed and photographed either in MeOH or after clearing in methyl salicylate.

## RESULTS

### *Effects of Distal Tip Excavation on Skeletal Pattern*

To test whether chick limb buds can reform in the presence of an intact AER, we removed distal mesenchyme from wing buds at stages 20/21 and 23/24, leaving the AER intact. Immediately following excavation of the distal region of mesenchymal cells, the AER is loosely attached to the remaining wing bud stump (Fig. 2a). Within a few hours, the AER contracts and comes into direct contact with mesenchymal cells of the amputated stump (Fig. 2c).

By 48 hr after excavation, the distal region of the wing bud reforms a dorsal-ventrally (DV) flattened bud and the marginal venous sinus reforms (Fig. 2d). In these experiments, all limbs underwent pattern regulation and all formed normal autopodia when examined after 6 days, regardless of the stage at excavation (Fig. 2b; Table 1). Some limb buds excavated at stage 20/21 (44%) either lacked or had a shortened radius. However, in every case, limb buds excavated at stage 23/24 formed completely normal patterns of skeletal elements.

When excavated stage 21/22 wing buds were used as graft sites for tissue from stage 24/25 leg buds (Fig. 2e), complete limbs formed in every case (Table 1). Without exception, the distal parts of the pattern were derived from leg tissue (Fig. 2f). In no cases were distal wing structures formed. Thus, although a few distal mesenchyme cells may remain attached to the AER following excavation, this result clearly shows that during PrDi pattern regulation, the resulting distal limb elements originate from proximal stump (leg) cells, rather than from any distal wing bud mesenchyme cells that may remain attached to the AER.

### *Effects of AER Removal and Distal Tip Amputation on Skeletal Pattern*

As reported previously, the AER does not reform following amputation of the wing bud. Limb outgrowth ceases and, as shown in Table 1, resulting limbs are truncated as predicted by established fate maps (Stark and Searls, 1973; Bowen *et al.*, 1989). Removal of the AER alone at stage 23/24 results in limbs that are truncated at similar levels (Table 1), as described in previous studies (Saunders, 1948; Hampé, 1959; Rowe and Fallon, 1982). Neither of these two control groups re-forms the distal part of the wing bud skeletal pattern.

TABLE 1  
PROXIMAL-DISTAL PATTERN REGULATION IN CHICK WING BUDS

Stage at operation	Type of operation	N	Truncated limbs	Level of Truncation <sup>a</sup>			Complete limbs
				Stylopod	Zeugopod	Autopod	
20/21	Amputation	10	10	7	3	—	0
	AER removal	7	7	1	6	—	0
	Excavation	9	0	—	—	—	9 <sup>b</sup>
23/24	Amputation	13	13	5	5	3	0
	AER removal	11	11	—	8	3	0
	Excavation	10	0	—	—	—	10
24/25	Leg tissue grafts	8	0	—	—	—	8

<sup>a</sup> Indicates the presence of some or all of the elements.

<sup>b</sup> Four of these limbs were slightly defective.

### *HoxD Gene Expression in Whole Mounts of Normal Limbs*

We first examined the expression patterns of *HoxD11* and *HoxD13* transcripts at various stages during normal limb development using whole-mount preparations of developing chick embryos. Early limb buds at stages 18 and 21 are illustrated in Fig. 3. Later stages are shown as the contralateral (left side) unoperated limbs in Figs. 4 and 5. In general, our results confirm those of previously published *in situ* hybridization studies of *HoxD* gene expression on histological sections of developing chick limbs (Izpisua-Belmonte *et al.*, 1991b; Nohno *et al.*, 1991; Yokouchi *et al.*, 1991).

As limbs develop, expression of both *HoxD11* and *HoxD13* genes is first detected in the posterior mesenchyme of wing and leg buds just prior to stage 18 (Figs. 3a and 3c). At later stages, *HoxD11* transcripts are intensely expressed in the posterior region of the limb buds from base to tip (Figs. 3b and 4a). At stage 23/24, the anterior boundary of *HoxD11* expression approximately bisects the limb bud along its AP axis, except at the very distal tip where the expression domain extends slightly into the anterior of the limb bud (Fig. 4a).

After stage 24, the intensity of *HoxD11* expression decreases. Consequently, the specimen shown in Fig. 4c was slightly "overstained" in order to better demonstrate the extent of *HoxD11* expression. However, the anterior boundary of *HoxD11* expression remains well demarcated, approximately dividing anterior and posterior halves of the limb from base to apex. Also shown in Fig. 4c, *HoxD11* expression at the distal tip of the limb bud at stages 25 to 28 (in the region strongly expressing *HoxD13*; see below) appears to be less intense than *HoxD11* expression more proximally.

At stages 18 to 24, *HoxD13* transcripts are expressed in more posterior and distally restricted domains (Figs.

3c, 3d, and 5a) than *HoxD11*. From stage 24, the proximal-posterior part of the *HoxD13* domain decreases markedly in intensity, while expression in the distal-most region of the limb bud increases in intensity and expands anteriorly (see Figs. 5c, 5e, and 5g). Cellular condensations for digits are first apparent at about stage 27. At this stage, *HoxD13* expression appears to be reduced in precartilaginous condensations and in interdigital spaces (Fig. 5i), but remains strongly expressed in perichondrial areas of digits 3 and 4.

### *Expression of HoxD11 Following Excavation*

Excavation of the distal end of the stage 23/24 wing bud removes the distal part of the *HoxD11* expression domain. Figure 4b shows *HoxD11* expression 6 hr after excavation of distal wing bud mesoderm at stage 23/24. The small region of more intense staining at the distal tip of the limb bud in Fig. 4b corresponds to an area of *HoxD11*-expressing mesenchyme that has not yet become re-covered by ectoderm during wound healing. As the limb bud grows out in the presence of an intact AER, *HoxD11* expression comparable to that seen proximally in the posterior half of the limb bud is observed in the posterior part of the distal tip as it reforms (Fig. 4d, 24 hr after excavation).

### *Reexpression of HoxD13 Following Excavation*

At stage 23/24, excavation of the distal tip of the wing bud results in removal of the distal region of intense *HoxD13* expression (compare Figs. 5a and 5b). *HoxD13* expression in whole mounts was examined in a total of 21 specimens fixed at 0, 4, 6, 16, 24, 36, 48, and 96 hr following excavation. At early time points, the distal expression domain of *HoxD13* is either not detectable or can be seen in only a few cells immediately subjacent to the AER (see Fig. 5b, at 6 hr). At subsequent time

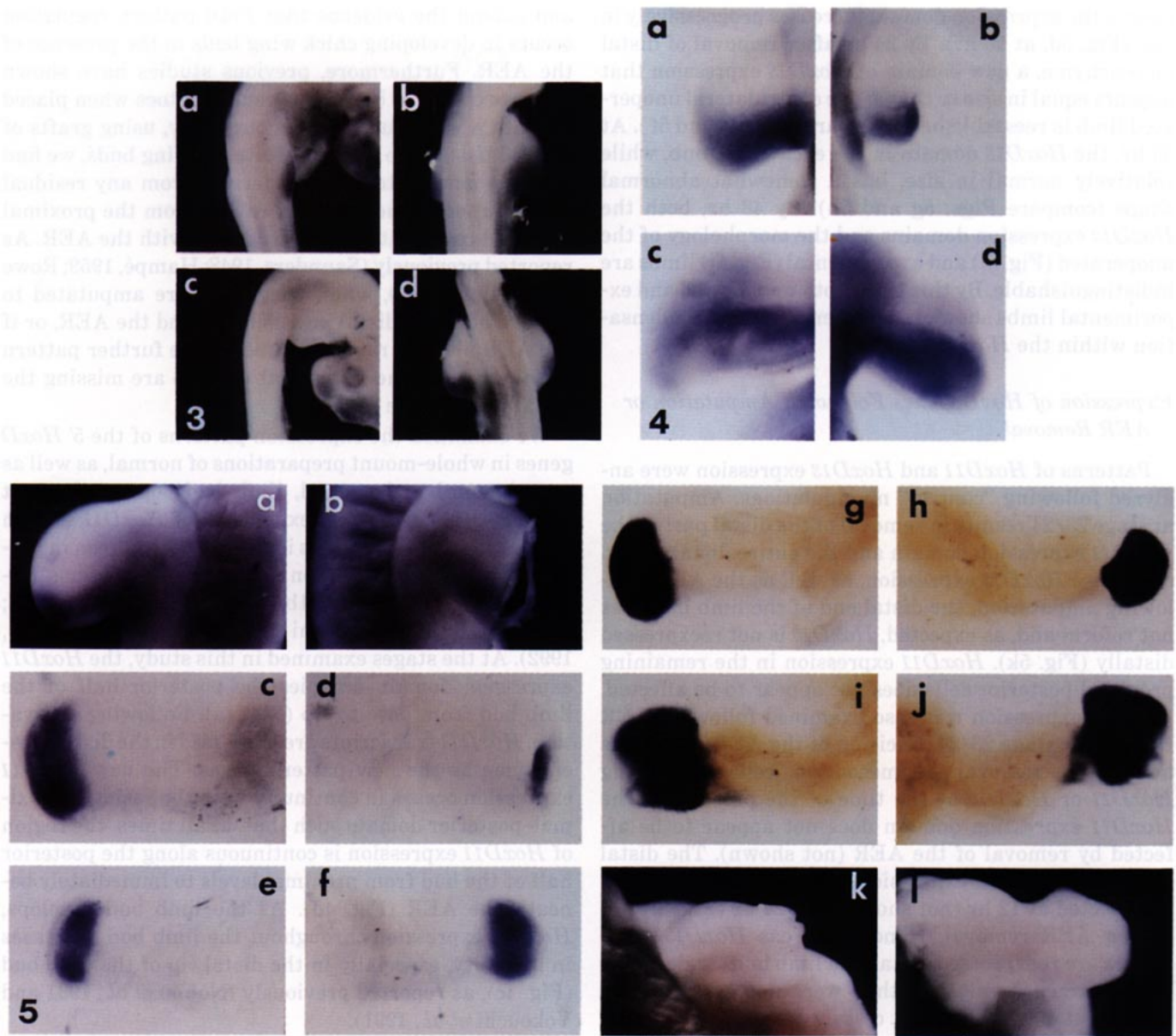


FIG. 3. Whole-mount *in situ* hybridization using *HoxD11* (a, b) and *HoxD13* (c, d) antisense RNA probes. Embryos at stage 18 (a, c) and stage 21 (b, d) showing domains of early *HoxD11* and *HoxD13* expression in the posterior parts of both wing and leg buds. Orientation of embryos is as described in the legend to Fig. 1.

FIG. 4. *HoxD11* expression following excavation. (b, d) Wing buds 6 hr (b) and 24 hr (d) after excavation at stage 23/24. The region of more intense staining at the distal end of the limb bud in (b) is an area of *HoxD11*-expressing mesenchyme that has not yet become re-covered by healing ectoderm. (a, c) Contralateral limb buds at stage 24 (a) and stage 25 (c). Orientation of limb buds is as in the legend to Fig. 1.

FIG. 5. Expression of *HoxD13* following excavation. (b) Wing bud 6 hr after excavation at stage 23/24; the AER has not yet retracted back onto proximal mesenchyme. The distal expression domain of *HoxD13* evident in the contralateral limb (a) has been removed. (d) Reexpression of *HoxD13* in excavated wing bud after 16 hr. (f) Wing bud 24 hr after excavation showing the regenerated *HoxD13* domain at the distal tip of the bud. (h) Wing bud 36 hr after excavation has an irregularly shaped domain of *HoxD13* expression, with a peak extending proximally in the center of the bud. The intensity of expression in the new domain appears to be equal to that of the control limb (e), now at stage 26. (i, j) After 48 hr, the excavated (j) and control (i) limbs are indistinguishable, both in *HoxD13* expression and morphology. Orientation of limb buds is as in the legend to Fig. 1. (k) Wing bud 24 hr after amputation of distal mesenchyme and AER. No *HoxD13* expression can be detected. (l) Wing bud 24 hr after AER removal at stage 24 showing no distal region of *HoxD13* expression. Orientation of limbs is as in the legend to Fig. 1.

points, the expression domain increases progressively in size (Fig. 5d, at 16 hr). By 24 hr after removal of distal mesenchyme, a new domain of *HoxD13* expression that appears equal in size to that of the contralateral unoperated limb is reestablished (compare Figs. 5e and 5f). At 36 hr, the *HoxD13* domain in the excavated limb, while relatively normal in size, has a somewhat abnormal shape (compare Figs. 5g and 5h). By 48 hr, both the *HoxD13* expression domains and the morphology of the unoperated (Fig. 5i) and experimental (Fig. 5j) limbs are indistinguishable. By this time, both unoperated and experimental limbs show clear evidence of digit condensation within the *HoxD13* domain.

#### *Expression of HoxD Genes Following Amputation or AER Removal*

Patterns of *HoxD11* and *HoxD13* expression were analyzed following "control" manipulations. Amputation at stage 23/24 results in removal of the distal part of the *HoxD11* expression domain and the entire distal region of intense *HoxD13* expression, as well as the AER. Following amputation, the distal end of the limb bud does not reform and, as expected, *HoxD13* is not reexpressed distally (Fig. 5k). *HoxD11* expression in the remaining proximal posterior cells does not appear to be affected.

*HoxD* expression was also examined following AER removal at stage 23/24. Excision of the AER alone does not entail removal of mesoderm cells expressing *HoxD11* or *HoxD13* at the time of the procedure. The *HoxD11* expression domain does not appear to be affected by removal of the AER (not shown). The distal domain of *HoxD13* expression, however, can no longer be detected at 12 hr (not shown) and 24 hr (Fig. 5l) following AER removal. Hence, whereas *HoxD13* transcripts are reexpressed distally in limb buds undergoing PrDi pattern regulation, they were not detectable in limbs that develop without distal pattern elements, regardless of whether or not distal mesenchyme was removed.

#### DISCUSSION

In this study, we examine the expression of *HoxD11* and *HoxD13* transcripts during pattern regulation occurring from a proximal limb bud stump that is supplied with an intact AER. Previous investigators (Hansborough, 1954; Hampé, 1959, 1960; Barasa, 1964) presented limited documentation that proximal limb bud mesenchyme can regulate to form normal limb pattern under the influence of the AER. In our study, we removed a region of distal wing bud mesenchyme which included the progress zone, while leaving the AER intact. We find that all limbs with an intact AER regulate to form a normal or near normal limb pattern. Thus, we confirm

and extend the evidence that PrDi pattern regulation occurs in developing chick wing buds in the presence of the AER. Furthermore, previous studies have shown that proximal leg bud tissue can form toes when placed beneath a wing bud AER. In our study, using grafts of leg bud tissue into excavated sites in wing buds, we find that the new pattern is not derived from any residual distal mesenchyme cells, but rather from the proximal limb bud cells that come into contact with the AER. As reported previously (Saunders, 1948; Hampé, 1959; Rowe and Fallon, 1982), when wing buds are amputated to remove both the distal mesenchyme and the AER, or if the AER alone is removed, limbs cease further pattern formation, and the limbs that develop are missing the distal parts of the pattern.

We examined the expression patterns of the 5' *HoxD* genes in whole-mount preparations of normal, as well as experimental and control, limb buds operated on at stage 23/24. The normal expression of *HoxD11* seen in whole-mount preparations is similar to that seen in previous *in situ* hybridization studies using sections (Izpisúa-Belmonte *et al.*, 1991b; Mackem and Mahon, 1991; Noji *et al.*, 1991; Yokouchi *et al.*, 1991; Morgan *et al.*, 1992). At the stages examined in this study, the *HoxD11* expression domain occupies the posterior half of the limb bud from base to tip (Fig. 4a). Following excavation, *HoxD11* transcripts are expressed in the distal mesenchyme as the new pattern forms. The new *HoxD11* expression occurs in continuity with the residual proximal-posterior domain such that, at all times, the region of *HoxD11* expression is continuous along the posterior half of the bud from proximal levels to immediately beneath the AER (Fig. 4d). As the limb bud develops, *HoxD11* expression throughout the limb bud decreases in intensity, especially in the distal tip of the limb bud (Fig. 4c), as reported previously (Nohno *et al.*, 1991 and Yokouchi *et al.*, 1991).

In comparison to the expression of *HoxD11*, *HoxD13* transcripts are restricted to a more posterior and distal domain (Fig. 5a; see also Izpisúa-Belmonte *et al.*, 1991b; Yokouchi *et al.*, 1991). Excavation of distal mesenchyme at stage 23/24 removes this domain. A new region of *HoxD13* expression is progressively reformed beneath the AER. By 24 hr, the region expressing *HoxD13* approximates the size of the normal contralateral domain. The shape of the region of *HoxD13* reexpression is slightly different than that of the normal domain, and it remains abnormal for more than 36 hr. By 48 hr, experimental and control wing buds appear indistinguishable in morphology and in the intensity of their 5' *HoxD* expression domains. Hence, proximal limb bud cells beneath an intact AER regulate to form normal patterns of skeletal structures. This PrDi pattern regulation is

accompanied by regeneration of the distal expression domains of the 5' *HoxD* genes.

The only other well-studied example of *HoxD* gene "induction" in the chick wing bud is seen during duplications along the AP axis, rather than during PrDi pattern regulation. That is, when cells are grafted from a posterior region of a donor bud (zone of polarizing activity, ZPA) to an anterior location in a host wing bud, domains of 5' *HoxD* gene expression appear between the graft and the AER (Nohno *et al.*, 1991; Izpisua-Belmonte *et al.*, 1991b, 1992a; Koyama *et al.*, 1993). Following a polarizing region graft, ectopic *HoxD11* expression in host wings can be seen by 16 hr (Izpisua-Belmonte *et al.*, 1992a); ectopic expression of *HoxD13* does not appear until 24–30 hr (Izpisua-Belmonte *et al.*, 1991b; Nohno *et al.*, 1991). When retinoic acid-containing beads are implanted anteriorly at doses that mimic the effect of ZPA grafts, ectopic expression of *HoxD11* is not seen until 20 hr following bead implantation (Izpisua-Belmonte *et al.*, 1991b); *HoxD13* expression is not seen until 24–48 hr (Izpisua-Belmonte *et al.*, 1991b; Nohno *et al.*, 1991). In our study of PrDi pattern regulation, when the entire domain of *HoxD13* expression is removed, a new normally sized domain has developed by 24 hr.

In contrast to the reexpression of *HoxD13* in limbs undergoing pattern regulation, control limbs which fail to form distal limb structures also fail to express *HoxD13* transcripts. In amputated limb buds, where the intense distal domain of *HoxD13* expression is removed, the domain is not replaced. When only the AER is removed at stage 23/24, leading to similarly truncated limbs, *HoxD13* expression at the distal tip also becomes undetectable. This result is similar to that reported by Koyama *et al.* (1993) for *HoxD12*. In addition, Izpisua-Belmonte *et al.* (1992b) report that, following AER removal, *HoxD13* expression becomes less intense, and the domain fails to expand as it does in normal limbs. Although mesenchymal cell death accompanies removal of the AER at stages 18 to 20, none occurs after AER removal at the stages used here (Rowe *et al.*, 1982). Hence, the finding that *HoxD13* expression is undetectable after AER removal at stage 23/24 cannot be accounted for by cell death. Instead, the data suggest that maintenance of distal expression of *HoxD13* at stage 24 is dependent on the AER.

In addition to being AER-dependent, *HoxD13* expression is also responsive to ZPA grafts and RA bead implants, as described above. Further, it has been reported that the domain of *HoxD13* expression correlates spatially and temporally with the ZPA (Izpisua-Belmonte *et al.*, 1991a). However, polarizing activity can be detected in pre-limb-bud flank mesoderm as early as stage 10 (Hornbruch and Wolpert, 1991), well prior to the onset of *HoxD13* expression in the limb bud at stage 17/18.

In addition, comparisons between the *HoxD13* expression domains in chick wing buds described here and in previous studies (Izpisua-Belmonte *et al.*, 1991b; Nohno *et al.*, 1991; Yokouchi *et al.*, 1991) and published ZPA "maps" (MacCabe *et al.*, 1973; Honig and Summerbell, 1985) reveal that, although these regions may overlap at some stages, they show distinct differences in their spatial distributions, especially at later stages. Finally, it has been demonstrated that polarizing activity does not require *HoxD* expression and, conversely, that intense *HoxD* expression does not necessarily translate into strong activity (Izpisua-Belmonte *et al.*, 1992a). Hence, both descriptive and experimental studies of *HoxD* gene expression indicate that the expression of *HoxD13* is not a marker for the ZPA.

In conclusion, we have shown that when chick limb buds undergo PrDi pattern regulation, the formation of normal distal structures is accompanied by the continued expression of *HoxD11* in the reforming distal region of the bud and by the re-formation of a posterior-distal domain of *HoxD13* expression. Conversely, under conditions in which the distal pattern will fail to form (after removal of the entire domain and AER or after removal of the AER alone), the domain of *HoxD13* expression is either not replaced or becomes undetectable. The cumulative evidence suggests that *HoxD13* expression correlates with formation of distal limb structures. Whether this relationship is causal or not can only be determined from studies in which the expression of *HoxD13* can be directly controlled.

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