DEVELOPMENTAL BIOLOGY **180,** 35–40 (1996)
ARTICLE NO. 0282

Surgical Removal of Limb Bud *Sonic hedgehog*

Sylvia M. Pagan,*[,1] Maria A. Ros,†[,1] Cliff Tabin,* and John F. Fallon‡[,2]

**Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115;*
†Departamento de Anatomia y Biologia Celular, Universidad de Cantabria, 39011 Santander, Spain; and ‡Department of Anatomy, University of Wisconsin, Madison, Wisconsin 53706

Using *Sonic Hedgehog* (*Shh*) as a marker for polarizing region cells we have repeated the experiments of MacCabe *et al.* (1973) and Fallon and Crosby (1975) in an attempt to reexamine the question of a continuous role for the polarizing region during limb development. We report that the earlier experiments probably left *Shh*-expressing cells after surgery. Our results show that *Shh*-expressing cells do not regenerate and complete removal of the polarizing region results in truncations along the anteroposterior (A–P) axis; further, A–P patterning cannot be restored when a bead soaked in FGF is implanted in the limb bud mesenchyme to maintain outgrowth after extirpation of the polarizing region. However, in order to reproducibly remove all *Shh*-positive cells, it is possible that cells with posterior limb skeletal fate also must be removed. Therefore, microsurgical approaches do not permit an unequivocal answer to the question raised in this and the earlier papers and it remains a reasonable possibility that at least up to stage 20–21 the polarizing region plays a continuous role in patterning of the limb bud during its development.

INTRODUCTION

The polarizing region (zone of polarizing activity or ZPA) was operationally defined more than 25 years ago as a restricted area of posterior limb mesoderm that caused mirror image duplications of digit patterns along the anteroposterior (A–P) axis when grafted into the anterior margin of a host wing bud (Saunders and Gasseling, 1968; Tickle *et al.*, 1975). It was assumed that the polarizing region played an important role in the anteroposterior patterning of the developing limb. One way to test this hypothesis was to surgically remove it, expecting either truncations along the A–P axis or uniform skeletal elements along the A–P axis if the polarizing region had an essential role in A–P specification during limb development. Results published by MacCabe *et al.* (1973) argued against this conclusion: when the polarizing region was removed from early limb buds, normal wings developed in about half of the cases, suggesting that it was not involved in normal anteroposterior patterning of the limb. Arguing that these data could also reflect the regeneration of polarizing activity after the surgeries, Fallon and Crosby (1975) took posterior tissue from wing buds 24 and 48 hr after polarizing region removal and assayed it for polarizing activity in a host limb bud. Negative results for the presence of polarizing activity in the operated buds allowed them to conclude that the polarizing region was not regenerated following removal. They also suggested that, given the fact that normal wing development still occurred in about 30% of the cases, this indicated that ''if the polarizing zone had any role during limb development, it must be at an early stage, as during limb induction, and any information from the zone is further acted upon throughout the morphogenetic field.'' Nevertheless, the presence of the polarizing region until nearly the end of limb development has been taken as a strong suggestion of it playing an active role in patterning as the limb bud elongates (Tickle *et al.*, 1975). However, there is no conclusive evidence arguing against the hypothesis that it exclusively acts early.

Three possibilities could explain why MacCabe *et al.* (1973) and Fallon and Crosby (1975) obtained a high percentage of normal wings after polarizing region removals. First, all of the polarizing region was removed after the surgeries and it is indeed dispensable for A–P patterning after limb induction stages, either because it has already fixed the positional identity of the future wing elements or because it

[1] The first two authors contributed equally to this work.

[2] To whom correspondence should be addressed at Department of Anatomy, University of Wisconsin, 1300 University Avenue, Madison WI 53706. Fax: 608/262-2327. E-mail: jffallon@facstaff.wisc.edu.

0012-1606/96 $18.00

has already set the cascade of events that ultimately result in A–P patterning. Second, all of the polarizing region was removed but it eventually regenerates, resulting in restoration of anteroposterior pattern. This alternative would imply that the negative results for polarizing activity obtained by Fallon and Crosby (1975) were due to assays that were not sensitive enough. Third, perhaps not all of the polarizing region was removed with the surgeries and the remaining amounts were sufficient to maintain normal A–P patterning of the developing bud, implying again that this residual activity must have been missed by the less sensitive polarizing activity assays. Discerning among these three possibilities requires a molecular marker for the polarizing region cells, which did not exist at the time that the earlier studies were carried out.

Several lines of evidence suggest that *Sonic Hedgehog* (*Shh*), a vertebrate homologue of the Drosophila *hedgehog* gene, is a crucial component of the polarizing region signaling pathway. Transcripts of *Shh* strongly localize to the polarizing region and its expression pattern strikingly correlates with maps of the polarizing region throughout development (Riddle *et al.*, 1993). Grafts of *Shh*-expressing cells (Riddle *et al.*, 1993; Chang *et al.*, 1994) as well as *SHH* protein in beads (López-Martínez *et al.*, 1995) have been shown to induce polarized digit duplications along the anteroposterior axis, which are indistinguishable from the classical polarizing region grafts. It thus seems likely that *Shh* is the molecule responsible for the patterning role of the polarizing region in the developing limb and that it would serve as an excellent marker for identifying the cells that belong to the polarizing region. We have reinvestigated the requirement for polarizing region tissue during limb development using *Shh* expression as a marker.

MATERIALS AND METHODS

White Leghorn chick embryos of stages 20–21 (Hamburger and Hamilton, 1951) were used for surgeries. The polarizing region was excised with tungsten wire needles and embryos were either harvested immediately or allowed to develop for 24 hr or until the 11th day of embryonic development. Whenever indicated, a bead (Affi-Gel Blue Gel, Bio-Rad) soaked in FGF-4 (0.85 mg/ml, a gift from the Genetics Institute) was inserted in a cut made in the limb bud mesenchyme after the polarizing region removal. Embryos harvested 0 and 24 hr after the surgeries were fixed in 4% paraformaldehyde, dehydrated in a graded methanol series, and used for *in situ* hybridization using the *Shh* probe as previously described (Riddle *et al.*, 1993). Embryos harvested at Day 11 were washed in PBS, fixed in 4% paraformaldehyde, and stained for cartilage with Alcian blue.

RESULTS AND DISCUSSION

Table 1 summarizes the results from surgeries performed on stage 20–21 chicken embryos. Our approach was to attempt to achieve a constant extent of cutting during each individual experiment, progressively trying to remove more tissue in each successive experiment until we obtained no *Shh* signal in the limbs as tested by *in situ* hybridizations. We scored the amount of remaining *Shh* in the limbs and compared these results with the wing patterns of Day 11 embryos from the same batch.

When we performed more conservative surgeries, we obtained results strikingly similar to those reported by MacCabe *et al.* (1973) and Fallon and Crosby (1975): at Day 11 normal wings were obtained in about 30% of the cases, with the rest exhibiting various postaxial defects, one of the most common being the presence of only a humerus, a radius, and digits 2 and 3, as was also the case for Fallon and Crosby (Table 1A, Fig. 1B). However, *in situ* hybridization of the stage 20–21 limbs revealed that only a small percentage had no *Shh* left, and a large fraction exhibited a considerable degree of remaining *Shh* signal in the operated limb (Table 1A, Fig. 1A). When we modified our surgeries such that 64% of the limbs had no *Shh* left and the remnant had very small amounts, the wing skeletal patterns of the Day 11 wings looked noticeably different: 100% exhibited extreme truncations along the A–P axis, most of them having only a humerus, a radius, and a digit 2 (Table 1B, Fig. 1C).

To investigate whether *Shh* regenerated after the polarizing region removals we performed *in situ* hybridizations of limbs from embryos harvested 24 hr after the surgeries. Our results show that the amount of *Shh* signal and the percentage of *Shh*-positive limbs after 24 hr is correlated with the amount of *Shh* and percentage of positive limbs at 0 hr. In an experiment where we analyzed limbs at several times after the surgery, it was clear that the *Shh* signal in the 24-hr limbs came from *Shh*-expressing cells that had not been removed during the surgery (data not shown). Table 1C summarizes an experiment where we removed a more distal portion of the polarizing region, purposefully leaving proximal *Shh*-expressing cells. Almost 90% of the limbs harvested at 0 hr after the surgeries had high remaining amounts of proximal *Shh*, close to the junction of the limb bud and body wall (Fig. 2). After 24 hr, 38% of the limb buds still showed high amounts of *Shh*, 50% had low levels of the signal, in each case confined to the proximal regions of the limb buds (Fig. 2, $n = 8$). In similar specimens, the skeletal patterns of the Day 11 wings showed a relatively high percentage (33%) of normal limbs; those remaining formed a humerus, radius, ulna, and digit 2. When we compared these skeletal patterns with those obtained from our previous experiments, the most obvious difference was the addition of an ulna in all limbs (compare Tables 1A and 1B with Table 1C). This is consistent with the *in situ* analysis of the 0- and 24-hr limbs, where the *Shh* signal was always proximal, i.e., not near the digit area, and correlated with the A–P pattern restoration at the zeugopod level. In contrast, we never observed an ulna in truncated limbs at Day 11 from surgeries done that were similar to those where no *Shh* was left proximally.

We conclude from these experiments that *Shh*, and thus the polarizing region, does not regenerate following polarizing region removal and that the degree of A–P patterning

TABLE 1

Remaining *Shh* Signal after Polarizing Region Removal and Corresponding Wing Structures Present at Day 11

Goal of surgery:	A (Leave *Shh*)	B (Leave no *Shh*)	C (Proximal *Shh*)	D (No *Shh* + FGF)
Shh expressions				
0 hr				
No *Shh*	17%	64%	0%	100%
Low levels *Shh*	33%	29%	0%	0%
Some *Shh*	25%	7%	11%	0%
High levels *Shh*	25%	0%	89%	0%
	$n = 12$	$n = 14$	$n = 9$	$n = 6$
Skeletal elements				
11 days				
R	0%	10%	0%	57%
R, 2	43%	90%	0%	0%
R, 2, 3	28.5%	0%	0%	0%
R, U, 2	0%	0%	66%	43%
R, U, 2, 3	0%	0%	0%	0%
R, U, 2, 3, 4	28.5%	0%	33%	0%
	$n = 7$	$n = 5$	$n = 3$	$n = 6$

Note. During each surgery, great care was taken to be consistent in the amount of tissue removed in embryos of each batch (A, B, C, and D). However, some variation is likely between surgeries done on different days; therefore, the phenotypic outcomes are only analyzed relative to the amount of *Shh* left in representative limbs done in parallel, and numbers are not combined between experiments. We performed a total of 203 polarizing region removals; a subset of representative experiments is shown here. The results obtained with those experiments not shown are consistent with the data presented in the table. R, radius; U, ulna; 2, digit 2; 3, digit 3; 4, digit 4.

in the Day 11 wings is correlated with the amount of *Shh* left in the operated buds. We reason that MacCabe *et al.* (1973) and Fallon and Crosby (1975) did not remove all of the polarizing region in their experiments, such that enough *Shh*-expressing cells were left to maintain patterning of the limbs. Negative assays for polarizing activity that allowed Fallon and Crosby (1975) to conclude that the polarizing region was not regenerated seem to contradict our result that *Shh* is found in some limbs 24 hr after polarizing region removals. This inconsistency can be reconciled by assuming that at the time, the assay for polarizing activity was not sensitive enough. Tickle (1981) has shown that placing a tissue graft beneath an intact apical ridge constitutes a significantly more sensitive test for polarizing activity, as opposed to placing the graft in a notch, the procedure that Fallon and Crosby followed. We speculate that assaying the 24-hr postsurgery limb buds using the more sensitive assay would have resulted in duplications along the A–P axis indicative of the residual polarizing activity.

We envisioned two possible explanations of why removing all of *Shh* resulted in severe pattern defects along the A–P axis of the limbs. One was that *Shh* could be necessary to actively pattern structures along the A–P axis as the wing grows and develops, in which case removing it at early stages results in postaxial defects. Alternatively, it was possible that *Shh* has completed its A–P patterning role after early limb induction stages and the reason truncations occur is simply because of the disruption of a feedback loop between *Shh* and FGF-4 (Laufer *et al.*, 1994; Niswander *et al.*, 1993). *Shh* maintains FGF-4 expression necessary for limb outgrowth and FGF-4, in turn, maintains *Shh* in the posterior wing mesoderm. Hence, after limb induction stages the positional fates along the A–P axis could have already been established, and the role of *Shh*—and the polarizing region—at that point would be to support limb outgrowth through its interaction with FGF-4 in the overlying ectoderm. These hypotheses were tested by attempting to maintain limb outgrowth by implanting a bead loaded with FGF in the limb mesoderm after polarizing region removal. Table 1D summarizes the results of one such experiment, where a bead of FGF-4 was implanted in the mesoderm of stage 20–21 limb buds after the polarizing region had been removed. As is shown in the table, no *Shh* signal was detectable in 100% of the limbs harvested right after the surgeries, and at Day 11 all of the wings showed extreme A–P truncations (Fig. 3). Interestingly, the ulna seemed to be the only structure that was to some extent restored with these experiments, since 43% of the limbs now had an ulna in addition to the humerus, the radius, and digit 2 (compare to 0% in experiment of Table 1B).

When we analyzed the limbs harvested 24 hr after polarizing region removal and FGF bead implants, we found that 100% showed *Shh* signal to various extents and always in a proximal position, away from the digit region (Fig. 3, $n = 6$). Given that we removed all of *Shh* at 0 hr and that the position of the *Shh* signal at 24 hr is always proximal, we assume that these *Shh*-expressing cells belonged to the flank at the time of polarizing region removal and that they

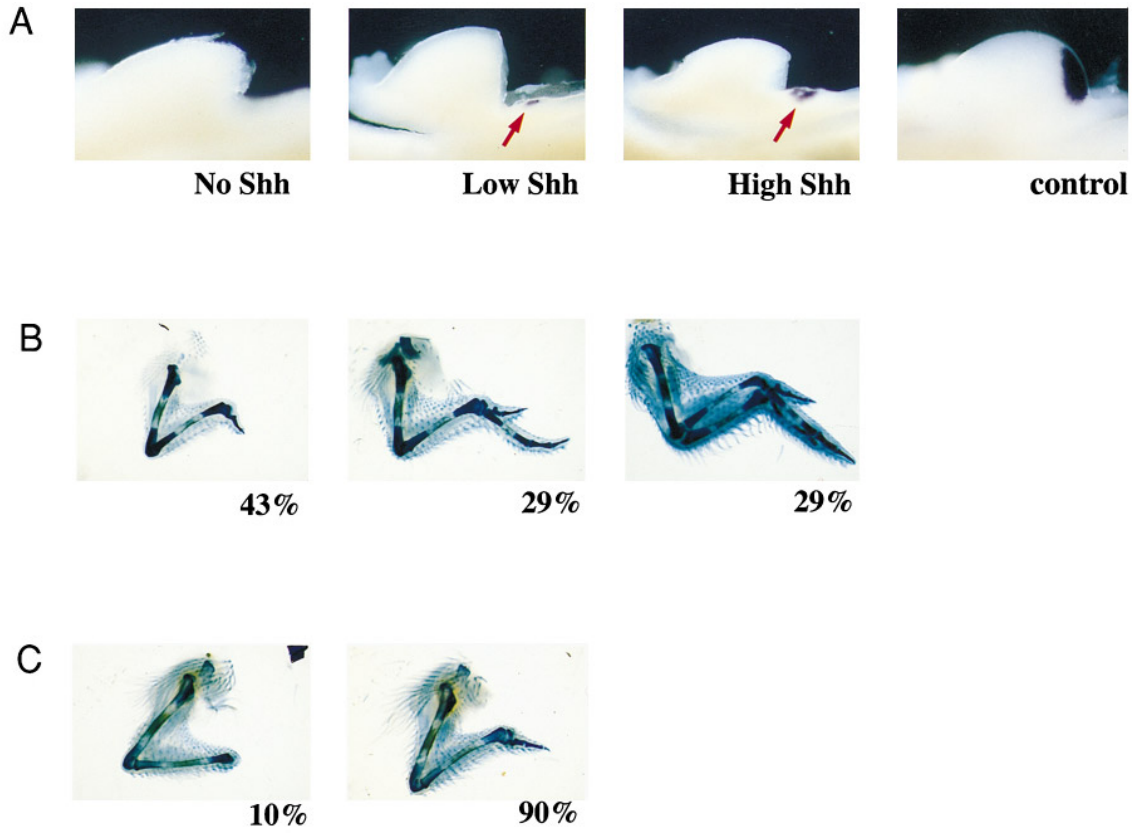

FIG. 1. (A) Tissue from the polarizing region was surgically removed and remaining polarizing region cells were visualized by *in situ* hybridizations with the *Shh* probe. Limbs in the figure show examples of what were categorized as low or high levels of remaining *Shh* as classified in Table 1. Control: *Shh* expression in an unoperated normal limb at stage 20–21. Arrows indicate remaining *Shh* signal in the limb buds. (B) Representative Day 11 (Table 1A) limbs show the range of wing skeletal patterns obtained after incomplete polarizing region removals. (C) When all or most of the polarizing region was removed the resulting Day 11 (Table 1B) wing skeletal patterns exhibited extreme truncations along the A–P axis.

are brought into proximity with the FGF bead as the limb bud grows and heals, inducing expression of *Shh* in a posterior region of the limb bud where it normally is not expressed at that stage of development. Similar results have been reported by Yang and Niswander (1995), where a bead of FGF-4 placed close to the limb flank enables cells in this region to express *Shh* when they normally would not. We propose that the proximal *Shh* expression permits regulation of patterning at the level of the zeugopod and the presence of an ulna in a high percentage of the wings. These results parallel those from our previous experiments (compare Tables 1C and 1D), where remaining proximal *Shh* after polarizing region removal equally resulted in regulation of the ulna. In conclusion, our FGF experiments indicate that *Shh* is probably needed to actively pattern the A–P axis throughout development of the bud. When we provided an FGF bead to maintain outgrowth, we did not observe any restoration of A–P pattern other than the appearance of an ulna, which can be explained by an induction of *Shh* expression in cells close to the limb flank.

We have shown that after complete polarizing region removal, posterior truncations along the anteroposterior axis result. The cause of truncations occurring in the absence of *Shh* remains to be addressed. First, it is possible that the surgery to remove all of *Shh*-expressing cells is radical enough to result in the observed deletions. Second, cells may no longer realize their fate without the signal from the polarizing region. Third, cell death may be induced in these cells after polarizing region removal, with the loss of posterior structures being a direct result of this cell death. This is the least likely since it would be expected that all the digits would be affected (Todt and Fallon, 1987). In summary, we have resolved a discrepancy in the literature by showing that the results previously obtained by MacCabe *et al.* (1973) and Fallon and Crosby (1975) probably were achieved after incomplete polarizing region removals. In order to consistently remove all *Shh*-positive cells it is possible that cells with posterior skeletal fates are also removed. Our data indicate that microsurgery cannot unambiguously answer the question of a continuous role for po-

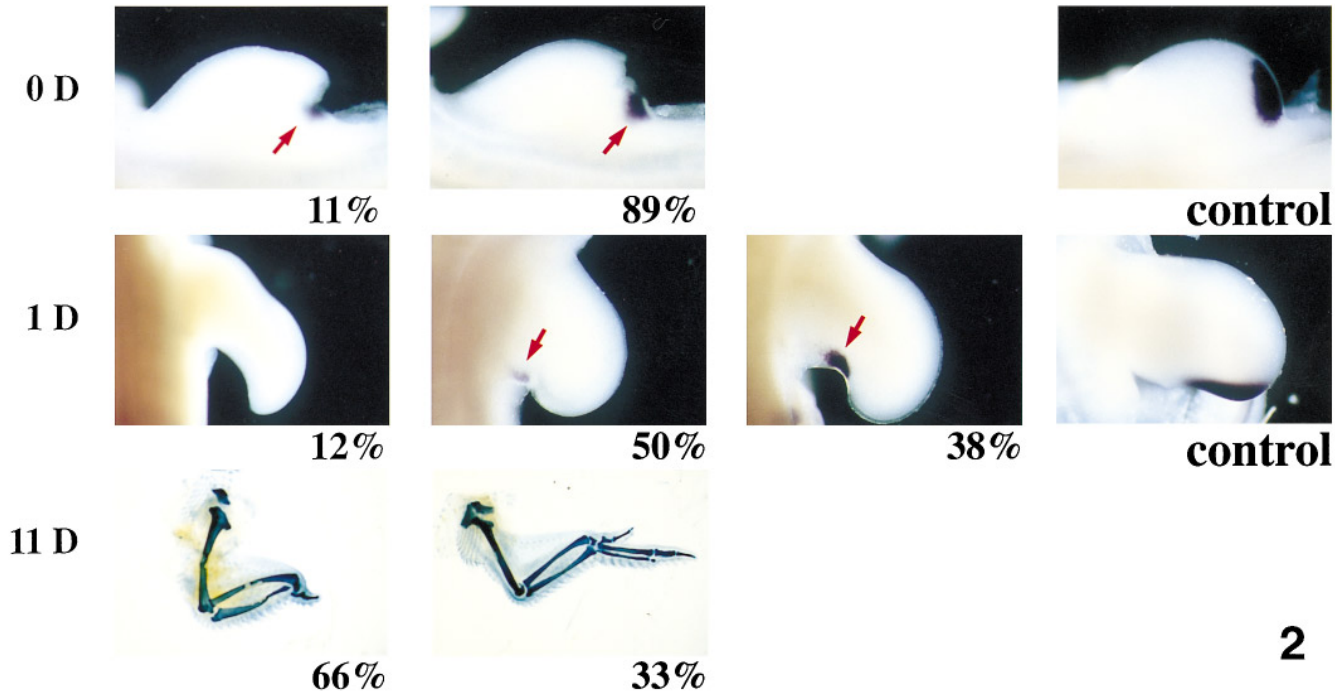

FIG. 2. When we performed polarizing region removals such that *Shh*-expressing cells were left proximally when examined on the day of surgery (0 D; D, day), the skeletal patterns were normal in a considerable number of wings, and the remaining wings showed extreme A–P truncations at the autopod level, but restoration of the ulna at the zeugopod level (11 D). This phenotype correlated with continued proximal *Shh* expression in a high degree of limbs analyzed 1 day after surgery (1 D). Control: *Shh* expression in unoperated limbs at 0 time and 1 day postsurgery. Arrows indicate remaining *Shh* signal in the limb buds.

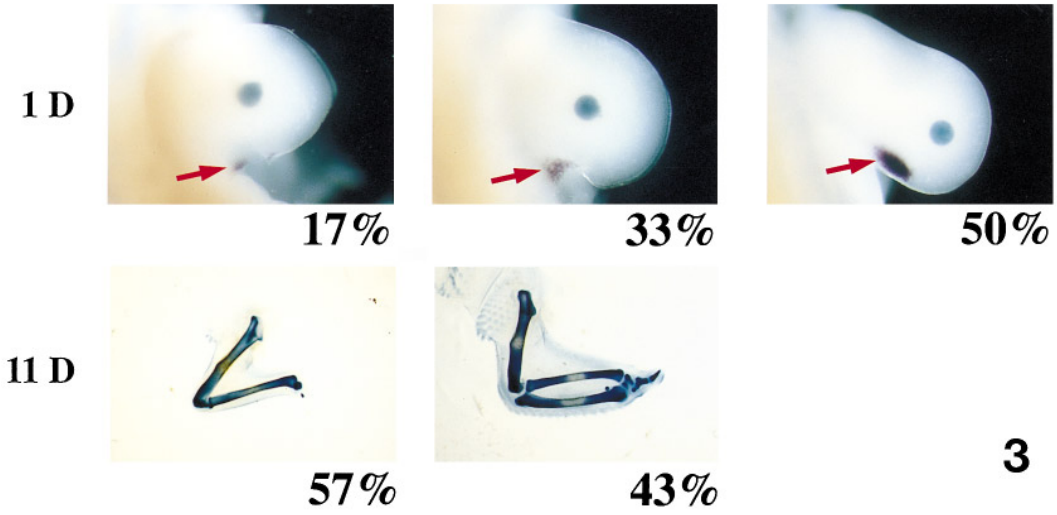

FIG. 3. Implanting an FGF-soaked bead in the operated limbs to maintain outgrowth did not restore A–P pattern at the autopod level (11 D). Regulation of the ulna in almost half of the cases correlates with induction of proximal *Shh* by FGF, as analyzed in limbs 1 day after surgery (1 D). Arrows indicate induced *Shh* signal.

larizing region during limb development posed by MacCabe *et al.* and Fallon and Crosby. Therefore, it remains a reasonable possibility that there is a continuous role for the polarizing region and *Shh* in A–P patterning of the developing limb at least up to stage 20–21 of development.

ACKNOWLEDGMENTS

This work was supported by Grant 95/0576 from FISS to M.A.R; NIH Grant HD32443 to C.T; and NIH Grant HD32551 to J.F.F. We thank B. Kay Simandl and Won-Sun Kim for comments on the manuscript.

REFERENCES

Chang, D. T., López, A., von Kessler, D. P., Chiang, C., Simandl, B. K., Zhao, R., Seldin, M. F., Fallon, J. F., and Beachy, P. A. (1994). Products, genetic linkage and limb patterning activity of a murine hedgehog gene. *Development* **120,** 3339–3353.

Fallon, J. F., and Crosby, G. M. (1975). Normal development of the chick wing following removal of the polarizing zone. *J. Exp. Zool.* **193,** 449–455.

Hamburger, V., and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Exp. Morphol.* **88,** 49–92.

Laufer, E., Nelson, C. E., Johnson, R. L., Morgan, B. A., and Tabin, C. (1994). *Sonic hedgehog* and *Fgf-4* act through a signalling cascade and a feedback loop to integrate growth and patterning of the developing limb bud. *Cell* **79,** 993–1003.

López-Martínez, A., Chang, D. T., Chiang, C., Porter, J. A., Ros, M. A., Simandl, B. K., Beachy, P. A., and Fallon, J. F. (1995). Limb-patterning activity and restricted posterior localization of the amino-terminal product of Sonic hedgehog cleavage. *Curr. Biol.* **5,** 791–796.

MacCabe, A. B., Gasseling, M. T., and Saunders Jr., J. W. (1973). Spatio-temporal distribution of mechanisms that control outgrowth and antero-posterior polarization of the limb bud in the chick embryo. *Mech. Ageing Dev.* **2,** 1–12.

Niswander, L., Tickle, C., Vogel, A., Booth, I., and Martin, G. R. (1993). FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell* **75,** 579–587.

Riddle, R. D., Johnson, R. L., Laufer, E., and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75,** 1401–1416.

Saunders, J. W., and Gasseling, M. T. (1968). Ectodermal-mesenchymal interactions in the origin of limb symmetry. *In* "Epithelial and Mesenchymal Interactions" (R. Fleischmajer and R. E. Billingham, Eds.), pp. 78–97. Williams & Wilkins, Baltimore.

Tickle, C. (1981). The number of polarizing region cells required to specify additional digits in the developing chick wing. *Nature* **289,** 295–298.

Tickle, C., Summerbell, D., and Wolpert, L. (1975). Positional signalling and specification of digits in chick limb morphogenesis. *Nature* **254,** 199–202.

Todt, W., and Fallon, J. F. (1987). Posterior apical ectodermal ridge removal in the chick wing bud triggers a series of events resulting in defective anterior pattern formation. *Development* **101,** 501–515.

Yang, Y., and Niswander, L. (1995). Interaction between the signaling molecules WNT7a and SHH during vertebrate limb development: Dorsal signals regulate anteroposterior patterning. *Cell* **80,** 939–947.

Received for publication June 6, 1996