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A model for anteroposterior patterning of the vertebrate limb based on sequential long- and short-range Shh signalling and Bmp signalling

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SUMMARY

It has been proposed that digit identity in chick limb bud is specified in a dose-dependent fashion by a long-range morphogen, produced by the polarising region. One candidate is Sonic hedgehog (Shh) protein, but it is not clear whether Shh acts long or short range or via Bmps. Here we dissect the relationship between Shh and Bmp signalling. We show that Shh is necessary not only for initiating *bmp*2 expression but also for sustaining its expression during the period when additional digits are being specified. We also show that we can reproduce much of the effect of Shh during this period by applying only Bmp2. We further demonstrate that it is Bmps that are responsible for digit specification by transiently adding Noggin or Bmp antibodies to limbs treated with Shh. In such limbs, multiple additional digits still form but they all have the same identity. We also explored time dependency and range of Shh signalling by examining *ptc* expression. We show that high-level *ptc* expression is induced rapidly when either Shh beads or polarising regions are grafted to a host limb. Furthermore, we find that high-level *ptc* expression is first widespread but later more restricted. All these data lead us to propose a new model for digit patterning. We suggest that Shh initially acts long range to prime the region of the limb competent to form digits and thus control digit number. Then later, Shh acts short range to induce expression of Bmps, whose morphogenetic action specifies digit identity.

Key words: Shh, Bmp, Limb development, Patched, Signalling, Chick

INTRODUCTION

The specification of the digits in the vertebrate limb is an excellent example of patterning in vertebrate embryos. The chick wing has three digits, designated **2**, **3** and **4**. These arise at different positions across the anteroposterior axis (digit **2** being most anterior and digit **4** most posterior) and are morphologically distinct. Anteroposterior digit pattern is controlled by the polarising region, a small region of mesenchyme cells at the posterior margin of the limb bud (Saunders and Gasseling, 1968). When the polarising region is grafted from one wing bud to the anterior margin of a second wing bud, a pattern of duplicated digits results, with an additional **432** in mirror-image symmetry with the normal set of digits **234** (Saunders and Gasseling, 1968; Tickle et al., 1975).

A gradient model has been proposed to account for signalling by the polarising region (Tickle et al., 1975). According to this model, the polarising region produces a diffusible morphogen that sets up a concentration gradient across the limb bud. Thus cells at different positions across the limb bud will be exposed to different concentrations of morphogen and can read the local morphogen concentration to find their position. Cells exposed to high concentrations of morphogen will form digit **4**, while cells exposed to a low concentration will form digit **2**. The essential features of this model are that the morphogen acts in a dose-dependent manner and is long range (Tickle et al., 1975).

A number of signalling molecules have been found to be associated with the polarising region. Retinoic acid was the first defined chemical to be discovered that could mimic the activity of the polarising region (Tickle et al., 1982, 1985). Retinoic acid is now known to induce expression of a gene encoding another signalling molecule, Sonic Hedgehog (Shh) (Riddle et al., 1993) and Shh can reproduce the effects of retinoic acid on digit patterning (Riddle et al., 1993; Lopez-Martinez et al., 1995; Yang et al., 1997). Genes encoding Bone Morphogenetic Proteins (Bmps) have also shown to be expressed in the polarising region (Francis et al., 1994) and their expression is induced by Shh (Laufer et al., 1994; Yang et al., 1997), but Bmps cannot reproduce the effects of Shh on digit patterning (Duprez et al., 1996). Thus Shh appears to be a good candidate for the polarising morphogen that specifies digit identity.

Specification of additional digits from anterior mesenchyme has been shown to occur in two phases (Smith, 1980; Eichele et al., 1985; Yang et al., 1997). The first phase lasts for about 12-14 hours (Smith, 1980; Eichele et al., 1985; Yang et al., 1997) after grafting a polarising region or applying a polarising signal (beads soaked in either retinoic acid or Shh). During this phase, no irreversible changes in digit patterns are induced and this has been called the priming phase (Eichele et al., 1985). In a second phase, lasting about 8-10 hours, digits are progressively specified and promoted so that more digits are formed and digits with more posterior identity. For example, if a Shh-soaked bead is removed after 16 hours (at the start of the promotion phase) only an additional digit **2** is formed, while if the bead is not removed until 24 hours, at the end of the specification phase, additional digit **2**, **3** and **4** result (Yang et al., 1997). One can explain the generation of this sequence of digit patterns by spread of a diffusible signal and progressive "promotion" of anterior digits into posterior digits (Tickle, 1995). *bmp2* expression in anterior mesenchyme can first be detected at 16 hours after Shh application, which is the start of digit specification and intensity of expression increases throughout the specification phase (Yang et al., 1997). Here we test whether Bmps are involved in digit specification and distinguish between the function of Shh and Bmps in specification of digit type.

One of the problems with the proposal that Shh is the polarising morphogen is whether it can act long range. Beads soaked in high concentrations of the N-terminal peptide of Shh give full digit duplications and the peptide diffuses widely from the beads into the limb bud (Yang et al., 1997). However, when Shh is produced by cells under physiological conditions, it undergoes autocatalytic processing with the addition of cholesterol and it is not clear whether this form of Shh is freely diffusible (Porter et al., 1996). Further, it has recently become apparent that there are several mechanisms to restrict the range of Hedgehog movement. In *Drosophila* (Chen and Struhl, 1996, 1998) and, presumably also in vertebrates, Hedgehog diffusion is contained by binding to Ptc, its receptor (Stone et al., 1996; Goodrich et al., 1996; Marigo et al., 1996a,b). Another recently discovered cell surface Hedgehog-binding protein, Hip (Hedgehog-interacting protein), could also limit Hedgehog signalling (Chuang and McMahon, 1999). High-level expression of the genes encoding these binding proteins is induced in response to Sonic Hedgehog signalling (Marigo et al., 1996b; Chuang and McMahon, 1999). Thus, the signalling molecule itself regulates expression of proteins that attenuate and limit the range of its action. In the polydactylous *talpid*^{3} chicken mutant, where there is no high-level expression of *ptc*, we have suggested that Shh protein diffuses widely (Lewis et al., 1999) and that this accounts for the extra digits.

Paradoxically, Ptc is not only the receptor for Hedgehog proteins but also acts in cells that have not received a Hedgehog signal to repress the signal transducing component of the multimeric Hedgehog receptor, Smoothened (Smo) (Ingham and Hidalgo, 1993; Chen and Struhl, 1996, 1998; reviewed by Ingham, 1998a). Constitutive activation of the Shh signalling pathway has been suggested to account for the extra digits in the polydactylous *talpid*^{2} mutant (Carrucio et al., 1999). The importance of these properties of Ptc is shown by the consequences of *ptc* mutations in humans, which lead to basal cell carcinomas (Hahn et al., 1996; Fan et al., 1997; reviewed by Ingham, 1998b).

The distribution of Shh protein has been revealed in developing limb and other regions of embryos by antibody labelling studies (Marti et al., 1995). However it is not clear that these methods are sensitive enough to detect small amounts of Shh protein. Therefore high-level *ptc* expression, which is normally induced in response to Shh, has been used as a reporter for Sonic Hedgehog protein distribution (Marigo et al., 1996b) and we use it here to examine the range of Shh signalling in the priming and promotion phases. Our results suggest a new model for digit specification in which two phases of Shh signalling are followed by the morphogenetic action of Bmps.

MATERIALS AND METHODS

Manipulation of chick embryos

Fertilised White Leghorn eggs were obtained from Poyndon Farm, Waltham Cross, Hertfordshire, UK. Eggs were incubated at 38±1°C and windowed on day 2 or 3 of incubation. Embryos were staged according to the method of Hamburger and Hamilton (1951).

Beads soaked in proteins or pellets of cells or fragments of chick limb-bud tissue were grafted to chick host limb buds. The implants were placed under a loop of apical ridge at the anterior margin of wing buds of stage 19/20 chick embryos and the beads, when necessary, secured with a platinum staple. The apical ridge was cut away from the underlying mesenchyme using a sharp tungsten needle. In some experiments, beads were removed at different time points after implantation using a sharp needle. When beads were removed and replaced by a new bead, the new beads were poked into the hole left by the first bead under the apical ridge. When a second bead or a piece of cell pellet was added to wings containing an Shh bead, they were inserted into a slit made under the apical ectodermal ridge just anterior and distal to the Shh bead.

Preparation of beads

(1) To prepare Shh beads, a 3 μl drop of Shh-N protein solution (14 mg/ml) was placed on a bacteriological grade Petri dish. Stock solutions were stored at –70°C and dilutions were made in 20 mM Tris-HCl, 120 mM NaCl buffer. Affi-gel blue CM beads (Biorad) (200-250 μm in diameter) were rinsed in the above buffer and then soaked in a 3 μl drop of Shh for 1-2 hours (or longer) at room temperature. On some occasions, beads were kept at 4°C in the drop of Shh protein and were used on subsequent days. An aliquot of Shh protein solution was not used for more than 2 weeks after thawing.

(2) Stock Bmp solutions were stored at –70°C and dilutions were made with 0.1% BSA in PBS (stock solution kept at 4°C) using siliconised pipette tips and Eppendorf tubes. A 2 μl drop of Bmp2 solution (1 mg/ml-0.01 mg/ml) (Genetics Institute) was placed in a bacteriological grade Petri dish. Heparin beads (Sigma Cat No: H-5236) 200-250 μm in diameter, were rinsed in PBS, then transferred to the 2 μl drop of Bmp2 solution and soaked for 1-2 hours.

(3) A stock solution of 1.74 μg/ml of Fgf4 (R&D systems) was stored at –20°C. Heparin beads were rinsed in PBS before being transferred to the 2 μl drop of Fgf4. They were soaked in Fgf4 for 1 hour at room temperature.

(4) Purified *Xenopus* Noggin protein (a kind gift of Richard Harland) was used at a concentration of 0.2 mg/ml. Affi-gel blue CM beads were soaked in a 2 μl drop of Noggin protein for 1 hour at room temperature and stored at 4°C for subsequent use.

(5) Anti-Bmp2/4 (A-20) polyclonal antibody (Santa Cruz Biotechnology Inc. catalogue sc-6267) was used at a concentration of

200 μg/ml. Affi-gel blue CM beads were soaked in the antibody for 1 hour at room temperature.

(6) Control beads (Affi-gel blue CM or heparin beads) were soaked in a 2 μl drop of PBS for 1 hour.

(7) 5×10^6 *bmp2*-expressing cells (QT6 cells, Duprez et al., 1996) were plated in a 100 mm bacteriological Petri dish (Nunc) 24 hours before grafting. Cell aggregates were incubated overnight in selection medium (F12) containing 800 μg/ml G418. The following day, the cell aggregates were washed twice with medium (with no serum and antibiotics) and then kept in medium supplemented with serum and antibiotics, but without any G418. Compact spherical cell aggregates of about 150-200 μm in diameter were selected using a stereomicroscope. Cell aggregates were transferred to the chick host using a Gilson pipette. These non-proliferative cell aggregates were used for grafting experiments on the same day only.

(8) Noggin-expressing B3 cells were produced in Dr Richard Harland's laboratory from a CHO cell-line transfected with a *Xenopus* Noggin expression vector (Lamb et al., 1993). Aliquots of cells were kept in liquid Nitrogen at −180°C until required. Cells were quickly thawed at 37°C in a water-bath and plated in 6 cm tissue-culture dishes. Cells were grown in MEM Alpha Medium (Gibco) without ribonucleosides and deoxyribonucleosides, supplemented with 10% dialysed foetal calf serum (FCS; Gibco), 1% penicillin/streptomycin and 80 μM methotrexate and incubated at 37°C in 5% CO_2 atmosphere. Parental CHO cells were used as controls. They were grown in MEM Alpha with ribonucleosides and deoxyribonucleosides, supplemented with 10% FCS and 1% penicillin/streptomycin (all from Gibco). Cells were plated in 6 cm dishes and, at confluency, they were washed twice in PBS and the medium replaced by growth medium (MEM 25 mM Hepes medium supplemented with 10% FCS, 2 mM glutamine and 1% penicillin/streptomycin). Cells were scraped off the plate with a small piece of silicon and small pellets were cut and transferred to a separate dish containing growth medium. Cell pellets were transferred to the embryo with a Gilson pipette and grafted into in a slit made in the limb buds.

Grafts of limb tissue and polarising region tissue

In all cases where limb mesenchymal tissue was grafted into host embryos, the same principles for preparing the tissue were applied. Tissue taken for polarising region grafts was based on the maps published by MacCabe et al. (1973).

Limb buds were dissected from chick embryos, trypsinised for 1 hour at 4°C and then the ectoderm removed. The required pieces of mesoderm were subsequently cut from the buds using tungsten needles. In some cases, dissected polarising region tissue was incubated in 10 μg/ml DiI in tissue culture medium (with serum) for 30 minutes at 37°C before grafting.

When tissue was removed from next to an Shh bead, a slit was made in the wing buds at the position of the bead, using a tungsten needle and the Shh bead was removed. The tissue around the bead extending up to 100-150 μm distally was cut out and trypsinised to remove the ectoderm. The isolated mesenchyme was then grafted into a new host wing bud under a loop of the apical ridge. Tissues were transferred to chick hosts using a Gilson pipette and placed into position in a loop under the anterior apical ridge using a sharp needle.

Alcian green staining for cartilage

Embryos were fixed in 5% trichloroacetic acid (TCA) overnight at room temperature, then stained with 0.1% (w/v) alcian green in acid alcohol (70% (v/v) ethanol, 1% HCl), for between 4 hours and overnight. The embryos were then washed in acid alcohol for between 3 hours and overnight and then several times in absolute ethanol for 3 hours minimum. Finally, the embryos were cleared and stored in methyl salicylate.

Calculation of polarising activity

In order to calculate polarising activities, we score limbs according to the most posterior additional digit that is specified. An extra digit **4** has a positional value of 4, a digit **3** has a positional value of 2, the value for a digit **2** is 1, while that for an extra *blip* is 0.5. For 100% polarising activity all the specimens should have an extra digit 4. Therefore, this gives the following formula:

$$\text{polarising activity} = \frac{(\text{digit } \mathbf{4})\times4 + (\text{digit } \mathbf{3})\times2 + (\text{digit } \mathbf{2})\times1 + (\textit{blip})\times0.5}{\text{Total number}\times4}\times100\,.$$

The brackets indicate the number of specimens with extra digit **4** (i.e. **4334**, **43234**), or **3** (i.e. **334**, **3234**), or **2** (**2234**).

DiI labelling

Chick limb buds with DiI-labelled polarising region grafts were fixed in 4% PFA overnight at 4°C and photographed in a Nikon microscope with rhodamine filter. Before being processed for in situ hybridisation (as mentioned below), they were washed in PBS several times and then refixed in 4% PFA, overnight at 4°C.

In situ hybridisation

Embryos were processed according to standard methods (Riddle et al., 1993; Francis-West et al., 1995). Probes were synthesised using following templates: *ptc* (Marigo et al., 1996b), *bmp2* (Francis et al., 1994), *shh* (Cohn et al., 1995) and *fgf4* (Cohn et al., 1995). Photographs of embryos on Kodak 64T Ektachrome slide film were scanned on a Sprintscan 35 mm slide scanner and processed using Adobe Photoshop software.

RESULTS

Relationship between Shh signalling and *bmp2* expression

Ectopic *bmp2* expression in anterior mesenchyme can first be detected 16 hours after an Shh bead implantation (Yang et al., 1997). When an Shh bead is removed at this time point, only an additional digit **2** is specified but, when an Shh bead is removed after 24 hours, an additional digit **4** is formed (Yang et al., 1997). We explored whether maintenance and enhancement of *bmp2* expression in anterior mesenchyme during digit promotion requires continuous signalling by Shh. We monitored *bmp2* expression either after removing the Shh bead at different time points between 16 and 24 hours or in anterior mesenchyme taken from next to the Shh bead and transplanted to the anterior margin of another wing bud (Table 1). When either a bead soaked in 14 mg/ml Shh was removed at 16 hours or in anterior mesenchyme transplanted at this time point, expression of *bmp2* could be readily detected (Fig. 1A and data not shown). However, no *bmp2* expression could be detected in the anterior mesenchyme or in the graft when monitored at 8 hours and 24 hours later, respectively (compare Fig. 1A and B, 1E). Nevertheless, it should be noted that, in 4/6 cases, strong expression of *bmp2* in anterior apical ridge was maintained following Shh bead removal (Fig. 1E, arrow). In contrast, when an Shh bead is removed at 20 hours and the embryos fixed 16 hours later, ectopic mesenchymal *bmp2* expression could still be detected (Table 1) and when the bead was removed or tissue taken at 24 hours, *bmp2* expression was maintained in anterior mesenchyme for at least 16-24 hours (compare Fig. 1C and D, 1F, 7/7 cases; Table 1) and, in most cases (6/7 cases), expression was strong. In addition, the grafts of mesenchyme, which continue to express *bmp2*, induce

Table 1. Maintenance of *bmp2* expression induced in anterior mesenchyme in response to Shh

(A) *bmp2* expression in anterior mesenchyme after Shh bead removed at different time points

Time in which bead is removed (hours)	Time at which expression is assayed after bead removal (hours)	*n*	*bmp2* expression			
			–	+/–	++	+++
16	Immediately	2				2
16	8	6	6*			
	24	3	3			
20	16	4			4	
24	16	4				4

*In 4 of these cases *bmp2* was still expressed in anterior ridge.

(B) *bmp2* expression in anterior mesenchyme taken next to an Shh bead and grafted to the anterior margin of a second wing bud

Time in which tissue is taken (hours)	Time at which expression is assayed in grafted tissue (hours)	*n*	*bmp2* expression			
			–	+/–	+	++
16	Immediately	1				1
	24	2	2			
24	Immediately	2			1	1
	24	3		1	1	1

+++Strong expression.
++Moderate expression.
+Some expression.
–No expression detected.

ectopic expression of *bmp2* in the apical ridge of host wings (Fig. 1D). These data show that the continuous presence of Shh is required to enhance and maintain *bmp2* expression in mesenchyme during the digit promotion phase but, by the end of this phase, *bmp2* expression becomes independent of Shh signalling. In addition, the length of time that *bmp2* is expressed and the intensities of mesenchymal expression correlate with the character of the digits that form. These data also suggest that a function of Shh during the promotion phase is maintenance of production of Bmps, which might then be involved in patterning the digits.

Sequential signalling by Shh and Bmps

If the sole function of Shh is to induce and maintain Bmp production, then one would expect that application of Bmps themselves should induce full digit duplications but this has not been found to be the case (Francis et al., 1994; Duprez et al., 1996). One possibility is that Shh is needed for another function in the priming phase and that this priming phase is essential before Bmps can act in the promotion phase. To test this possibility, we implanted beads soaked in Shh (14 mg/ml) at the anterior margin of chick wing buds, then 16 hours later removed the beads and replaced them with a pellet of *bmp2*-expressing cells or beads soaked in different concentrations of Bmp2 protein (Table 2).

Wings, in which the Shh bead was not removed, usually had an additional digit **4** (Fig. 2A; *n*=4/5; polarising activity 80%) and wings in which no Bmp2 was added after removal of an Shh bead had mostly an extra digit **2** (Fig. 2B, 10/24 cases; Table 2 see also Yang et al., 1997; polarising activity 19.7%). However, when Shh beads were replaced by *bmp2*-expressing cells or beads soaked in high concentrations of Bmp protein, polarising activity increased to more than 30% and more digits developed. Thus with *bmp2*-expressing cells, many wings had an extra digit **3** (Fig. 2C,D, 5/11 cases; overall polarising activity 31%), and with beads soaked in either 1 mg/ml or 0.1 mg/ml Bmp2 protein, again additional digit **3**s were formed (Table 2; Fig. 2E). It is also important to note that, in several of these wings in which Bmp was added (Fig. 2D,E), there were cartilaginous elements anterior to the extra digit **3**, which could be rudimentary digit **4**s and if this was taken into account in the calculations, the polarising activity would be even higher. When Shh beads were replaced with beads soaked in

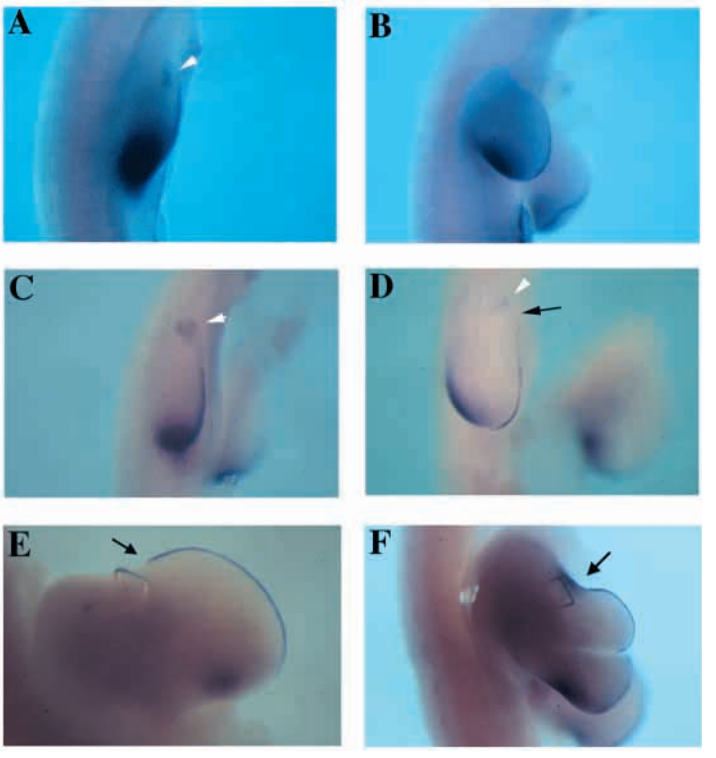

Fig. 1. Relationship between Shh signalling and *bmp2* expression. Beads soaked in 14 mg/ml Shh were implanted at the anterior margin of stage 20 chick wing buds. (A,B) Mesenchyme taken from next to the bead 16 hours after implantation was grafted to the anterior margin of a new host wing bud and *bmp2* expression was examined at 0 hours (A) or 24 hours (B) later. White arrowhead in A points to the grafted tissue which is expressing *bmp2* whereas in B, *bmp2* expression is lost from the graft. (C,D) The tissue was removed 24 hours after bead implantation and *bmp2* expression was again analysed 0 hours (C) or 24 hours (D) later. *bmp2* expression in graft is detected in C and maintained for 24 hours in D (white arrowheads). Furthermore *bmp2* expression in host apical ridge is expanded towards grafted tissue (as indicated by black arrow in D). (E,F) Shh beads were removed and *bmp2* expression was monitored at different time points later. (E) Shh bead was removed at 16 hours and embryo fixed 8 hours later. Ectopic anterior *bmp2* expression is still present in the apical ridge (arrow) but not detected in the mesenchyme. (F) Shh bead was removed at 24 hours and embryo fixed 16 hours later. Ectopic *bmp2* expression is maintained both in mesenchyme and in apical ridge (arrow).

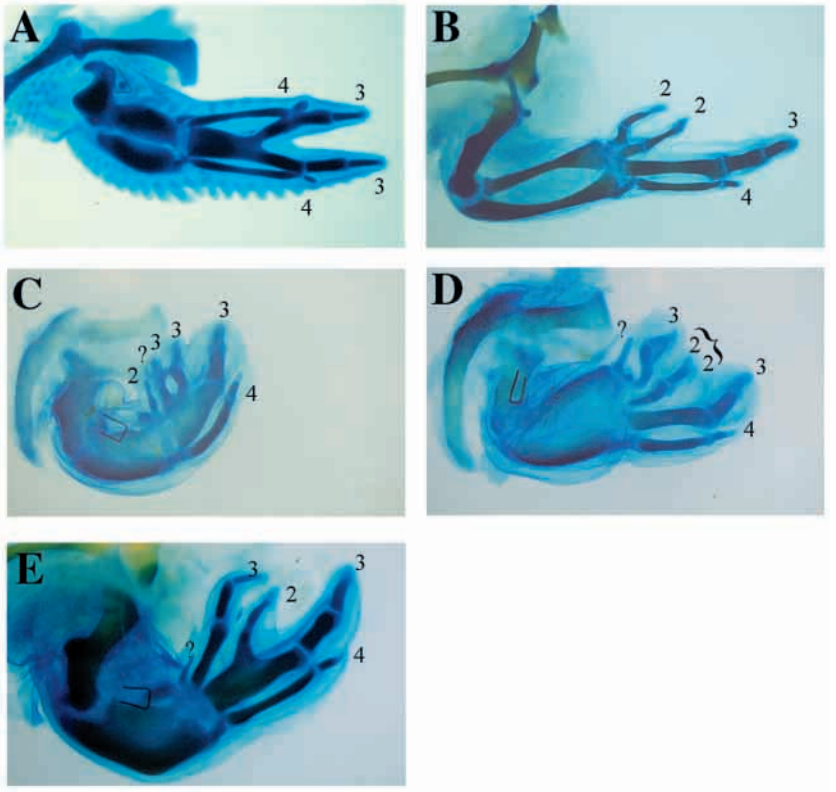

Fig. 2. Effects on chick wing digit pattern of sequential application of Shh and Bmp2. (A) Implantation of an Shh bead (14 mg/ml) at the anterior margin of stage 20 chick wing buds results in a **4334** digit pattern when the bead is left in place. (B) Removal of the Shh bead 16 hours after implantation results in a **2234** digit pattern. (C,D) Shh bead removed at 16 hours and replaced by pellet of *bmp2*-expressing cells. Extra digits **3** are induced. (Note the small cartilaginous element (?) anterior to the extra digit **3** in D). (E) Shh bead removed at 16 hours and replaced with a bead soaked in 1mg/ml Bmp2 protein. A **3234** digit pattern is produced. Note the small cartilaginous element (?) formed anteriorly to the extra **3**.

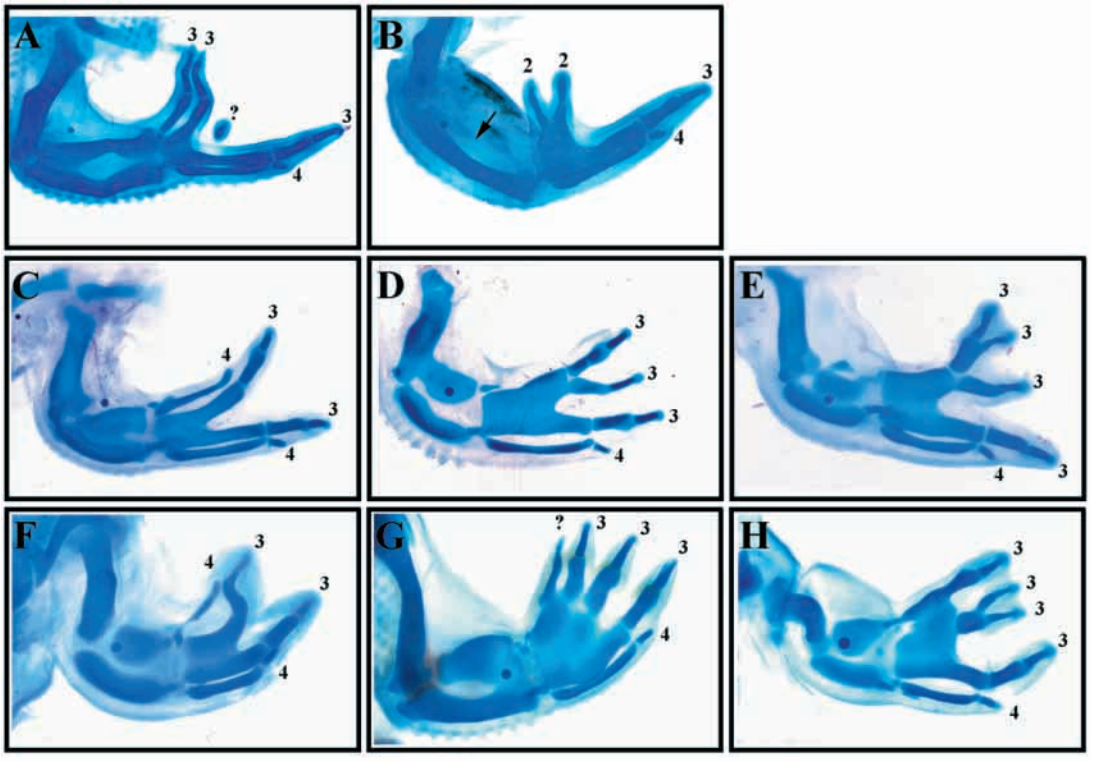

Fig. 3. Effects of Noggin or anti-Bmp antibody application during the promotion phase. Shh beads (14 mg/ml) were implanted at the anterior margin of stage 20 wing buds. (A,B) 18 hours after bead implantation a pellet of control CHO cells (A) or *noggin*-expressing CHO cells (B) was added. Whereas the wing that received control cells had a digit pattern **33***?***34**, the wing that received *noggin*-expressing cells only had extra digits **2** (**2234**). Note also the absence of the radius in B (arrow). (C-E) 16 hours after the Shh bead implantation, a second bead was implanted that was removed 9 hours later. A representative example of a wing that received an Shh bead plus a control bead is shown in C, with a duplicated digit pattern including an extra digit **4** (**4334**). (D,E) Wings treated with an Shh bead plus a Noggin bead and with attenuated duplication of digits. Typically more additional digits **3***s* were present (**3334** in D, **33334** in E) but with no extra digits **4**. (F-H) 16 hours after the Shh bead implantation a second bead was implanted. (F) A control bead; (G,H) a bead soaked in an anti-Bmp antibody. (F) A wing that received a second control bead shows a pattern of duplicated digits **4334**. (G,H) Two wings treated with anti-Bmp antiboby beads show a pattern of repeated extra digits **3** having been induced and no additional digits **4**s. Note than in D, E and in G, H the radius is present in each case (compare with B).

Table 2. Effects on digit pattern of sequential application of Shh and Bmp2

Shh bead removed at 16 hours	*n*	Digits specified: normal **234**	extra ***blip*** ***blip*****234**	extra **2** **2234**	extra **3** **3…34**	extra **4** **4…34**	Polarising activity (%)
Shh bead removed only	24	8	2	10	4	0	20
Shh bead replaced by pellet of *bmp2*-expressing cells	11	2	1	3	5	0	31
Shh bead replaced by bead soaked in 1 mg/ml Bmp2	8	0	1	3	4	0	36
Shh bead replaced by bead soaked in 0.1 mg/ml Bmp2	7	2	0	3	2	0	32
Shh bead replaced by bead soaked in 0.01 mg/ml Bmp2	6	3	0	3	0	0	12

n, number of cases.

lower concentrations of Bmp (0.01 mg/ml), this did not enhance polarising activity (Table 2). Thus application of high concentrations of Bmps to cells that have already been exposed to Shh clearly leads to the formation of an increased number of digits and digits with a more posterior identity, i.e. extra **3**s rather than **2**s.

Application of Bmp2 after Shh can increase polarising activity and this is consistent with the idea that it is Bmps in the promotion phase that determine digit character. In order to examine this possibility further, we used either the secreted protein Noggin (Zimmerman et al., 1996; Dosch et al., 1997; Merino et al., 1998) or anti-Bmp antibodies as reagents to interfere with Bmp signalling. Previous work has shown that overexpression of *noggin* by means of a retroviral vector can inhibit chondrogenesis and block the duplications induced by a polarising region graft to the anterior margin of a limb bud (Capdevila and Johnson, 1998). Therefore we designed experiments to interfere only transiently with Bmp signalling so that we could distinguish between direct effects on digit specification and later side effects on chondrogenesis.

In the first set of experiments, we used pellets of cells that express *Xenopus noggin* (Lamb et al., 1993). *noggin* expression in the grafted cells is strong at 4 hours after implantation in a chick wing bud but, by 16 hours, expression is barely detectable (data not shown) and thus these grafts provide a transient source of Noggin. We implanted beads soaked in Shh at the anterior margin of a chick wing bud and then, during the promotion phase between 16 and 20 hours later, we added a small pellet of cells. Addition of *noggin*-expressing cells at 16-20 hours after Shh bead implantation clearly prevented digit promotion (Table 3A; Fig. 3A,B). An additional digit **3** or **4** was obtained when the Shh bead alone was left in place, or when control (non-*noggin*-expressing) cells were added. However, when *noggin*-expressing cells were grafted, even though the Shh bead was still present, posterior digits were obtained much more infrequently and polarising activity was reduced to 25% (compared to 75% with Shh bead alone). We observed, however, that the radius was absent in the Noggin-treated embryos and therefore the *noggin*-expressing cells are still able to have some effects on chondrogenesis (Fig. 3B, arrow).

In a second set of experiments, we used beads soaked in Noggin protein, which we could remove later, thus only treating the anterior mesenchyme tissue during digit specification. We implanted beads soaked in Shh at the anterior margin of a chick wing bud and then, 16 hours later, at the beginning of the promotion phase, either a control or a Noggin-soaked second bead was implanted close to the Shh bead. Then 9 hours later, just after the end of the promotion phase, the second bead was removed. When Shh plus control beads were used, extra digits **4** were obtained in 33% of the embryos (3/9) with a polarising activity of 67%, whereas when Shh plus Noggin beads were implanted, only 12% of the embryos (1/8) had an extra digit **4** with a reduction in the overall polarising activity to 56% (Table 3B; Fig. 3C-E). Moreover, instead of having just a single additional digit **3**, many of the wings had two (or occasionally even three) additional digits **3**s usually branching distally from a common proximal origin (Table 3B; Fig. 3D,E). This phenotype was never seen in control duplications induced by Shh alone.

To confirm the hypothesis that inhibition of Bmp signalling can affect the specification of digit identity, we made use of an anti-Bmp antibody to block Bmps. Shh beads were implanted anteriorly in stage 20 limb buds and 16 hours later a second bead (a control bead or a bead soaked in anti-Bmp antibody) was implanted. In these experiments, 50% (4/8) of embryos that received Shh plus control beads had an extra digit **4** with a total polarising activity of 71%. However, only 31% (4/13) of limbs treated with Shh plus anti-Bmp antibody had an extra digit **4** with an overall polarising activity of 63%. (Table 3C; Fig. 3F-H). Again, patterns of repeated extra digit **3**s (Fig. 3G) or distally forked digit **3**s (Fig. 3F) were obtained that looked very similar to those obtained in the Noggin bead experiments. The same results were obtained whether the anti-Bmp bead was removed after 8 hours or not (data not shown) but, in all cases, as in the wings with the Noggin beads, no effects on chondrogenesis were seen and the radius was always present. Taken together, all these results support the idea that it is Bmp signalling that determines digit identity during the promotion phase. With just a short Noggin treatment or with anti-Bmp antibody more additional digits are formed but these are mostly digits **3**'s (Table 3B,C).

Although replacement of Shh at the beginning of the promotion phase with Bmp2 enhances polarising activity, it does not fully mimic the effect of leaving the Shh bead in place. Other signals in addition to Bmp2 may be required and one of these could be Fgf4 (Niswander et al., 1994; Laufer et

Table 3

Shh beads implanted	*n*	Digit patterns: *normal 234*	*extra blip b234*	*extra 2 2234*	*extra 3 3…34*	*extra 4 4…34*	Polarising activity (%)
A. Effects of adding *noggin*-expressing cells during the promotion phase of Shh signalling							
Control cells added at 18-20 hours after Shh beads	3				2	1	75
Noggin-cells added at 16-20 hours after Shh beads	11	5	3	2	2	1	25

Shh beads implanted	*n*	Digit patterns: *normal 234*	*extra blip b234*	*extra 2 2234*	*extra 3* *3…34*	*extra 3* *3334 or 33334*	*extra 4 4…34*	Polarising activity (%)
B. Effects of adding Noggin beads only during the promotion phase of Shh signalling, removing the beads at the end of the promotion phase								
Control beads added at 16 hours after Shh beads then removed 9 hours later	9				5	1	3	67
Noggin beads added at 16 hours after Shh beads then removed 9 hours later	8				2	5	1	56

Shh beads implanted	*n*	Digit patterns: *normal 234*	*extra blip b234*	*extra 2 2234*	*extra 3* *3…34*	*extra 3* *3334 or 33334*	*extra 4 4…34*	Polarising activity (%)
C. Effects of adding anti-Bmp antibody beads during the promotion phase of Shh signalling								
Control beads added at 16 hours after Shh beads	8			1	3	0	4	72
anti-Bmp antibody beads added at 16 hours after Shh beads	13			1	4	4	4	63

al., 1994). The apical ridge is necessary for additional digit formation and *fgf4* expression in the apical ridge is necessary for Shh to activate some downstream targets such as *Hoxd13* (Niswander et al., 1994; Laufer et al., 1994). *fgf4* expression is induced in anterior ridge at 16 hours after Shh application (Yang et al., 1997). When Shh beads are removed at 16 hours, *fgf4* expression was maintained for 8 hours, i.e., throughout the promotion phase (4/4 cases). *Fgf4* expression was also maintained in anterior ridge for 8 hours after we replaced an Shh bead at 16 hours with a bead soaked in 0.1 mg/ml Bmp2 (3/4 cases). Therefore, failure of complete digit promotion does not appear to be due to lack of *fgf4* expression.

High-level *ptc* expression to monitor temporal and spatial aspect of Shh signalling

In order to follow the response to Shh during the priming and promotion phases, we used high-level *patched* expression as an indicator that cells have received a Shh signal and responded to it. Induction of high-level *ptc* expression could be detected as early as 2 hours after bead implantation (Table 4; Fig. 4B). Ectopic high-level *ptc* expression was not found, however, in cells immediately next to the bead; instead there was an expansion of the posterior high-level *ptc* domain towards the Shh bead (3/4 cases). A similar effect was observed at 4 hours in 5/6 cases (in one case, there was no significant change in *ptc* expression). At 6 and 8 hours, (Fig. 4C; 6/6 cases) the high-

Table 4. Gene expression patterns of *ptc* and *shh* at different time points after grafting a polarising region at the anterior margin of a chick limb bud

	Time at which gene expression was assayed: 4 hours		8 hours		12 hours		14 hours		16 hours		24 hours	
	Graft tissue	Host tissue	Graft tissue	Host tissue	Graft tissue	Host tissue	Graft tissue	Host tissue	Graft tissue	Host tissue	Graft tissue	Host tissue
ptc	+++ (5)	+++ (5)	– (4)	+ (4)	ND	ND	ND	ND	++ (2)	++ (2)	++ (4)	+++ (4)
*shh**	+++ (6)	– (6)	+++ (6)	– (6)	+++ (2)	– (2)	+++ (5)	– (5)	+++ (4)	– (4)	+++ (3)	– (3)

Numbers in brackets represent the number of cases examined.
ND, not determined.
+++ Strong expression.
++ Moderate expression.
+ Some expression.
– No expression detected.
*Intensity of *Shh* expression in the grafted tissue does not change but in most cases only part of the graft expresses *Shh*.

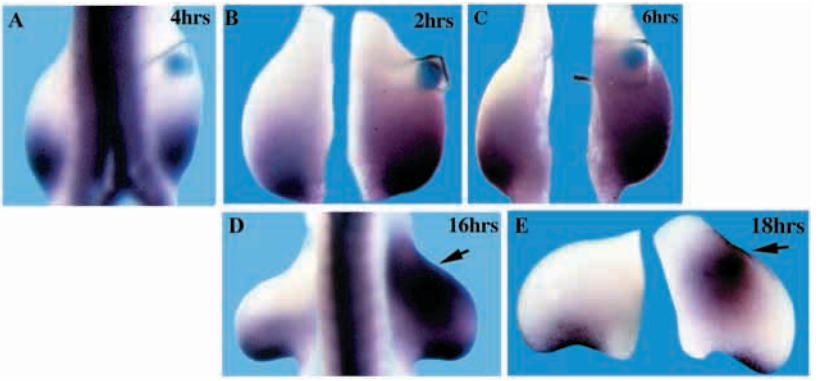

Fig. 4. *ptc* expression after implantation of Shh soaked beads. (A) 4 hours after control bead implantation. No change in *ptc* expression can be detected. (B) 2 hours after Shh bead implantation, *ptc* expression extends anteriorly towards the bead. (C) 6 hours after Shh bead implantation; note that *ptc* domain is covering the implanted bead. (D) 16 hours after Shh bead implanted; extensive upregulation of *ptc* expression in entire wing bud. (E) Restricted ectopic *ptc* expression domain around Shh bead is established by 18 hours.

level *ptc* domain was again expanded towards the anterior but, in three specimens, there was a small ectopic high-level *ptc* domain just over the bead. At 16 and 18 hours, instead of an expansion of the posterior high-level *ptc* domain, there is now a discrete ectopic *ptc* domain extending a few cell diameters from the bead (Fig. 4D,E). Control beads soaked in buffer had no effect on *ptc* expression in host wing buds (Fig. 4A; 0/3 at 4 hours, 0/3 at 16 hours). Thus, anterior mesenchyme cells respond rapidly to applied Shh protein at the start of the priming phase. High-level *ptc* expression persists throughout both priming and promotion phases although the pattern is dynamic, extending initially away from the Shh bead and later becoming more localised.

In order to check how rapidly high-level *ptc* expression can respond to changes in Shh protein distribution, we removed Shh beads at different times after implantation. When beads were removed at 4 hours and embryos fixed 12 hours later or beads were removed at 16 hours and embryos fixed 8 hours later, *ptc* expression in most buds has returned to normal (4/6 and 6/7 cases respectively). However, in one specimen, in which the bead was removed at 16 hours, there was a very small ectopic domain of high-level *ptc* just above where the bead used to be. In addition, when the Shh bead was removed at 24 hours and the embryo fixed 12-15 hours later, a strong and rather broad ectopic high-level *ptc* domain persisted at the anterior wing margin (three cases). Thus, ectopic high-level *ptc* expression seems to disappear when the source of Shh is removed during the priming phase suggesting that it depends on continuous presence of Shh but may persist in absence of Shh once digit promotion has been completed.

Diffusion of the unmodified N-terminal Shh peptide from beads will not reproduce the way in which Shh produced by the polarising region spreads in the limb bud (Yang et al., 1997). Therefore, we tested directly how grafts of polarising region mesenchyme placed at the anterior margin affect the expression pattern of high-level *ptc* in host limb buds. Surprisingly, we found even more dynamic patterns of *ptc* expression than we had found with Shh beads.

At 4 hours after grafting a polarising region, high-level *ptc* is induced in anterior host tissue (5/5 cases) and the graft itself still expresses very high levels of *ptc* (Fig. 5A; Table 4). The ectopic *ptc* domain shows levels of expression comparable to those in the posterior region, and encompasses almost the

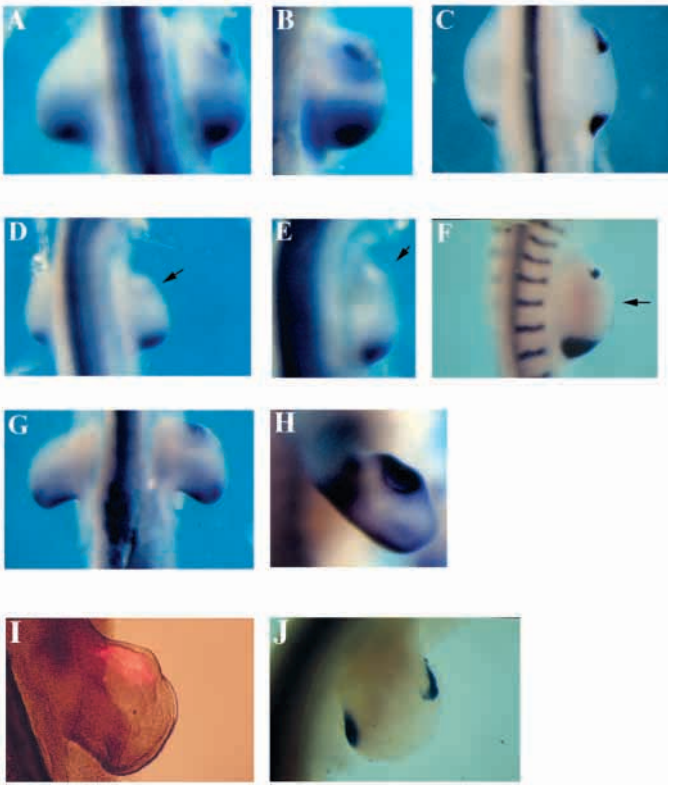

Fig. 5. *ptc* and *shh* expression patterns following polarising region grafts at the anterior margin of stage 20 chick wing buds. (A-C) 4 hours after grafting. (A) *ptc* expression is induced in the host tissue next to the graft; host expression is weaker than in graft (B). Another specimen showing induced *ptc* domain surrounding the graft. (C) *shh* expression maintained in graft. (D-F) 8 hours after grafting. (D,E) *ptc* expression in grafted polarising region is lost and induced *ptc* domain in host tissue is markedly reduced. Arrows point to thin rim of restricted ectopic *ptc* expression surrounding the non-expressing grafted polarising region (whitish tissue). (F) *shh* is expressed in part of the grafted polarising region, as can be seen in this double *in situ* hybridisation with *shh* and *fgf4* (arrow indicates *fgf4* expression in the ridge). (G) 16 hours after grafting. Ectopic *ptc* expression is re-established in host mesenchyme, just surrounding the graft. Part of the graft also expresses *ptc*. (H-J) 24 hours after grafting. (H) Entire grafted polarising region is expressing *ptc* in addition to surrounding host tissue. Note that at this time point, the induced *ptc* expression domain is not restricted to just around the graft but extends further into host tissue. (I) DiI labelling shows grafted polarising region. (J) Only the part of the graft closest to the anterior apical ridge is still expressing *shh*.

whole anterior third of the limb bud (Fig. 5A,B). By 8 hours, *ptc* expression has changed dramatically. The ectopic domain of *ptc* expression in the host tissue is now reduced to a thin rim of expressing cells surrounding the graft. (Table 4; Fig. 5D,E). Furthermore, there is no longer any high-level *ptc* expression in the graft. At 16 hours, (2/2 cases), there is strong *ptc* expression around the graft and a weaker ectopic domain extending a little further away, but still not extending as far as at 4 hours (Fig. 5G). Some grafted cells now express high-level *ptc*. By 24 hours, there is a well-established ectopic domain, expressing *ptc* at the same high level of intensity as the normal posterior domain (Fig. 5H). Some cells in the centre of the graft express high-level *ptc*, although some in the outer regions do not (Fig. 5H).

These results show that there are several phases of high-level *ptc* expression in anterior mesenchyme cells in response to a polarising region graft. In an initial phase, a broad region of anterior mesenchyme expresses high-level *ptc*. Then this *ptc* expression disappears. A second phase of high-level *ptc* expression follows in which *ptc* is restricted to the region around the grafted polarising region. It should also be noted that *ptc* expression in the graft is not stable.

One possible explanation for these changes in high-level *ptc* expression in anterior mesenchyme and in the polarising region is that *shh* expression switches off in the polarising region graft and then switches back on again. However we found that *shh* is expressed continuously in grafted polarising regions. *shh* transcripts could be detected in the grafts, usually in the part of the graft near the apical ridge, but not in the host at 4, 8, 12, 16 and 24 hours (Table 4; Fig. 5C,F,J). In some cases, polarising region mesenchyme was isolated and then stained with DiI before grafting into host wings in order to be able to locate the graft and confirm that *shh* expression is in the grafted cells (Fig. 5I).

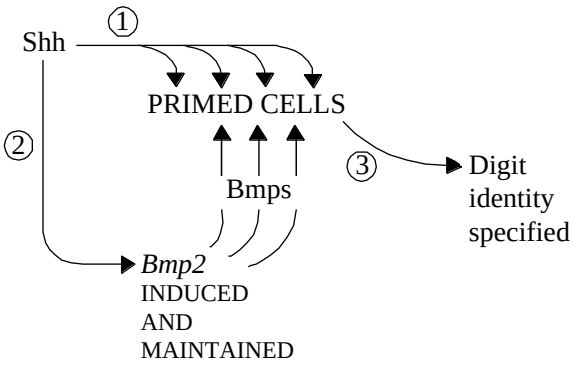

Fig. 6. Schematic diagram illustrating the proposed model of sequential Shh and Bmp signalling. The function of Shh in the promotion phase (1) is to define the region of the bud that is competent to form digits. During this phase Shh acts long range. During the promotion phase (2), Shh induces and maintains *bmp2* expression. Bmps then act on the primed cells in order to specify digit pattern (3).

DISCUSSION

We have found that Shh not only induces *bmp* expression in anterior mesenchyme during digit specification but is also necessary to maintain it. The extent of *bmp* expression correlates with digit character. Bmp2, if added after Shh, enhances digit patterning and results in more additional digits and digits with more posterior identity. Interference with Bmp signalling attenuates specification and multiple digits with the same identity are found. High-level *ptc* expression is induced rapidly when either Shh beads or polarising regions are grafted and occurs in two phases, initially widespread and later more restricted.

Two-step model for digit patterning

These findings suggest that Shh acts in two steps, first, long range, and then, short range, to control digit pattern (Fig. 6). We suggest that whether Shh acts short range or long range will be determined by whether or not high-level expression of binding molecules such as *ptc* has been induced in responding cells. We propose that, in the first step, Shh acts long range to "prime" limb mesenchyme cells and thus make them competent to form digits. This in essence will determine the number of digits that can form and will be related to the length of the apical ridge. In the second step, Shh acts short range to induce and maintain *bmp2* expression. Bmp2 then acts in a dose-dependent fashion on the competent limb mesenchyme cells to specify digit identity. Thus, in terms of classical models for polarising region signalling, we propose that Bmps act as polarising morphogens and progressively diffuse into adjacent mesenchyme. This establishes a concentration gradient and cells that are first specified as anterior digits are later promoted to form posterior digits (Tickle, 1995; Yang et al., 1997).

This model accounts for the truncations found in the *shh* knockout (Chiang et al., 1996), because, in the absence of Shh, no cells will be competent to form digits. The model also seems to be able to account for all previous results on the polarising region and Shh induction of digits. For example, the dose-dependent effects of Shh on digit pattern are mediated by dose- and time-dependent induction of *bmp2* expression, just as the dose-dependent effects of retinoic acid are mediated by dose-dependent induction of *shh* expression (Y. Yang and L. Niswander, personal communication). The only exception is the finding that *shh* expressed tethered to the membrane protein CD4 appears to be able to induce full duplications (Yang et al., 1997). However, it is still possible that there is some kind of active diffusion of this Shh::CD4 involving cleavage and/or intracellular transport.

The model can also account both for the polydactyly and the uniformity of digit type in the *talpid*^{3} mutant in which high-level *ptc* expression cannot be induced (Lewis et al., 1999). Polydactyly will occur because Shh diffusion will not be restrained and more of the bud will be competent to form digits. All the digits will be the same because *bmp2* is uniformly expressed. In this context, it is striking that we found, when limbs were treated with antagonists of Bmp signalling, which could act to homogenise signal levels, a series of morphologically similar digits develop anteriorly, reminiscent of the digits in *talpid*^{3} mutants.

This type of model could be widely applicable. The two phases of Sonic Hedgehog signalling are reminiscent of the way in which Hedgehog patterns the insect eye (Dominguez and Hafen, 1997) and sequential signalling, short range by Hh, followed by Dpp long range, is a well-established model

in *Drosophila* wing patterning (Basler and Struhl, 1994). In vertebrates, it has been shown that Shh signalling acts in two different phases to specify motor neuron identity during neural tube development (Ericson et al., 1996) and, furthermore, sequential signalling by Shh and Bmps has been proposed to be involved in sclerotome specification in somites (Murtaugh et al., 1999). Finally, Bmps have recently been shown to be responsible for specifying tooth identity (Tucker et al., 1998).

Long- and short-range Shh signalling

We propose that Shh initially acts long range to define the region of the bud that is competent to form digits. According to our recent fate map, this comprises the posterior half of the limb bud (Vargesson et al., 1997). Our evidence for this initial widespread Shh diffusion comes from our high-level *ptc* expression studies. With both Shh beads and polarising region grafts, a broad initial domain of high-level *ptc* expression is induced. With polarising region grafts, this broad domain of expression encompasses almost the whole anterior third of the limb and therefore includes the cells that we have shown form the additional digits (Yang et al., 1997). We suggest that the key to this initial long-range diffusion of Shh protein could be the absence of high-level expression of Shh-binding proteins in the naïve mesenchyme. Thus it would be easy for Shh to overcome repression and activate the signalling pathway over a large distance. This idea stems from our recent work on the *talpid*^{3} mutant (Lewis et al., 1999) where we have argued that failure to express high levels of *ptc* might have led to widespread diffusion of Shh. In normal embryos, high-level *ptc* expression is activated in response to Shh and seems to serve as a brake to Shh diffusion. The gene encoding Hip, Hedgehog interacting protein, is also expressed in response to Shh (Chuang and McMahon, 1999). Thus one could imagine that prior to high-level expression of these binding proteins, Shh protein will be able to diffuse much more freely. Other recent work seems to support this interpretation (Milenkovic et al., 1999). Transgenic overexpression of *ptc* in wild-type mice results in loss of anterior digits, one possible explanation being that extra Ptc restricts the diffusion of Shh in the first phase thus producing a smaller area of the limb competent to form digits. Conversely, low level expression of *ptc* in a *ptc*$^{-/-}$ mutant mice induces polydactyly, a result compatible with less Ptc allowing Shh to diffuse further away. However, this interpretation is complicated by the fact that ectopic anterior *shh* expression was observed in the limb buds. Finally, overexpression of *ptc* in an *extratoe* (*gli3* deletion) mutant mice, which has ectopic anterior Shh expression leading to polydactyly, rescues this phenotype restoring a normally patterned limb. These results suggest that a balance between Ptc and Shh function is essential for proper limb patterning and fit with our findings.

It should be noted that several other mechanisms of regulating the distribution of signalling molecules have been proposed recently. For instance, it has been shown that the gene *tout velu* is involved in the control of Hedgehog protein movement during *Drosophila* limb development (Bellaiche et al., 1998) by affecting the synthesis of heparan sulphate proteoglycan (The et al., 1999). Likewise, cellular processes called cytonemes have been described in *Drosophila* wing imaginal discs, which may transport morphogens such as Hedgehog and Dpp (a Bmp2/4 homologue) (Ramirez-Weber and Kornberg, 1999).

We found that initial widespread high-level *ptc* expression in the host chick wing bud in response to an Shh source was followed by a reduction in *ptc* expression, then local high-level expression was seen again. This can be explained by the high levels of Ptc induced at first, repressing the pathway. Thus *ptc* expression levels will then go down. High-level Ptc protein will also localise Shh protein. Thus an equilibrium will be set up where there are high levels of *ptc* expression near the Shh source because Shh is localised there at high enough levels to overcome Ptc repression. This would also explain why co-localisation of Shh protein and *shh* transcripts has been reported in limb buds at stages when high-level *ptc* is expressed (Marti et al., 1995). It should be noted that Shh beads induce more extensive high-level *ptc* expression than polarising region grafts (see also Yang et al., 1997). It seems likely that this is due to the Shh produced by the polarising region being modified by covalent attachment of cholesterol (Porter et al., 1996) and thus being less diffusible.

Priming phase

There is a considerable lag between the first response to Shh (high-level *ptc* expression) and the start of the digit specification phase. This time lag may reflect the fact that re-specification of anterior mesenchyme may involve not only activation of genes normally expressed posteriorly but also inhibition of genes normally expressed anteriorly. In the neural tube, for instance, it has been shown that an early effect of Shh is the downregulation of the expression of *pax3* and *pax7* and that this is necessary for a second step in Shh signalling which specifies neuron type identity (Ericson et al., 1996). In the limb bud, *alx4*, which is expressed anteriorly, is switched off 6 h after Shh is applied (Takahasi et al., 1998). *gli3* is also expressed in anterior mesenchyme. When Shh is applied, *gli3* is also switched off but not until 24 hours (Takahashi et al., 1998), suggesting that this is not responsible for the lag in induction of *bmp2* expression. There is some evidence for reciprocal interactions between these genes and Shh because in mice with mutations in either *alx4* or *gli3*, there is ectopic *shh* expression (Qu et al., 1997, 1998).

A curious finding is that *ptc* expression in a grafted polarising region fluctuates and cannot be detected 8 hours after the cells have been transplanted at the anterior margin of another limb bud. However, *shh* transcripts are still expressed in the graft. Therefore the reason for this fluctuation in *ptc* expression is not clear. The absence of *ptc* expression could reflect absence of Shh protein in the graft. Alternatively it has been suggested that *ptc* expression is switched off in *shh*-expressing cells in the limb when the Shh protein concentration becomes very high (Marigo and Tabin, 1996c). This is also seen in floor plate of the neural tube in chick, mouse and fish embryos (Concordet et al., 1996; Goodrich et al., 1996; Marigo and Tabin, 1996c).

Digit specification phase

The model proposes that the function of Shh in the digit specification phase is to induce and maintain expression of Bmp2 (and/or other Bmps), which then act in competent tissue to specify digit pattern. Our results show that Bmp2 alone in this phase can substantially enhance digit specification but we

have not been able to reproduce completely the effects of polarising region grafts or of leaving a Shh bead in place throughout the promotion phase. Our results suggest this is not due to lack of Fgf4 signalling. It could be that other Bmps and/or Bmp heterodimers would lead to specification of the most posterior identity.

The effect of application of Bmps to cells that have been exposed to Shh contrasts with the effects of simply applying Bmps to the anterior mesenchyme. Far from promoting additional digit formation, application of Bmps leads to cell death and loss of anterior skeletal elements (Macias et al., 1997). The fact that Bmps can act only in primed cells explains why application of Bmps, on their own, cannot produce full digit duplications. Furthermore, it also explains why polarising activity is almost invariably associated with Hedgehog signalling. Pellets of *bmp2*-expressing cells (Duprez et al., 1996) and overexpression of *fgf2* (Riley et al., 1993) have been reported to lead to induction of an extra digit **2** but it is not clear in these cases that this involves a change in positional values.

According to *ptc* expression patterns, Shh acts more locally in this second phase and this would be important in order to induce local expression of *bmp*. Subsequently, diffusion of Bmps could set up a classical gradient in competent cells, which would determine digit pattern. This morphogen mechanism would be similar to the long-range gradient action of Dpp during wing imaginal disc development (Nellen et al., 1996). Furthermore, it is tempting to speculate that downstream Bmp targets could act as negative regulators to modulate the interpretation of such a morphogen gradient, like the *brinker* gene in *Drosophila* (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999).

Attenuation of Bmp signalling by Noggin is consistent with the idea that low concentrations of Bmps give digit **2**, high concentrations of Bmps give digit **4**. This also tallies with the preferential loss of posterior structures when Bmp signalling is blocked during limb pattern formation by co-expression of dominant-negative forms of Bmp receptors (Kawakami et al., 1996). Recent work on *gremlin*, another extracellular inhibitor of Bmps, highlights the importance of the tight regulation of Bmp activity in limb bud development (Zuniga et al., 1999; Capdevila et al., 1999; Merino et al., 1999; see also Pizette and Niswander, 1999). *Gremlin* is expressed in the posterior limb bud of mouse and chicken embryos and has been shown to be necessary for the relay of the Shh signal to the apical ectodermal ridge. This leads to an antagonism of Bmp signalling in the ridge thus allowing expression of *fgf4*. We suggest that Gremlin could fine tune Bmp concentration in the mesenchyme and thus ensure that the levels of Bmps appropriate for specification of each digit are established. In addition, this would provide a mechanism to regulate digit promotion and thus prevent every digit becoming a digit **4**.

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