Relationship between dose, distance and time in *Sonic Hedgehog*-mediated regulation of anteroposterior polarity in the chick limb

Y. Yang[1,2],*, G. Drossopoulou[3],*, P.-T. Chuang[1], D. Duprez[3],†, E. Marti[1], D. Bumcrot[1], N. Vargesson[3], J. Clarke[4], L. Niswander[2], A. McMahon[1] and C. Tickle[3],‡

[1]Department of Molecular and Cellular Biology, The Biolabs, Harvard University, 16 Divinity Avenue, Cambridge, Massachusetts 02138, USA
[2]Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York 10021, USA
[3]Department of Anatomy and Developmental Biology, University College London, Medawar Building, London WC1E 6BT, UK
[4]Department of Anatomy and Developmental Biology, University College London, The Anatomy Building, London WC1E 6BT, UK
*These authors are to be considered as joint first authors
†Present address: Institut D'Embryologie Cellulaire et Moleculaire du CNRS and College de France, 49 bis Avenue de la Belle Gabrielle, 94736 Nogent-Sur-Marne, France
‡Author for correspondence (ucgacmt@ucl.ac.uk)

SUMMARY

Anteroposterior polarity in the vertebrate limb is thought to be regulated in response to signals derived from a specialized region of distal posterior mesenchyme, the zone of polarizing activity. *Sonic Hedgehog (Shh)* is expressed in the zone of polarizing activity and appears to mediate the action of the zone of polarizing activity. Here we have manipulated Shh signal in the limb to assess whether it acts as a long-range signal to directly pattern all the digits. Firstly, we demonstrate that alterations in digit development are dependent upon the dose of Shh applied. DiI-labeling experiments indicate that cells giving rise to the extra digits lie within a 300 μm radius of a Shh bead and that the most posterior digits come from cells that lie very close to the bead. A response to Shh involves a 12-16 hour period in which no irreversible changes in digit pattern occur. Increasing the time of exposure to Shh leads to specification of additional digits, firstly digit 2, then 3, then 4. Cell marking experiments demonstrate that cells giving rise to posterior digits are first specified as anterior digits and later adopt a more posterior character. To monitor the direct range of Shh signalling, we developed sensitive assays for localizing Shh by attaching alkaline phosphatase to Shh and introducing cells expressing these forms into the limb bud. These experiments demonstrate that long-range diffusion across the

anteroposterior axis of the limb is possible. However, despite a dramatic difference in their diffusibility in the limb mesenchyme, the two forms of alkaline phosphatase-tagged Shh proteins share similar polarizing activity. Moreover, Shh-N (aminoterminal peptide of Shh)-coated beads and *Shh-expressing* cells also exhibit similar patterning activity despite a significant difference in the diffusibility of Shh from these two sources. Finally, we demonstrate that when Shh-N is attached to an integral membrane protein, cells transfected with this anchored signal also induce mirror-image pattern duplications in a dose-dependent fashion similar to the zone of polarizing activity itself. These data suggest that it is unlikely that Shh itself signals digit formation at a distance. Beads soaked in Shh-N do not induce *Shh* in anterior limb mesenchyme ruling out direct propagation of a Shh signal. However, Shh induces dose-dependent expression of *Bmp* genes in anterior mesenchyme at the start of the promotion phase. Taken together, these results argue that the dose-dependent effects of Shh in the regulation of anteroposterior pattern in the limb may be mediated by some other signal(s). BMPs are plausible candidates.

Key words: *Sonic Hedgehog*, chick embryo, vertebrate limb development, bone morphogenetic proteins, positional signalling

INTRODUCTION

A fundamental step in vertebrate limb development is the establishment of limb pattern that leads to the correct arrangement of differentiated cells and tissues. Signalling by the zone of polarizing region (ZPA), which is located in the distal posterior mesenchyme, ensures that each digit arises in its correct position. When the ZPA of a chick wing bud is grafted to the anterior margin of a second chick wing bud, this results in mirror-image duplicated patterns of digits (Saunders and

Gasseling, 1968). In the normal chick wing, the digits are **234** (anterior to posterior) while, following a ZPA graft, the digit pattern is **432234**. From results of extensive grafting experiments, it has been shown that behavior of cells (e.g. identity of the digit that they will form) depends on distance from the ZPA (Tickle et al., 1975). Shh is a good candidate for a ZPA signal. *Shh* transcripts map to the ZPA (Riddle et al., 1993) and grafts of *Shh*-expressing cells induce digit duplication (Riddle et al., 1993). All signalling activity appears to reside in the aminoterminal peptide of Shh (Shh-N) (Marti et al., 1995a; Roelink et

al., 1995; Fan et al., 1995), including the ability to specify additional digits in the wing bud (Lopez-Martinez et al., 1995).

Signalling by the ZPA is dose-dependent as the identity of additional digits specified depends on number of ZPA cells grafted. For example, grafts that include only small numbers of ZPA cells specify digit **2** while increasing numbers of cells give an additional digit **3** and finally digit **4** (Tickle, 1981). Further, in both chick and mouse limb buds, reduced levels of *Shh* expression are correlated with absence of posterior digits (Yang and Niswander, 1995; Parr and McMahon, 1995). This suggests that the number of *Shh*-expressing cells is related to digit specification. In one model that can account for such dose-dependent signalling, a morphogen produced by the ZPA diffuses across the bud to set up a concentration gradient (Tickle et al., 1975). According to this model, high concentrations of morphogen would specify posterior digits and low concentrations of morphogen more anterior digits. Such signalling is long-range and would act over a distance of 200 µm or so (Honig, 1981). There is some evidence that the ZPA can act at a distance and induce formation of a digit **2** in cells that have never been next to the ZPA (Honig, 1981). A short-range signal could also act in a dose-dependent fashion if, upon exposure to the signal, cells themselves produced the same signal but at an attenuated level. A second model suggests that the ZPA activates a series of different short-range signalling molecules and that each signal controls local patterning at a particular threshold concentration (Riddle et al., 1993). In this model, one signal would control digit **4** identity, another digit **3** and yet another digit **2**. It is also possible that the ZPA triggers the expression of secondary signals that interact to pattern the anteroposterior (A-P) axis. In this scenario, none of the secondary signalling molecules on their own would be able to mimic the function of the ZPA in specifying digit pattern.

Cells may respond in several ways to polarizing activity. They may respond to the highest concentration of signal that they experience or they may sum the total amount that they see over a certain period. Thus, length of exposure to the morphogen as well as its concentration at any one time may be important in limb patterning. This is illustrated by experiments in which a ZPA graft was removed after different time periods; grafts left in place for short intervals induced no additional digits but grafts left in place for about 16 hours induced an additional digit **2** and more posterior digits after longer periods (Smith, 1980). However, it is not clear whether the same cells are promoted to more posterior fates after longer exposure to ZPA signalling or that different digits are formed from distinct populations.

A major question that has not been resolved is whether Shh acts directly or indirectly to specify additional digits. Direct immunostaining only detects Shh in close association with expressing cells (Marti et al., 1995b) arguing against long-range diffusion. In other systems, Shh signalling appears to have both local and long-range components (Marti et al., 1995a; Roelink et al., 1995; Fan et al., 1995; Ericson et al., 1995). The best evidence for direct long-range effects comes from studies of motor neuron specification (Ericson et al., 1996). However, in the limb there is evidence that Shh may activate secondary signals. For example, *Shh*-expressing cells lead to activation of *Fgf-4* in the anterior ectodermal apical ridge and *Bmps* in the anterior mesenchyme (Niswander et al., 1994; Laufer et al., 1994).

Here we have explored the role of Shh in patterning the chick limb. Our results are most consistent with a model in which the concentration of Shh is the primary determinant of A-P polarity in the limb. Our results also suggest that Shh is unlikely to act as a long-range signal directly regulating all digit identities.

MATERIALS AND METHODS

Preparation of beads

FGF beads

Heparin-acrylic beads were soaked in 1 mg/ml FGF-4 as previously described (Niswander et al., 1993).

Shh beads

To prepare Shh beads, a 3 µl drop of Shh protein solution (see text for concentrations) was placed on a bacteriological grade Petri dish. Stock solutions were stored at 4°C and dilutions made in Tris chloride/sodium chloride buffer. 10-15 Affigel CM beads (200-250 µm in diameter) were rinsed in Tris chloride/sodium chloride buffer and then transferred to the 3 µl drop and allowed to soak in Shh for between 1 and 2 hours at room temperature.

Shh protein preparation

A DNA fragment containing mouse *Shh*-coding sequences (amino acids 25-198) (Echelard et al., 1993) was cloned into the expression vector pEt11d 25 so that the *Shh* coding sequences were downstream of sequences encoding six histidine residues and a recognition sequence for the restriction protease thrombin. In these experiments, the hexahistidine sequences were not removed. The protein was induced and purified as previously described (Marti et al., 1995a). Following purification, Shh protein was extensively dialyzed against storage buffer (20 mM Tris-HCl (pH 7.9), 250 mM NaCl, 1 mM DTT, 5% (v/v) glycerol), concentrated and stored at −80°C.

Preparation of Shh-expressing cells

COS cell lines that stably express *Shh* were obtained by transfection with pcDNA1-*Shh* followed by Neo selection. Grafts of mixtures of *Shh*-expressing cells and chick embryo fibroblasts (CEF) were prepared from confluent cultures. CEF or COS cells from one 100 mm dish were trypsinized and resuspended in 10 ml M199 medium supplemented with 9% fetal calf serum and 2% chicken serum. The cells were counted and *Shh*-expressing COS cells were mixed in known proportions with CEF cells. The cells were then centrifuged at 1000 revs/minute for 5 minutes and resuspended in 0.1 ml of medium (around 5×10^7/ml). A 30 µl drop of resuspended cells was placed in a 35 mm Petri dish which was flipped upside down to form a hanging drop and then incubated for about 3 hours at 37°C. The cell aggregates that formed were cut into small pieces (100-200 µm in diameter containing about 3×10^4 cells) and grafted to chick limb buds.

Grafts

Fertile White Leghorn chicken eggs were purchased from SPAFAS, Inc (Norwich, CT) or from Poyndon egg farm, Hertfordshire, UK. Staging of chick embryos was according to Hamburger and Hamilton (1951). Shh beads or cell pellets were placed under a loop of apical ectodermal ridge at the anterior margin of wing buds of stage 19/20 embryos and where necessary secured with a platinum staple. In a small series of experiments, Shh beads were placed at the apex or at the posterior margin of stage 19/20 chick wing buds. In another series of experiments Shh beads were implanted at the anterior margin of stage 20 wing buds and, at intervals between 12 hours and 24 hours after implantation, beads were removed with a sharp needle, embryos were left to continue to develop to 10 days and skeletal patterns were assessed as described (Yang and Niswander, 1995). Wing buds were also fixed at the same time intervals in 4% paraformaldehyde (PFA) for whole-mount in situ hybridization to detect *Fgf-4*, *Hoxd-13* and *Bmp-2* expression. In a series of experiments to test for induction of polarizing activity, anterior mesenchyme adjacent to implanted Shh beads (beads soaked in 16 mg/ml Shh) was removed either 16 or 24

hours after bead implantation and grafted to a new host under the anterior apical ectodermal ridge region of the wing bud. Control polarizing region grafts from manipulated or contralateral wings of the embryos were performed as previously described (Tickle, 1981). A few embryos were also left to grow up to 10 days with implanted Shh beads in place and out of a total of 11 embryos more than half had wing duplications with additional digits **4** and **3** (Table 2B).

In situ hybridization

In situ hybridization was performed as previously described (Wilkinson, 1992; Nieto et al., 1996).

DiI and DiA injections

DiI and DiA injections were performed through glass micropipettes using a pressure injector (Picospritzer, General Valve Company). A dilution of 3 mg/ml in dimethyl formamide was used for each dye and a spot of dye was injected (diameter of 20-25 µm) to mark cells at different distances from the implanted Shh beads. Shh beads were soaked in 8 mg/ml or 16 mg/ml Shh and grafted at the anterior margin of the wing bud as above. Injections were performed 1 hour after bead implantation and distances were measured with an eyepiece graticule and with reference to the somites. Most embryos were fixed 96 hours later (while some were fixed immediately after the injections or after 36 hours) in 4% PFA. Photography was performed using a Nikon microscope with rhodamine filter while all bud widths were measured under a dissecting microscope using an eyepiece graticule.

Construction and expression of AP fusion proteins and membrane-tethered Shh-N protein

Clones encoding Shh-N or full-length Shh lacking the signal peptide (amino acids 1-24) were cloned into the expression vector AP-tag4 (Cheng et al. 1995) to generate two constructs (AP::Shh-N and AP::Shh) which express either alkaline phosphatase (AP)-tagged Shh-N or Shh. In both cases, Shh-N or Shh was fused in frame to the C terminus of AP in AP-tag4. To generate a membrane-tethered Shh-N, cDNA sequences encoding Shh-N were fused with those encoding the

transmembrane protein CD4 in the expression vector pcDNA3 (Invitrogen). This fusion protein (Shh-N::CD4) appends the CD4 sequences to the signalling moiety of Shh.

To produce AP-tagged Shh proteins or membrane-tethered Shh-N, AP::Shh-N, AP::Shh or Shh-N::CD4 were transiently transfected into COS cells with Lipofectamine (Life Technologies, Inc) according to manufacturer's instructions. The TCA-precipitated medium and cell lysate were analyzed by SDS-PAGE and immunoblotted with anti-Shh antibodies as previously described (Bumcrot et al., 1995). Suramin treatment was performed as described (Bumcrot et al., 1995).

Detection of AP-tagged Shh proteins in chick embryos

COS cells transfected with constructs expressing AP-tagged Shh-N or Shh were harvested 48 hours post-transfection, mixed with 10% CEF cells and allowed to aggregate for 12 hours (Yang and Niswander, 1995). Cell pellets about 150 µm in diameter were implanted under the anterior apical ectodermal ridge of stage 20 chick wing buds. Operated chick embryos were incubated for 24 hours. Diffusion of AP-tagged Shh proteins was detected by whole-mount AP staining as described (Cheng and Flanagan, 1994).

RESULTS

(A) Is signalling by Shh dose-dependent?

(a) *Shh*-expressing cells

To test whether the number of *Shh*-expressing cells governs the A-P identity of digit duplications, pellets of COS cells expressing full-length Shh were diluted with different proportions of primary chick embryo fibroblasts and grafted to the anterior margin of stage 19/20 wing buds (Table 1A; Fig. 1A). With 95% *Shh*-expressing COS cells, an additional digit **4** was specified in most cases (digit patterns **43234**, **4334**) (Fig. 1Aa); with between 20% and 80% *Shh*-expressing cells, a digit **3** is formed most often (Fig. 1Ab,c); whereas with 10% only an

Table 1. Digit patterns following (A) grafts of *Shh* expressing cells and (B) implanted beads soaked in Shh

(A) % of *Shh* expressing cells	*n*	**234** normal	blip b**234**	extra**2** **2234**	extra**3** **3234**	extra**4** **43234**
95%	4	–	–	–	2	2
80%	7	–	–	–	7	–
50%	4	–	–	1	3	–
20%	4	–	–	–	4	–
10%	6	1	–	5	-	–

(B) Shh concentration in mg/ml	*n*	**234** normal	blip b**234**	extra**2** **2234** (22234)*	extra**3** **3334** (334, 3234)*	extra**4** **4334** (43234)*
16	4	–	–	–	1	3
14	4	–	–	–	1	3
8	5	1	2	–	1	1
5	6	2	1	1	1	1
2	7	1	1	2	3	–
1	20	10	2	1	7	–
0.75	12	5	1	4	2	–
(bead replacement)	(11)	(6)	(2)	(2)	(1)	–
0.5	4	1	2	1	–	–
0.1	18	14	2	1	1	–
0.01	5	5	–	–	–	–

*The digit patterns in brackets specify the less common patterns obtained.

Numbers in brackets in B show cases where beads soaked in 0.75 mg/ml *Shh* were replaced after 16 hours with beads freshly soaked in the same concentration of *Shh*. *n*= number of cases.

additional digit **2** was formed (Fig. 1Ad). Thus the number of *Shh*-expressing cells grafted determines the identity of the additional digits that are specified.

(b) Application of Shh protein

To directly test the dose effects of Shh, beads soaked in different concentrations of a bacterially expressed aminoterminal peptide of Shh (Shh-N) were implanted beneath the anterior apical ectodermal ridge of stage 19/20 chick wing buds (Table 1B). With beads soaked in high concentrations of Shh (16 mg/ml, 14 mg/ml, 8 mg/ml or 5 mg/ml), most wings had an additional digit **4** (Fig. 1Ba). With beads soaked in 2 mg/ml or 1 mg/ml Shh, an additional digit **3** was the most frequently observed digit duplication and no additional digit **4**s were produced (Fig. 1Bb). When beads soaked in 0.75 mg/ml Shh were implanted, however, the most frequently observed additional digits obtained were digit **2**s (Fig. 1Bc). Although beads soaked in lower concentrations of Shh occasionally gave an extra digit **2**, most wings had a normal digit pattern (Fig. 1Bd). These results show that low concentrations of Shh can lead to the formation of the anterior digit, digit **2**, and its induction can occur without inducing more posterior digits.

When a bead soaked in 16 mg/ml is removed 24 hours after implantation and placed at the anterior margin of a wing bud of a new host, additional **2**s (two cases) or an additional digit **3** (one case with pattern **3234**) can be specified in that host. This indicates that Shh protein in the grafted bead is still active after being incubated in chick embryos for 24 hours and that the absence of digit **2** in wings treated with the highest Shh concentrations are likely to be due to high levels of Shh throughout the bud.

To test how cells in different parts of a wing bud respond to Shh protein, Shh beads were also implanted either apically or posteriorly. When beads soaked in either 1 mg/ml or 16 mg/ml Shh (concentrations that usually give additional digit **3**s or **4**s, respectively, when placed anteriorly) were grafted at the apex of wing buds, an extra digit **3** could be formed in the region where the bead was placed, resulting in a **2334** pattern (3/3 cases, 16 mg/ml Shh; 3/6 cases, 1 mg/ml Shh). When beads soaked in high concentrations of Shh were placed posteriorly, most wings had a normal digit pattern (5/5 cases, 16 mg/ml Shh; 3/4 cases, 1 mg/ml Shh). Thus Shh only induces additional digits to form when the bead was grafted outside of the resident ZPA.

In almost all wings treated with Shh, proximal elements were thick and short. In 8% of normal limbs (i.e. limbs with no digit duplications) that developed after Shh application anteriorly (Fig. 1Bd) and in limbs with Shh beads implanted posteriorly, the shape of the humerus and occasionally the radius/ulna were distorted. One possibility is that Shh could mimic Indian hedgehog (*Ihh*), another vertebrate hedgehog gene that is expressed in early cartilage (Bitgood and McMahon, 1995; Vortkamp et al., 1996), and thereby influence cartilage morphogenesis (Vortkamp et al., 1996).

(B) Timing of specification of additional digits by Shh

Digit specification depends not only on signal strength but also on length of exposure to the signal. When grafts of the ZPA are removed after 16 hours, only an additional digit **2** is specified. However, when grafts are left in place for longer, posterior digits are also formed (Smith, 1980). In order to find out whether length of exposure to Shh affects digit specification, beads soaked in 16 mg/ml Shh were implanted at the anterior margin of wing buds and then removed at different times (Table 2A). When beads were removed at 12 hours and 14 hours after implantation, no additional digits were formed. In contrast, when beads were removed at 16 hours, an additional digit **2** developed. Progressively longer periods resulted in more extensive duplications so that after a 24 hour treatment,
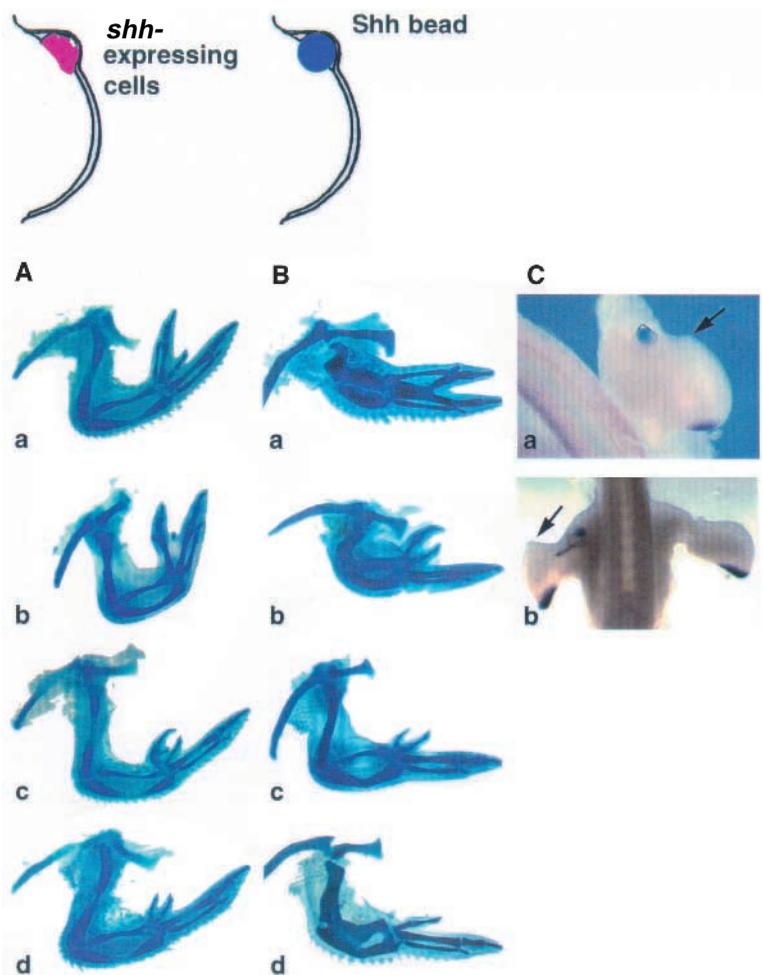

Fig. 1. *Shh*-expressing cells and Shh protein beads (as shown in diagram) produce dose-dependent changes in wing pattern but do not activate Shh expression. (A) Wing skeletal patterns after grafts of *Shh*-expressing cells mixed with chick embryo fibroblasts. (a) 95% *Shh* cells; digit pattern **43234**. (b) 80% *Shh* cells; digit pattern **334**. (c) 20% *Shh* cells; digit pattern **3234**. (d) 10% *Shh* cells; digit pattern **2234**. (B) Wing skeletal patterns after implantation of beads soaked in Shh. (a) 16 mg/ml; digit pattern **4334.** (b) 2 mg/ml; digit pattern **3234**. (c) 0.75 mg/ml; digit pattern **2234**. (d) 1 mg/ml; digit pattern **234**. (C) *Shh* expression after implantation of beads soaked in Shh. (a) 24 hours (bead soaked in 16 mg/ml Shh). (b) 48 hours (bead soaked in 1 mg/ml Shh) no induction of *Shh* expression in anterior cells. Note broadening of buds (indicated by arrow).

an additional digit **4** was formed. Thus there appears to be two phases to digit specification; a phase lasting around 14 hours in which no irreversible changes in digit pattern are induced

and a second promotion phase, about 8 hours long, in which additional digits are progressively specified.

A high concentration of Shh on beads (16 mg/ml) applied
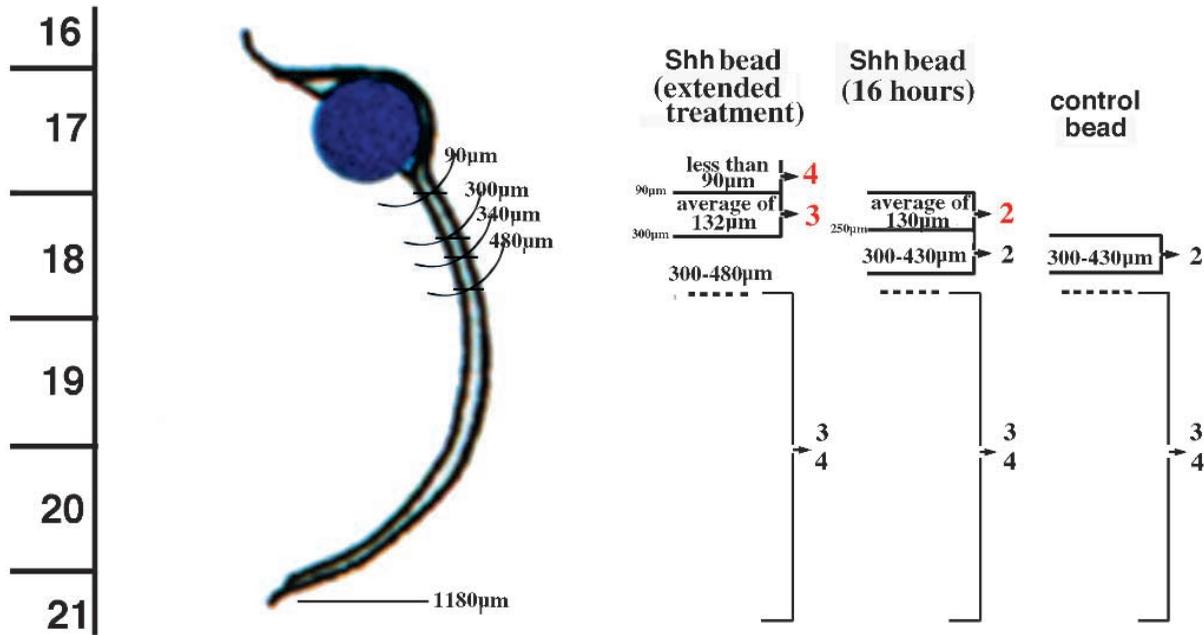
Somite number

Fig. 2. DiI and DiA injections were performed to mark cells at different distances either from beads soaked in high concentrations of Shh (16 mg/ml and 8 mg/ml) or control beads. Upper part of the figure summarises data obtained that show which cells participate in forming particular digits. (Left) Outline of wing bud relative to the somites, with a bead implanted at the anterior margin and regions of bud at different distances from the bead periphery (indicated in µm) are shown by the arcs; (right) the fate of these regions, in terms of contributing to particular digits at 96 hours is indicated; in each case this represents accumulated data from between 3 and 10 marked specimens. The columns show data for wing buds with Shh beads that were left in place (extended treatment); wing buds in which Shh beads were removed after 16 hours, and control wing buds with beads soaked in buffer. Lower part of figure shows examples of labelled wing buds. (A) Wing bud injected at time 0. One mark was at 90 µm from the bead and the other was at 300 µm. (B) Wing bud about 36 hours after implanting Shh bead and marking cells with DiI and DiA. Two stripes of labelled cells extend from the apical ridge; cells labelled with DiI, closest to the bead (90 µm from it at time 0), are now 100 µm from the anterior margin of the bud; cells labelled with DiA (300 µm from bead at time 0) are now 350 µm from the anterior margin of the bud. The whole bud width is about 1300 µm. (C-F) After 96 hours, condensing cartilage can be seen and digits can be identified. (C) Control bead; cells marked with DiA at 90 µm from bead soaked in control buffer (big arrow) did not contribute to distal structures while cells marked with DiI 375 µm away from bead (small arrow) contributed to the normal anterior digit **2**. (D) Bead soaked in 16 mg/ml Shh was removed 16 hours after application resulting in a digit pattern **2234**. Cells marked with DiI (big arrow) initially at 90 µm from the implanted bead contributed to the extra digit **2** while cells marked with DiA at 300 µm away (small arrow) ended up in the normal digit **2** (normal and extra digit **2** indicated by asterisks). (E,F) Beads soaked in 16 mg/ml (extended treatment). (E) Cells marked with DiA at 90 µm and with DiI at 300 µm from implanted bead (big arrow and small arrow) contribute to the extra structures. (F) Cells marked with DiI at 90 µm away contribute to extra structures (big arrow) while cells marked with DiA at 340 µm away do not (small arrow).
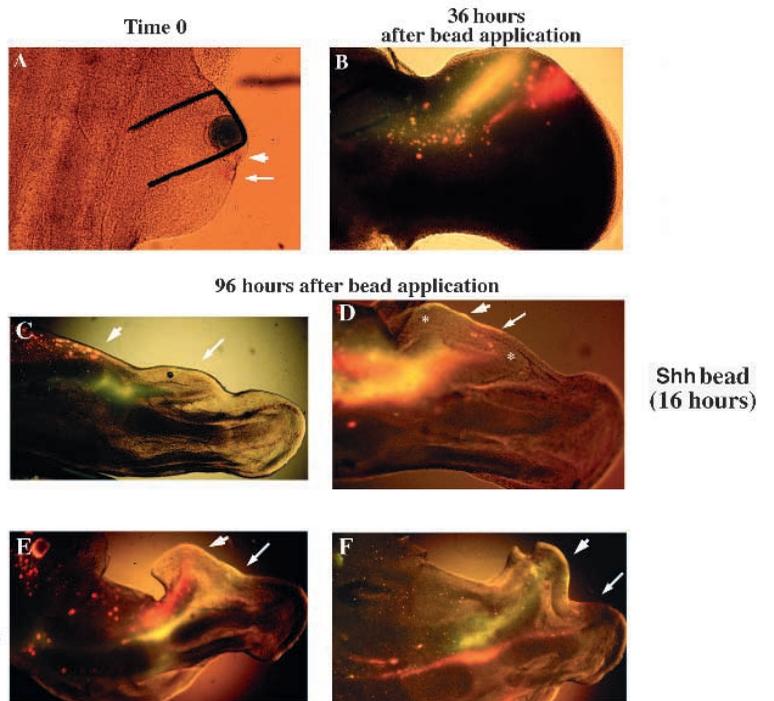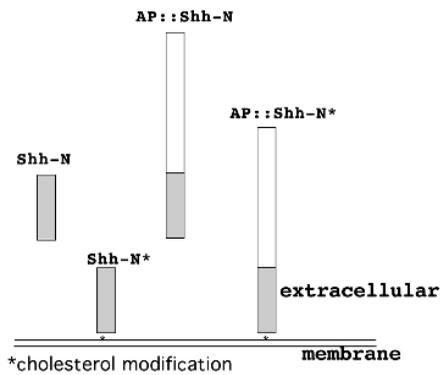
Fig. 3. Schematic diagram of expression constructs encoding alkaline phosphatase (AP)-tagged Shh proteins. Full-length Shh protein undergoes proteolytic cleavage to generate a 19 KDa N-terminal fragment that also undergoes cholesterol modification (indicated by * in diagram), resulting in membrane attachment. AP-tagged Shh proteins were generated by fusing the N-terminal fragment of Shh (Shh-N) or the full-length Shh in frame to the C terminus of AP and resulting constructs were designated AP::Shh-N and AP::Shh respectively. AP::Shh undergoes proper proteolytic cleavage and presumably cholesterol modification to generate AP-Shh-N* as shown in Fig. 4. The AP protein is shown as open bar and the N-terminal Shh protein is shown as shaded bar.

Fig. 4. Expression of AP-tagged Shh proteins in COS cells. COS cells were transfected with constructs encoding either AP::Shh-N or AP::Shh. 48 hours later medium (sup) and cell lysate (pellet) were analysed by SDS-PAGE and immunoblotted with anti-Shh antibodies. A single band corresponding to the predicted relative molecular mass of AP::Shh-N is detected in the medium and cell lysate from cells transfected with AP::Shh-N. AP-Shh produces two species; the higher M_r band corresponds to the unprocessed AP::Shh and the lower band, which migrated at the same position as AP-Shh-N, represents AP::Shh-N* that was cleaved from AP::Shh and cholesterol modified. Both proteins were only detected in the cell lysate in the absence of suramin and AP-Shh-N* was released into the medium upon suramin treatment, a property previously described for Shh protein which undergoes proteolytic cleavage. Molecular size standards are indicated on the left ($\times 10^{-3}$).

for a shorter time (16 hours) can give the same results as low concentration Shh beads (0.75 mg/ml) applied for a longer time. However, when exposure of the wing bud to low Shh beads was prolonged, by replacing a low Shh bead at 16 hours with a second bead freshly soaked in low Shh, this failed to reproduce the effect of a higher concentration Shh bead. In only 1 out of 11 cases, was a digit **3** formed and the pattern of remaining limbs was either **2234** or normal (Table 1B).

In neural tube specification, application of Shh protein can itself induce *Shh* expression (Marti et al., 1995a). When we examined *Shh* expression in limb buds treated with Shh beads,
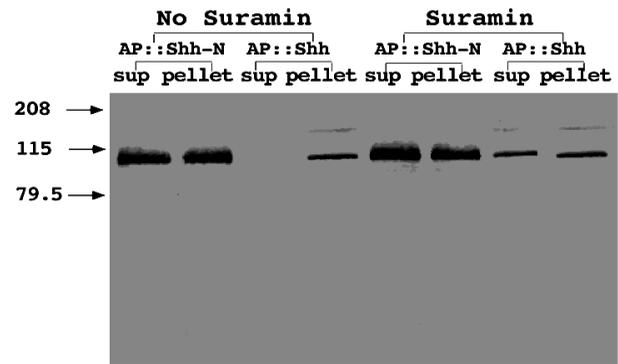
no detectable *Shh* expression was induced in anterior mesenchymal cells near Shh beads (Fig. 1Ca,b; Table 4). Thus we can exclude an autoinductive Shh relay as a patterning mechanism.

(C) Distance over which signalling occurs

In order to determine which cells in the limb form extra digits in response to Shh beads, DiI and DiA injections were performed to mark mesenchymal cells at different distances from the bead. Beads were soaked in either 8 mg/ml or 16 mg/ml of Shh protein

Table 2. (A) Digit patterns of wings following removal of beads soaked in 16 mg/ml Shh at different times after implantation to anterior margin of stage 20 chick wing buds

Time when bead was removed	n	**234** normal	blip b**234**	extra**2** **2234**	extra**3** 3334 (334)*	extra4 **4334**
Bead not removed	45	7	1	3	23	11
24 hours	8	1	1	–	5	1
20 hours	5	3	–	–	2	–
16 hours	9	3	3	3	–	–
15 hours	4	1	2	1	–	–
14 hours	3	3	–	–	–	–
12 hours	1	1	–	–	–	–

(B) Test of polarising activity of anterior mesenchyme taken from next to implanted Shh beads

Wing patterns following:	n	**234**	blip**23** 4	**2234**	**3334** (**3234**)*	**43234** (**4334**)*
Grafts of anterior mesenchyme taken from next to a Shh bead	21	19	2	–	–	–
Grafts of posterior mesenchyme (polarising region from the same buds or contralateral buds)	9	1	1	2	2	3
Implantation of Shh beads in the same series of experiments as controls	11	4	1	–	5	1

*The digit patterns in brackets specify the less common patterns obtained.
n=number of cases

Table 3. Positions in which cells labelled at different distances from a bead soaked in Shh are found 96 hours later

Initial distance of labelled cells from bead*	Shh bead (extended treatment)		Shh bead (16 hours)		Control beads soaked in buffer	
	Mean distance of labelled cell population from anterior margin*	(n)	Mean distance of labelled cell population from anterior margin*	(n)	Mean distance of labelled cell population from anterior margin*	(n)
90	460	(10)	278	(3)	NDF†	(4)
125	509	(6)	–		–	
195	600	(5)	–		NDF†	(1)
250	785	(3)	392	(1)	–	
300	814	(6)	710	(2)	310	(3)
340	1444	(3)	–		–	
375	–	–	–		320	(3)
430	1000	(1)	–		–	
480	1328	(1)	–		700	(1)
Mean width of bud	1914 μm		1756 μm		1387 μm	
Mean width of duplicated part	814 μm		440 μm			

*All distances are measured in μm.
†Non digit-forming regions.
n=number of cases.

and the injections were performed 1 hour later. Cells under the apical ridge near the posterior edge of the bead were marked 90 μm away from the bead and then at intervals up to 480 μm away from the bead. In some wing buds, just a single mark of DiI was applied; in other cases, two spots, one of DiI and one of DiA, were applied at different distances from the bead in the same bud.

Fig. 2A shows an example of a wing bud with an Shh bead injected at time 0. Two spots of dye which are about 20-25 μm in diameter can be seen in the bud. Fig. 2B shows a similar bud about 36 hours later; marked cell populations have given rise to two thick stripes of labelled cells extending from the apical ridge. Table 3 shows the position in which cells initially marked near the bead, end up relative to the anterior margin of duplicated wing buds at 96 hours and indicates the proliferation that has occurred to generate the new digits. At this time, the condensing cartilage of the digits can be seen and contribution of labelled cells to the extra digits assessed (Fig. 2C-F).

Most of the duplicated wings had a pattern of **4334** as expected, while others had extra **3**s (pattern **334** and **3334**). As shown in the summary diagram at the top of Fig. 2, an additional digit **4** came from cells initially within a 90 μm radius of the bead edge, while the additional digit **3** came from cells initially between 90 μm and 300 μm away; the normal digit **3** came from cells that lie more than 480 μm from the bead (Fig. 2E,F). In contrast, in control wing buds in which buffer-soaked beads were implanted, cells initially marked between 90 and 195 μm from the beads did not contribute to distal mesenchyme, while cells initially marked between 300 and 430 μm contribute to digit **2** (Fig. 2C; see also Table 3). Therefore anterior cells not normally destined to form digits can be induced to give rise to digits following implantation of a Shh bead.

To find out whether posterior digits arise by promotion from cells specified first as anterior digits, beads soaked in 16 mg/ml Shh were implanted, injections were performed as before but beads were removed 16 hours after implantation, which gives the digit pattern **2234**. In these limb buds, cells labelled between 90 and 250 μm away from the bead contribute to the extra digit **2** while labelled cells between 300 and 430 μm contribute to the normal digit **2** (Fig. 2D). Thus the same cells initially (at a mean distance of about 130 μm from the bead) can give rise to either

a digit **2** or a more posterior digit according to the length of time that the Shh bead has been left in place. However, it should be noted that the most posterior digit (the anterior digit **4** in a **4334** duplication) does not appear to arise by promotion and came from cells initially within 90 μm of the bead (i.e. less than 9-cell diameters away). Cells marked at 90 μm end up nearly twice as far away from the anterior margin when the Shh bead is left in place compared with when the bead is removed at 16 hours and this is related to the formation of an extra digit **4** (Table 3).

In a few cases, cells were marked either anterior or proximal to the bead, not immediately adjacent to the apical ridge. These labelled cell populations remained close to the bead and,

Table 4. Gene expression in anterior wing bud following (A) grafts of *Shh*-expressing cells and (B) implantation of beads soaked in Shh protein

(A) % *Shh*-expressing cells	Bmp-7	Hoxd-13	Hoxd-11	
95%	+++(2)	+++(3)	++(2)	
80%	++(3)	++(3)	+(2)	
20%	+/−(3)	+(2)	+/−(2)	

(B) Time after bead implantation (hours)	Shh	Fgf-4	Bmp-2	Hoxd-13
12-14		−(2)	−(2)	
16-18		++(2)	++(4)	++(1)
			−(2)	−(1)
20		++(1)	−(2)*	+++(1)
24	−(6)	+++(2)	+(1)	+(1)
			−(1)*	−(1)
48	−(3)		−(1)*	

+++: strong ectopic expression
++: moderate ectopic expression
+: some ectopic expression
+/−: very weak ectopic expression detected
−: no ectopic expression detected
*: strong expression in anterior ridge but no detectable expression in anterior mesenchyme
Beads were soaked in 16 mg/ml Shh protein except for buds probed for *Shh* in which, in 3 cases at 24 hours and 3 cases at 48 hours, beads soaked in 1 mg/ml Shh protein were implanted.

although they expanded a little, they remained fairly rounded and did not end up in digits.

(D) Diffusion of Shh in the wing bud

Previous experiments using existing antibody reagents have not detected endogenous Shh protein outside the polarizing region where it is processed (Marti et al., 1995b) but this technique may not be sufficiently sensitive. To develop a more sensitive assay for localizing Shh in limb buds, two constructs were generated which were tagged identically by the addition of alkaline phosphatase at the N terminus of the secreted Shh (Fig. 3). When alkaline phosphatase (AP) is attached to full-length Shh, this generates AP-tagged Shh-N* which is modified so that it is associated with cell membrane presumably due to addition of cholesterol (Porter et al., 1996). In addition, we engineered a second form that produces an unmodified AP-tagged Shh-N. Both tagged forms are processed equally well in producing cells in culture but the latter is secreted whilst the former is on the surface of the cells (Fig. 4). Activities of the tagged fusion proteins produced from Shh-N* and from Shh-N were determined by examining resulting digit patterns after grafting pellets of COS cells producing the two forms of Shh. In both cases, digit **2** was duplicated ($n>5$). No difference in polarizing activity could be detected between the two tagged forms of Shh although tagging the protein with alkaline phosphatase does attenuate polarizing activity because cells transfected with non-AP-tagged Shh-N or Shh induce in most cases digits that are more posterior in character.

Despite the fact that cells producing AP::Shh-N* and AP::Shh-N have similar polarizing activities, AP::Shh-N* behaves differently in the limb compared to AP::Shh-N. No significant diffusion of AP::Shh-N* could be detected by directly monitoring alkaline phosphatase activity while AP::Shh-N diffused extensively across the limb within 20 hours (Fig. 5A,D). Thus we can detect diffusion of an AP-tagged form of Shh, suggesting that long-range diffusion gradients of large peptides are at least a possibility in limb development. However, normal modification of Shh-N* is likely to prevent long-range diffusion. Furthermore, by whole-mount antibody staining, we found that Shh-N coated on the bead diffused in a broad region in the limb mesenchyme (Fig. 6B) whereas Shh-N* produced by COS cells expressing full-length Shh does not appear to diffuse (Fig. 6A). The expression of *Ptc* in both cases correlates with that of Shh. Induction of *Ptc* expression by *Shh*-expressing cells is localized to regions immediately adjacent to the cell graft (Fig. 6D) while the domain of *Ptc* expression induced by Shh-N released from the bead is fairly broad and symmetric to that of the endogenous *Ptc* (Fig. 6E). Nevertheless, in both cases, digit **3** and **4** were duplicated. These results taken together suggest that normal processing of Shh leads to a non-diffusible protein. One possibility is that diffusion of Shh is not necessary to polarize the limb because polarizing activity of both tethered and non-tethered Shh appears to be essentially the same.

To rule out the possibility that the broad diffusion observed is a result of cell migration from the graft, COS cells were labelled with DiI before being implanted into the chick limb bud to allow us to detect their final destination. Twenty hours after grafting, embryos were harvested, stained with AP substrate and sectioned through the limb buds. A striking difference in distribution of AP::Shh-N and modified AP::Shh-N* was observed. The distribution of AP::Shh-N as detected by AP staining extends far beyond that of DiI staining which marks the position of the grafted cells (Fig. 5B,C). In contrast, DiI and AP staining colocalises within the grafted cells that express AP::Shh-N* (Fig. 5E,F). These results indicate that cell migration from the graft did not occur and cannot account for the observed broad diffusion. They also confirm that tagged Shh-N* processed by a cell and placed into the environment of the limb bud does not diffuse to any appreciable level.

(E) Membrane-tethered Shh-N exhibits the same patterning activity as wild-type Shh

Though we have shown that Shh does not appear to diffuse in vivo, it is possible that an extremely low level of diffusion is sufficient to account for its activity at a distance. In this case, we reasoned that if Shh became completely membrane-tethered, its long-range activity should be abolished. To test this, we fused Shh-N to the integral membrane protein CD4 (Shh-N::CD4) in an attempt to generate a membrane-anchored Shh-N (Fig. 7). We first demonstrated that Shh-N::CD4 expressed in COS cells is completely membrane-tethered. Shh-N::CD4 is detected only in the cell lysate from cells transfected with Shh-N::CD4 and was
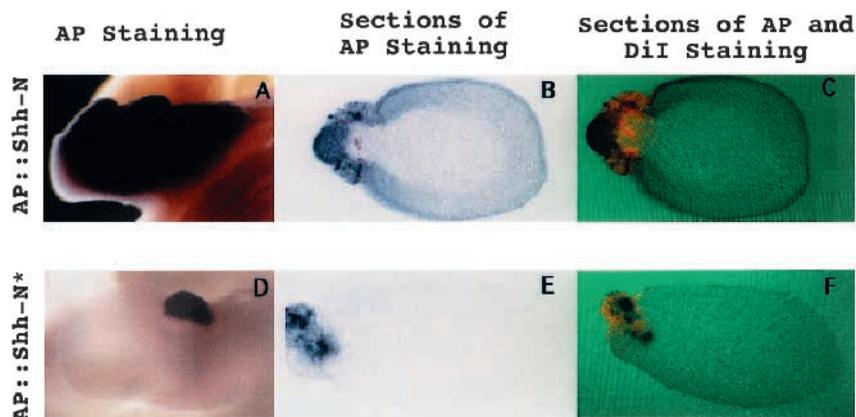

Fig. 5. Diffusion of Shh protein in chick limb. COS cell pellets expressing AP-tagged Shh proteins were grafted under the anterior apical ectodermal ridge, and distribution of Shh was analysed by whole-mount AP staining 20 hours after implantation (A,D). AP::Shh-N (A) traverses a substantial distance (>200 μm/20 hours) from its site of production while no diffusion of AP::Shh-N* was detected (D). Vibratome sections of these AP-stained chick wing buds were also performed to visualise the extent of diffusion. A broad distribution of AP staining through limb mesenchyme was observed in the limb bud implanted with cells expressing AP::Shh-N (B). In contrast, AP staining was only detected at the site of cell implantation in the limb bud implanted with cells expressing AP::Shh-N* (E). Lack of staining in the central region of the limb (B) is mostly likely due to poor penetration of staining solutions during whole-mount AP staining procedure. To follow the final destination of grafted COS cells in the chick wing bud, they were DiI-labelled before being grafted. (C,F) DiI fluorescent images of limb sections superimposed with the AP-staining images (B,E). Thus grafted COS cells stay at the position where they were implanted while AP-staining in C, but not in F, extends far beyond the DiI signals. (A,D) Dorsal view of limb buds, anterior is up and posterior down; (B,C,E,F) anterior is to left and posterior right; dorsal is up and ventral down.
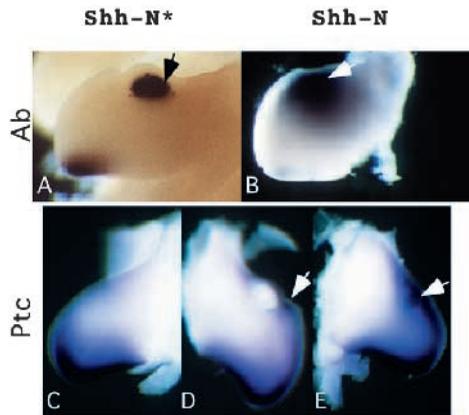
Fig. 6. Induction of *Ptc* expression by diffusible and non-diffusible Shh signal. Shh protein expressed from grafted COS cells is localized to the cell pellet (A) whereas Shh-N coated on the implanted bead diffused broadly in the limb mesenchyme (B) as revealed by immunostaining with Shh antibodies. Similarly, induction of *Ptc* transcripts by *Shh*-expressing cells is localized in regions immediately adjacent to the cell graft (D) while in contrast, Shh-N released from the bead induced a fairly broad domain of Ptc expression (E). (C) *Ptc* expression in the unoperated contralateral limb. Arrows in A and B point to the exogenously supplied Shh-N revealed by antibody staining. Arrows in D and E point to the induced *Ptc* transcripts.

not found in the medium even in the presence of suramin, indicating that it is membrane-tethered (Fig. 8). The activity of Shh-N::CD4 was then determined by examining resulting digit patterns after grafting pellets of COS cells as described above. We found that cell aggregates with 90-80% transfected COS cells were able to induce mirror-image digit duplication in a pattern **4334** (*n*=3, Fig. 9A,B). These data demonstrated that Shh-N when membrane-anchored is able to induce the most posterior digit, digit **4**, an indication that it is fully active. Furthermore, this localized Shh-N signal is able to specify ectopic digits with at least two different positional values i.e., **4334** (*n*=3, Fig. 9A,B) or **32234** (*n*=2, Fig. 9C). In addition, when the percentage of transfected COS cells was reduced to 20%, the resulted digit pattern became **2234** in most cases (*n*=3, Fig. 9D,E). Occasionally, a digit pattern **3234** (*n*=1, Fig. 9F) was observed. This dose-response relationship is similar to that observed using the wild-type Shh. Taken together, these results indicate that Shh-N when completely membrane-tethered exhibits a similar activity to that of wild-type Shh and suggest that Shh acts short range.

(F) Downstream signals induced by Shh

If Shh is not a direct long-range signal, how may the dose-dependent properties of Shh in limb patterning be explained? Two possibilities are that other signalling molecule(s) may be activated in limb bud cells in response to Shh or that cooperation between Shh and other signalling molecules could lead to dose-dependent digit specification and long-range effects on cells. *Fgf-4* was induced in anterior apical ridge following grafts containing *Shh* cells and appears to be independent of cell number (data not shown). In contrast, activation of *Bmp-7* in anterior mesenchyme was dose-dependent (Table 4; Fig. 10); an extensive domain of *Bmp-7* expression was induced with high numbers (95%) of *Shh*-expressing cells (Fig. 10b). With fewer *Shh*-expressing cells (20%), activation of *Bmp-7* in anterior mesenchyme was only weakly detectable (Fig. 10d).
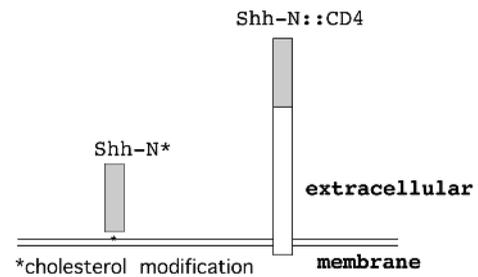

Fig. 7. Schematic diagram of expression constructs encoding membrane-tethered Shh proteins. As described in Fig. 3, Shh-N* is attached to the membrane presumably through cholesterol modification. Another membrane-tethered Shh protein was generated by fusing the integral membrane protein CD4 to the C terminus of Shh-N and the resulting construct was designated Shh-N::CD4. The CD4 protein is shown as open bar and Shh-N shown as shaded bar.

In contrast, expression of *Bmp-7* in anterior apical ectodermal ridge was very strong in all wings grafted with *Shh*-expressing cells (Fig. 10d). *Hox-d13* and *Hox-d11* gene expression also displayed a dose-response to Shh (Fig. 10f-l).

Changes in gene expression (Fig. 11A-C) occur at the start of the promotion phase suggesting that expression of at least some of these genes may be involved in digit specification (Table 4). By 16 hours after application of Shh protein, the normal domains of expression of *Fgf-4*, *Bmp-2* and *Hoxd-13* were all extended anteriorly and ectopic expression was clearly seen at 20-24 hours (Fig. 11D-F).

To explore whether expression of these genes or other as yet unidentified signalling molecules operate downstream of Shh as
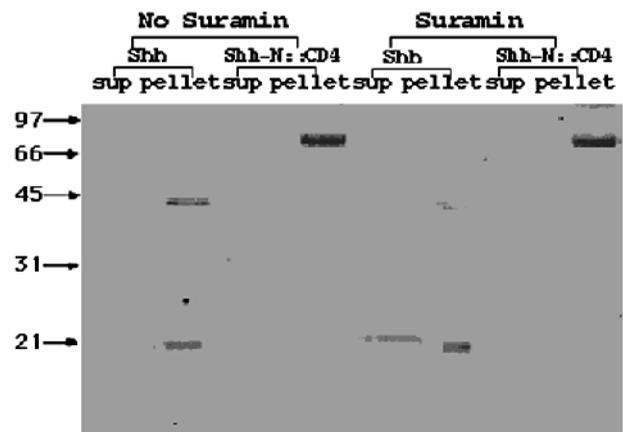

Fig. 8. Expression of membrane-tethered Shh-N in COS cells. COS cells were transfected with constructs encoding either Shh or Shh-N::CD4, and medium (sup) and cell lysate (pellet) were analyzed by SDS-PAGE and immunoblotted with anti-Shh antibodies. Shh produces two species; the higher M_r band corresponds to the unprocessed Shh and the lower band represents Shh-N* that was cleaved from Shh and cholesterol modified. Both proteins were only detected in the cell lysate in the absence of suramin, and Shh-N* was released into the medium upon suramin treatment as described above. A single band corresponding to the predicted M_r of Shh-N::CD4 is detected only in the cell lysate from cells transfected with Shh-N::CD4 and was not found in the medium even in the presence of suramin, indicating that Shh-N::CD4 is membrane-tethered. Molecular size standards are indicated on the left ($\times 10^{-3}$).

Fig. 9. Wing skeletal patterns after grafts of Shh-N::CD4-expressing cells mixed with different proportions of CEFs. (A-C) With 80% Shh-N::CD4-expressing cells, mirror-image digit duplication was observed. The pattern varies from **4334** (A) to **43** (a partial **3**) **34** (B) to **32234** (C). In all cases, more than one ectopic digit was induced. Arrow in C points to a small cartilaginous blip at a position that will form digit **4**. (D-F) With 20% Shh-N::CD4-expressing cells, only a duplicated digit **2** was induced (D,E) in most cases and occasionally digit **3** was induced (F). The percentages here refer to the relative proportions of COS and CEF cells and not the percentage of COS cells expressing Shh after transient transfection which was usually around 40% of cells.
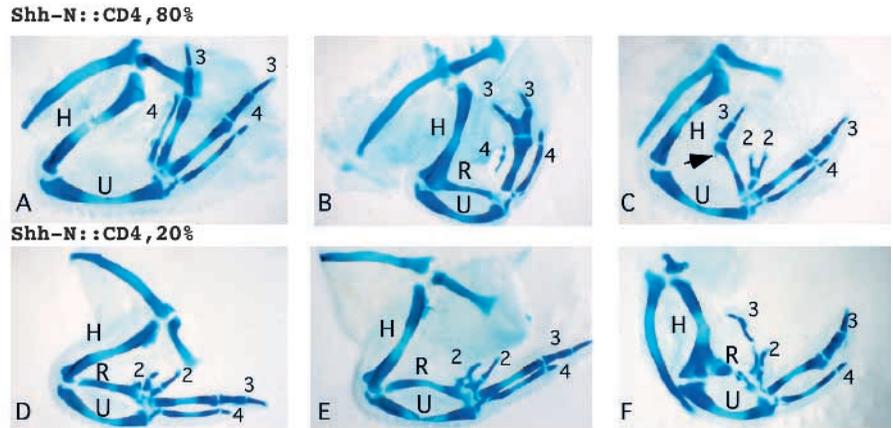
polarizing signals, we investigated whether anterior tissue next to a Shh bead acquires polarizing activity. Either 16-17 hours or 20-24 hours after implantation of the bead, we removed mesenchyme next to the Shh-soaked bead and grafted it to the anterior margin of a wing bud (stage 20). In 19 out of 21 cases,

Fig. 10. Dose-dependent effects of *Shh*-expressing cells (shown in diagram) on gene expression in chick wing buds. (a) Normal expression of *Bmp-7*. (b) 95% *Shh-expressing* cells induce an extensive expression domain of *Bmp-7*. (c) 80% *Shh*-expressing cells induce a medium expression domain of *Bmp-7*. (d) 20% *Shh*-expressing cells induce a small expression domain of *Bmp-7*. (e) Normal expression of *Hoxd-11*. (f) 95% *Shh*-expressing cells induce extensive domain of *Hoxd-11*. (g) 80% *Shh*-expressing cells induce medium expression domain of *Hoxd-11*. (h) 20% *Shh*-expressing cells induce small expression domain of *Hoxd-11*. (i) Normal expression of *Hoxd-13*. (j) 95% *Shh*-expressing cells induce extensive expression domain of *Hoxd-13*. (k) 80% *Shh*-expressing cells induce medium expression domain of *Hoxd-13*. (l) 20% *Shh*-expressing cells induce small expression domain of *Hoxd-13*. Arrows indicate ectopic gene expression.

the grafted wings were normal, whereas 2 had a small cartilaginous fragment (blip) (Table 2B). In contrast, 50% of grafts of the polarizing region from manipulated or contralateral limb buds gave duplications with additional posterior digits (Table 2B). Therefore although Shh-soaked beads induced duplications, adjacent cells have no detectable polarizing activity when assayed 16-24 hours after bead implantation.
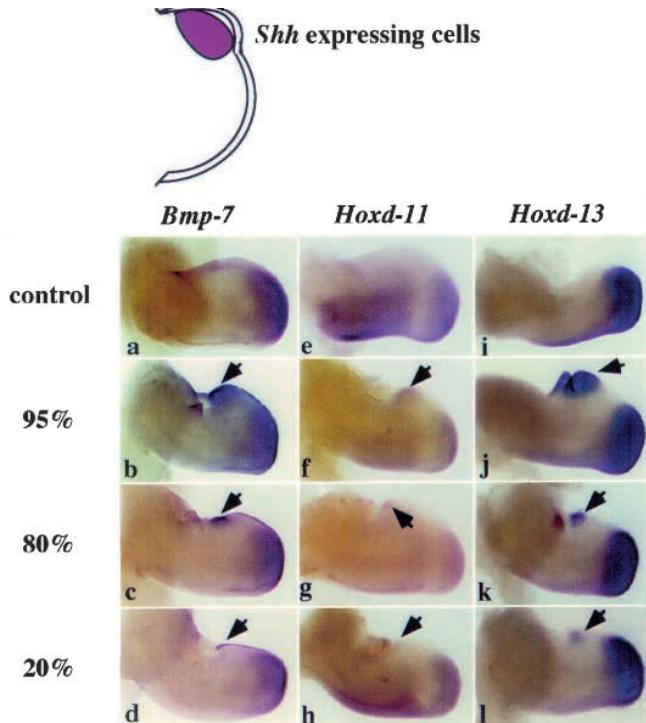
DISCUSSION

Our main finding is that Shh can produce dose-dependent changes in digit pattern. Thus the primary determinant of antero-posterior pattern in the limb is likely to be the concentration of Shh. Cells from a maximum distance of about 300 μm from a Shh source can be recruited to form additional digits but Shh processed by cells does not appear to be able to diffuse this far. Shh-N released from beads and Shh processed by COS cells exhibit dramatic difference in diffusibility in the limb mesenchyme. This correlates with the much broader domain of *Ptc* expression induced by the widely diffused Shh-N as compared to that induced by the apparently non-diffusible Shh-N*. *Ptc* has been suggested to be an indicator of exposure of cells to Hh signalling in both *Drosophila* and vertebrates (Perrimon, 1995; Goodrich et al., 1996). Therefore, since polarizing activities of diffusible and non-diffusible forms of Shh are similar, the critical action of Shh appears to be short range. Consistent with this, a membrane-tethered Shh exhibits similar activity to that of wild-type Shh in digit induction although we cannot rule out the possibility of some uncharacterized and undetected proteolytic cleavage of membrane-tethered Shh in vivo. Short-range signalling by Shh could still directly specify each digit in a dose-dependent fashion if production of Shh was propagated by a relay mechanism with progressive attenuation. However, this possibility is excluded because application of Shh does not induce *Shh* expression. Therefore, we conclude that it is unlikely that endogenous Shh produced by the polarizing region in the chick limb bud directly governs the behavior of cells at different distances from the polarizing region but instead it acts short range.

If the Shh signal does not directly act on cells to determine which digit they will form, there must be other downstream signal(s) activated by Shh that act in a dose-dependent manner. We showed that *Bmp*s are activated in a dose-dependent fashion in response to *Shh*-expressing cells. However, it has not been possible to duplicate the effects of Shh signalling on digit pattern by application of BMP-2 protein (Francis et al., 1994)
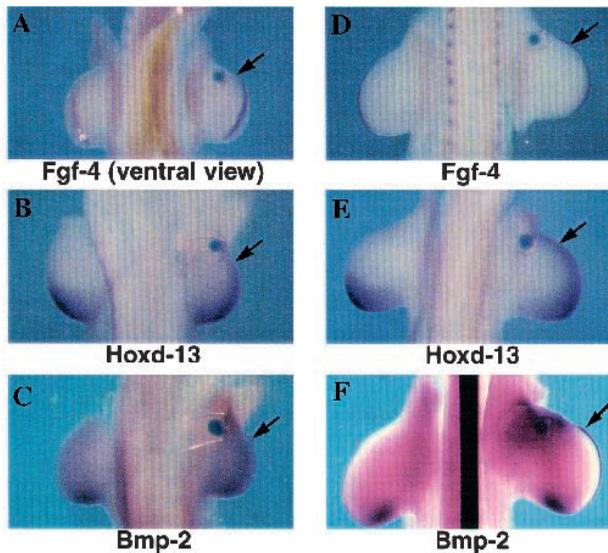
Fig. 11. Effects of different lengths of exposure to Shh on gene expression in anterior tissues of chick wing buds. Beads soaked in 16 mg/ml Shh protein were implanted under anterior apical ectodermal ridge and the embryos were subsequently fixed at different time intervals. (A-C) Embryos fixed 16 hours after bead application. Arrows show extended domains of expression of *Fgf-4*, *Hoxd-13* and *Bmp-2*, respectively. (D-F) Embryos fixed 20-24 hours after bead application. There is now marked ectopic expression of *Fgf-4*, *Hoxd-13* and *Bmp-2* in anterior tissues (compare with A-C and with normal contralateral wings). Note that expression of *Bmp-2* is induced in ectoderm as well as in mesenchyme.

and grafts of cells expressing *Bmp-2* also have only weak polarizing activity (Francis-West et al., 1995; Duprez et al., 1996). Furthermore, we have not been able to detect polarizing activity in cells that have been adjacent to an Shh bead. These observations suggest that Shh may activate more than one secondary signal and that continual Shh input may be required to produce secondary signal(s).

An important feature of Shh signalling is its time dependency. Irreversible digit pattern changes are not brought about until 16 hours after Shh application. Digit **2** is specified first and then progressively more posterior digits are specified over the next 8 hours or so. Since the mean number of digits remains almost constant but the character of additional digits changes with time, this suggests that cells, which would have initially formed anterior digits, are promoted with continued exposure to form more posterior digits (see also model by Eichele et al., 1985). Our data with DiI- and DiA-labelled cell populations indicate that this indeed appears to be the case; cells that will participate in forming a more posterior digit when the Shh bead is left in place, form digit **2** when the bead is removed early. In the promotion phase, cells may respond according to the highest concentration of signalling molecule(s) that they see. Another possibility is that cells integrate over time the strength of a Shh signal or a Shh-dependent signal to which they have been exposed. In this model, cells in the limb would not sense morphogen concentration at a particular time but instead continuously interpret the morphogen signal. Precedence for this comes from recent observations of positional signalling in frog embryos (Gurdon et al., 1995).

Specification of a series of digits across the anteroposterior

pattern of the limb appears to involve a cascade of signals generated at the posterior margin of the limb bud. Retinoic acid can activate *Shh* expression in a dose-dependent fashion (Yang and Niswander, 1995) and we have shown that Shh signalling in turn is dose-dependent. Blocking of retinoic acid synthesis at pre-limb bud stages disrupts limb initiation (Stratford et al., 1996; Helms et al., 1996), while inactivation of Shh does not prevent limb initiation but instead results in limb truncation (Chiang et al., 1996). This suggests that retinoic acid may function in early polarizing signalling and Shh may signal later. Our bead removal experiments also indicate that continuous Shh signalling does not appear to be essential for patterning the most distal parts of the skeleton since these are not specified until at least 24 hours after bead removal (Summerbell, 1974). The idea that Shh is not required continuously is consistent with the observation that, when *Shh* expression is reduced in chick limb buds as a consequence of inhibiting retinoic acid synthesis at the bud stage (Stratford et al., 1996), digit development is normal. Also, some digit development still occurs in the ld mutant which has weak and transient *Shh* expression (Haramis et al., 1995; Kuhlman and Niswander, 1997).

We have shown here that *Bmp*s are activated in a dose-dependent manner by Shh at the time that digit promotion occurs. It is possible that establishment of a morphogen gradient occurs during this short phase of digit promotion rather than during the first priming phase. Thus, it is possible that secondary signals such as BMPs act as dose-dependent signals to promote digit specification once the limb bud has been primed in some way by a short-range Shh signal. It should be noted that the cell-marking experiments show that the most posterior digits come from cells very close to the bead. Diffusion of BMPs could perhaps extend the range of a polarizing signal and perhaps even specify the most anterior digit in the absence of Shh priming as has been observed by grafting *Bmp-2*-expressing cells to anterior mesenchyme (Duprez et al., 1996). Since the potential ability of BMP to specify posterior digits may depend on prior Shh priming and cells primed by Shh may produce several different BMPs and signalling molecules other than BMPs, this could explain, for example, why BMP-2 protein (or *Bmp-2* expression) alone cannot provide a complete polarizing signal.

Finally, it should be noted that it has proved very difficult in a number of systems to distinguish between long-range and short-range signalling. Although in chick spinal cord development, it now seems that floor plate and motor neuron development involves both long-range and short-range Shh signalling (Ericson et al, 1996). In insect wing development, direct long-range signalling has been demonstrated by expression of mutated receptors that block formation of structures at a distance from the signalling source (Lecuit et al., 1996), while there is also evidence for simultaneous short-range signalling (Nellen et al., 1996). In *Xenopus*, different experiments have implicated both long-range and short-range signalling in mesoderm specification (Reilly and Melton, 1996; Jones et al., 1996). Specification of limb digits also appears to be just as complex and to involve multiple signals and there may also be different downstream targets that differ in their requirements for activation.

REFERENCES

Bitgood, M. J. and McMahon, A. P. (1995). Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. *Dev. Biol.* **172**, 126-138.

Bumcrot, D. A., Takada, R. and McMahon, A. P. (1995). Proteolytic processing yields two secreted forms of sonic hedgehog. *Molec. Cell. Biol.* **15**, 2294-303.

Cheng, H.J. and Flanagan, J. G. (1994). Identification and cloning of ELF-1, a developmentally expressed ligand for the Mek4 and Sek receptor tyrosine kinases. *Cell* **79**, 157-168.

Cheng, H.J., Nakamoto, M., Bergmann, A.D. and Flanagan, J.G. (1995). Complementary gradients in expression and binding of ELF-1 and Mek4 in development of the topographic retinotectal projection map. *Cell* **82**, 371-381.

Chiang, C., Litingtung, Y., Lee, E., Young.K. E., Cordent, J. L., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383**, 407-413.

Duprez, D. M., Kostakopoulou, K., Francis-West, P. H., Tickle, C. and Brickell, P. M. (1996). Activation of expression of FGF-4 and HoxD gene expression by BMP-2 expressing cells in the developing chick limb. *Development* **122**, 1821-8.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). Sonic hedgehog a member of a family of putative signalling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-1430.

Eichele, G., Tickle, C. and Alberts, B. M. (1985). Studies on the mechanism of retinoid-induced pattern duplications in the early chick limb bud: temporal and spatial aspects. J. *Cell Biol.* **101**, 1913-1920.

Ericson, J., Muhr, J., Jessell, T. M. and Edlund, T. (1995). Sonic hedgehog a common signal for ventral patterning along the rostrocaudal axis of the neural tube. *Int. J. Dev. Biol.* **39**, 809-16.

Ericson, J., Morton, S., Kawakami, A., Roelink, H. and Jessell, T.M. (1996). Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. *Cell* **87**, 661-673.

Fan, C. M., Porter, J. A., Chiang, C., Chang, D.T., Beachy, P.A., and Tessier-Lavigne, M. (1995). Long-range sclerotome induction by *Sonic hedgehog*: direct role of the amino-terminal cleavage product and the modulation by the cyclic AMP signalling pathway. *Cell* **81**, 457-465.

Francis, P. H., Richardson, M. K., Brickell, P. M. and Tickle, C. (1994). Bone morphogenetic proteins and a signalling pathway that controls patterning in the developing chick limb bud. *Development* **120**, 209-218.

Francis-West, P. H., Robertson, K. E., Ede, D. A., Rodriguez, C. Ipsizua-Belmonte, J. C., Houston, B., Burt, D. W., Gribbin, C., Brickell, P. M. and Tickle, C. (1995). Expression of genes encoding bone morphogenetic proteins and sonic hedgehog in talpid (ta³) limb buds: their relationships in the signalling cascade involved in limb patterning. *Dev. Dyn.* **203**, 187-197.

Goodrich, L. V., Johnson, R. L., Milenkovic, L., McMahon, J. A., and Scott, M.P. (1996). Consrvation of the hedgehog/patched signalling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. *Genes Dev.* **10**, 301-312.

Gurdon, J. B., Mitchell, A. and Mahony, D. (1995). Direct and continuous assessment of cells of their position in a morphogen gradient. *Nature* **376**, 520-521.

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

Haramis, A. G., Brown, J. M. and Zeller, R. (1995). The limb deformity mutation disrupts the SHH/FGF-4 feedback loop and regulation of 5′ HoxD genes during limb pattern formation. *Development* **121**, 4237-45.

Helms, J. A., Kim, C. H., Eichele, G. and Thaller, C. (1996). Retinoic acid is required during early chick limb development. *Development* **122**, 1385-94.

Honig, L. (1981). Positional signal transmission in the developing chick limb bud. *Nature* **291**, 72-73.

Jones, J. M., Armes, N. and Smith, J. C. (1996). Signalling by TGF-ß family members: short-range effects of Xnr-2 and BMP-4 contrasts with the long-range effects of activin. *Curr. Biol.* **6**, 1468-1476.

Kuhlman, J. and Niswander, L. (1997). Limb deformity proteins: role in mesodermal induction of apical ridge. *Development* **124**, 133-139.

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 feedback loop to integrate growth and patterning of the developing limb bud. *Cell* **79**, 993-1003.

Lecuit, T., Brook, W. J., Ng, M., Calleja, M., Sun, H. and Cohen, S. M. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the Drosophila wing. *Nature* **381**, 367-93.

Lopez-Martinez, 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.

Marti, E., Bumcrot, D., Takada, R. and McMahon, A. P. (1995a). Requirement of 19K form of Sonic Hedgehog for induction of distinct ventral cell types in CNS explants. *Nature* **375**, 322-325.

Marti, E., Takada, R., Bumcrot, D. A., Sasaki, H. and McMahon, P. A. (1995b). Distribution of Sonic hedgehog peptides in the developing chick and mouse embryo. *Development* **121**, 2537-2547.

Nellen, D., Burke, R., Struhl, G. and Basler, K. (1996). Direct and long-range action of a DPP gradient. *Cell* **85**, 357-68.

Nieto, M. A., Patel, K. and Wilkinson, D. G. (1996). *In situ* hybridisation analysis of chick embryos in whole mount and tissue cultures. *Methods in Cell Biology* **51**, 219-35.

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

Niswander, L., Jeffrey, S., Martin, G. and Tickle, C. (1994). Positive feedback loop in signalling in vertebrate limb development. *Nature* **371**, 609-612.

Parr, B. A. and McMahon, A. P. (1995). Dorsalising signal Wnt-7a required for normal polarity of D-V and A-P axes of mouse limb. *Nature* **374**, 350-353.

Perrimon, N. (1995). Hedgehog and beyond. *Cell* **80**, 517-520.

Porter, J. A., Ekker, S. C., Pard, W. J., Von-Kessler, D.P., Young, K. E., Chen, C. H., Ma, Y., Wood, A. S., Cotter, R. J., Koonin, E. V. and Beachy, P. A. (1996). Hedgehog patterning activity: a role of a lipophilic modification mediated by the carboxy-terminal autoprocessing domain. *Cell* **86**, 21-34.

Reilly, K. M. and Melton, D. A. (1996). Short-range signalling by candidate morphogens of the TGF beta family and evidence for a relay mechanism of induction. *Cell* **86**, 743-54.

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

Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A. and Jessell, T. M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. *Cell* **81**, 445-455.

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

Smith, J. C. (1980). The time required for positional signalling in the chick wing bud. *J. Embryol. Exp. Morph.* **60**, 321-328.

Stratford, T., Horton, C. and Maden, M. (1996). Retinoic acid is required for the initiation of outgrowth in the chick limb bud. *Current Biol.* **6**, 1124-33.

Summerbell, D. (1974). A quantitative analysis of the effect of the excision of the AER from the chick limb bud. *J. Embryol. Exp. Morph.* **32**, 651-660.

Tickle, C. (1981). The number of polarising 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.

Vortkamp, A., Lee, K., Segre, G. V., Kronenberg, H. M. and Tabin, C. J. (1996). Regulation of rate of cartilage differentiation by Indian-hedgehog and PTH-related protein. *Science* **273**, 613-22.

Wilkinson, D. G. (1992). *In Situ Hybridisation. A Practical Approach.* (ed. D. Rickwood and B. D. Hames) Oxford: IRL Press/ Oxford University Press.

Yang, Y. and Niswander, L. (1995). Interaction between the signalling molecules WNT and SHH during vertebrate limb development: dorsal signals regulate anteroposterior patterning. *Cell* **50**, 939-947.