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Morphogenesis of Digits in the Avian Limb Is Controlled by FGFs, TGFβs, and Noggin through BMP Signaling

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In the final stages of limb morphogenesis, autopodial cells leaving the progress zone differentiate into cartilage or undergo apoptotic cell death, depending on whether they are incorporated into the digital rays or interdigital spaces. Most evidence indicates that these two opposite fates of the autopodial mesoderm are controlled by BMP signaling. However, the molecular basis for these two distinct actions of BMPs, including the receptors involved in the process, is controversial. In this study we have addressed this question by exploring the presence in the developing autopod of diffusible signals able to modulate BMP function and by analyzing the effects of their exogenous administration on the pattern of expression of BMP receptor genes. Our findings show that *tgfβ2* and *noggin* genes are expressed in the condensing region of the developing digital rays in addition to the well-known distribution in the autopodial tissues of FGFs (apical ectodermal ridge, AER) and BMPs (AER, progress zone mesoderm, and interdigital regions). Exogenous administration of all the factors causes changes in the expression of the *bmpR-1b* gene which are followed by parallel alterations of the skeletal phenotype: FGFs inhibit the expression of *bmpR-1b* compatible with their function in the maintenance of the progress zone mesoderm in an undifferentiated state; and TGFβs induce the expression of *bmpR-1b* and promote ectopic chondrogenesis, compatible with a function in the establishment of the position of the digital rays. In addition we provide evidence for the occurrence of an interactive loop between BMPs and noggin accounting for the spatial distribution of *bmpR-1b* which may control the size and shape of the skeletal pieces. In contrast to the *bmpR-1b* gene, the *bmpR-1a* gene is expressed at low levels in the autopodial mesoderm and its expression is not modified by any of the tested factors regardless of their effects on chondrogenesis or cell death. Finally, the role of BMPs in programmed cell death is confirmed here by the intense inhibitory effect of noggin on apoptosis, but the lack of correlation between changes in the pattern of cell death induced by treatment with the studied factors and the expression of either *bmpR-1a* or *bmpR-1b* genes suggest that a still-unidentified BMP receptor may account for this BMP function.

Key Words: **chick embryo; limb development; apoptosis; chondrogenesis; BMP receptor; TGFβ2.**

INTRODUCTION

In the final stages of limb morphogenesis, the cells leaving the progress zone in the distal region of the limb bud (autopod) can have two different fates, chondrogenesis or apoptotic cell death, depending on whether they are incorporated into the digital rays or into the interdigital regions. In the past few years, considerable evidence has been found indicating that BMPs are essential mediators in the specification of these two fates of the autopodial mesoderm. Three members of this family, BMP-2, BMP-4, and BMP-7 (OP-1), are coexpressed in the interdigital regions and in the progress zone mesoderm of the autopod (Francis *et al.*, 1994; Lyons *et al.*, 1995; Macias *et al.*, 1997; Laufer *et al.*, 1997). Blocking BMP signaling in limb buds after

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infection with retroviruses carrying dominant negative BMP receptors results in digit truncation and inhibition of interdigital cell death (Zou and Niswander, 1996; Yokouchi *et al.,* 1996). In addition, exogenous administration of human BMP recombinant proteins promotes cell death or cartilage overgrowth depending on whether they are applied to the undifferentiated limb mesoderm or to the prechondrogenic blastemas (Macias *et al.,* 1997). The basis for these two distinct actions of BMPs remains unclear. The involvement of different types of BMP receptors and/or possible interactions between BMPs and other local soluble factors might account for such different actions of BMPs.

BMP signaling requires the formation of heterodimers between protein kinase type I and type II BMP receptors. At present, two type I receptors, BMPR-1a (BRK-1) and BMPR-1b (BRK-2), and one type II receptor (BRK-3) have been identified although BMPs may also signal through other TGFβ-related receptors (Yamashita *et al.,* 1995; Chang *et al.,* 1997). The *BmpR-1a* gene is expressed at low levels in the undifferentiated limb mesoderm (Kawakami *et al.,* 1996; Zou *et al.,* 1997), while the *bmpR-1b* gene exhibits well-defined domains of expression in the prechondrogenic blastemas (Kawakami *et al.,* 1996; Zou *et al.,* 1997). It has been proposed that BMPR-1a receptors are involved in interdigital cell death while BMPR-1b receptors participate in chondrogenesis (Kawakami *et al.,* 1996; Enomoto-Iwamoto *et al.,* 1998). However, experiments blocking BMP signaling by retrovirus-induced overexpression of dominant negative constructs of either one of the two type I BMP receptors (Zou and Niswander, 1996; Yokouchi *et al.,* 1996) produce similar effects, including defective digit growth and inhibition of interdigital cell death. Whether this apparently contradictory finding might be explained by secondary sequestration of BMP molecules by the overexpressed receptor remains to be clarified. More recently, Zou *et al.* (1997) using retroviruses carrying a dominant active or negative BMP type I receptor gene have reported a double role for *bmpR-1b* controlling both chondrogenesis and cell death. In these experiments misexpression of either dominant active *bmpR-1a* or dominant active *bmpR-1b* promoted intense chondrogenesis, but only misexpression of the dominant active *bmpR-1b* increased cell death in the undifferentiated mesenchyme. However, this last effect was only observed when the limbs were infected at very early stages (before stage 17), although interdigital regions appeared to regress precociously in limbs infected at later stages. Unlike the chondrogenic blastemas, the prospective areas of cell death lack specific domains of *bmpR-1b* expression identifiable by *in situ* hybridization. Therefore, a question that requires clarification is whether cell death induced in those experiments reflects a physiological function of the *bmpR-1b* gene.

This study explores a possible explanation for the dual role of BMPs, promoting chondrogenesis in the digital rays and cell death in the interdigits, namely that BMP function is regulated locally by other diffusible molecules able to influence cell death and/or chondrogenic differentiation. Investigation of these hypothetical interactions between BMPs and other diffusible signals present in the autopod and their effects on the regulation of BMP-receptor gene expression may help to clarify the BMP receptor responsible for each BMP function. Among the possible signaling molecules which may influence the action of BMPs in the autopod are FGFs, TGFβs, and secreted proteins antagonistic to BMP function. FGFs, which are delivered by the AER, maintain the progress zone mesoderm in an undifferentiated state (Niswander *et al.,* 1993; Fallon *et al.,* 1994; Mahmood *et al.,* 1995; Crossley *et al.,* 1996) and local administration of FGFs to the autopod prevents induced ectopic chondrogenesis (Gañan *et al.,* 1996) and delays interdigital cell death (Macias *et al.,* 1996). TGFβs are strong chondrogenic signals (Kulyk *et al.,* 1989; Schofield and Wolpert, 1990; Leonard *et al.,* 1991) and their local application to the interdigital regions inhibits interdigital cell death and induces the formation of extra digits (Gañan *et al.,* 1996). Thus, TGFβs are good candidate signals for switching BMP function to chondrogenesis instead of apoptosis. However, it is not yet clear if the pattern of expression of *tgfβs* in the autopod is compatible with that function or if the chondrogenic effect of TGFβs is related with BMP signaling. Finally, several BMP antagonistic secreted proteins, such as noggin (Zimmerman *et al.,* 1996), chordin (Piccolo *et al.,* 1996), and follistatin (Feijen *et al.,* 1994; Fainsod *et al.,* 1997) have been identified in a variety of embryonic organs. These proteins appear to operate by binding to specific BMPs, thus avoiding interaction with their cognate receptors. Whether any of these proteins play a role in limb morphogenesis modulating BMP function awaits clarification.

In this study, we have analyzed whether changes in the pattern of differentiation of the autopodial mesoderm caused by ectopic administration of growth factors with a presumed physiological function in limb bud morphogenesis, including FGFs, TGFβs, and BMPs themselves, influence the pattern of expression of BMP receptor genes. In addition, we have explored the possible involvement of noggin as a modulator of BMP function during the stages of digit formation.

MATERIAL AND METHODS

We have employed Rhode Island chick embryos ranging from days 3.5 to 9 of incubation (stages 22–35; Hamburger and Hamilton, 1951). Eggs were windowed at the desired stages and the right leg bud was exposed. Heparin or Affi-Gel blue beads incubated in PBS or in the selected recombinant human protein solutions (see below) were implanted into the limb mesenchyme. Most experiments were performed at stages 28–29 and the beads were implanted at the tip of digit III or in the third interdigital space.

The morphology of the limbs was studied after cartilage staining with Alcian green as described previously (Gañan *et al.,* 1996). The pattern of cell death was analyzed by vital staining with neutral red and by Tdt-mediated dUTP nick end labeling (TUNEL) in tissue sections as described previously (Macias *et al.,* 1997).

Preparation of Beads

Affi-Gel blue (Bio-Rad) or heparin acrylic beads (Sigma) were employed as carriers for administration of the selected proteins. Beads of diameter ranging between 80 and 150 μm were selected, washed in PBS, and incubated for 1 h at room temperature in the selected protein solution. Recombinant human TGFβ1 and TGFβ2 (both from R & D Systems) were employed at a concentration of 10 μg/ml. Recombinant human FGF-2 and FGF-4 (R & D Systems) were employed at 1 mg/ml. The following human recombinant BMPs (Creative Biomolecules, Hopkinton, MA) were employed at the indicated concentrations: BMP-2, 1 mg/ml; BMP-4, 0.33 mg/ml; and OP-1 (BMP-7), 0.5 mg/ml. Human recombinant noggin (Regeneron Pharm Inc., Tarrytown, NY; Lot No. 970127) was employed at 1.05 mg/ml.

Probes and in Situ Hybridization

Specific probes for *bmpR-1a* (*BRK-1*) and *bmpR-1b* (*BRK-2*) were provided by L. Niswander and T. Nohno. *Ck-erg* probe was provided by M. Duterque. Fragments of chicken *tgfβ2* (743 bp), *tgfβ3* (742 bp), and *noggin* (667 bp) genes were obtained by RT-PCR. First-strand cDNA was synthesized with a mixture of random hexamers (Promega) and 1 μg of total RNA from a day 7.5 autopod. The following primers (5′ to 3′) were used: for chicken *tgfβ2* gene, 5′ primer 5′-ATGCACTGCTATCTCCTGAG-3′ and 3′ primer 5′-CAGGCAGCAATTATCCTGCA-3′; for chicken *tgfβ3* gene, 5′ primer 5′-TGACAGTGAAGATGACTATG-3′ and 3′ primer 5′-TCTTCCGCATCAACTGT CCA-3′; and for chicken *noggin* gene, 5′ primer 5′-AAGGATGGATCATTCCCAGT-3′ and 3′ primer 5′-CTAGCAGGAGCACTTGCACT-3′. PCR were performed in a total volume of 100 μl using *Taq* DNA polymerase (Gibco BRL). The cycling conditions were 1 min at 94°C for denaturation, 2 min at 55°C for annealing, 3 min at 72°C for elongation, and then 10 min at 72°C after the last cycle (35 cycles). The PCR products were subsequently cloned into pBluescript SK (Strategene) and the authenticity of the fragments was confirmed by dideoxy sequencing.

In situ hybridization was performed on whole-mount specimens (see details in Gañan *et al.*, 1998) and in tissue sections. For whole-mount *in situ* hybridization, samples were treated with concentrations of proteinase K ranging from 10 to 40 μg/ml for 30 min at 20°C. Best results were obtained with proteinase K at 30 μg/ml. Hybridization with digoxigenin-labeled antisense RNA probes was performed at 68°C. Reactions were developed with BCIP/NBT substrate or with purple AP substrate (Boehringer-Mannheim). *In situ* hybridization in tissue sections was performed using digoxigenin-labeled antisense RNA probes as described by Zou *et al.* (1997). Specificity of labeling was controlled using sense RNA probes.

RESULTS

1. Regulation of BMP-Receptor Gene Expression by TGFβs, FGFs, and BMPs

In the stages of digit formation *bmpR-1a* and *bmpR-1b* genes exhibit distinct patterns of expression in the autopod. *BmpR-1a* is expressed at low levels in the undifferentiated mesenchyme subjacent to the AER, both at the level of the digital rays and in the interdigital regions (Fig. 1A). *bmpR-1b* exhibits well-defined domains of expression in the condensing mesenchyme of the digital rays but is not expressed in the interdigital regions (Figs. 1B–1D). All the growth factors tested in this study showed prominent effects on the pattern of expression of *bmpR-1b* consistent with their effects on the differentiation of cartilage. By contrast, none of these factors caused significant modifications in the expression of *bmpR-1a*.

At stages 28–29, implantation of beads bearing TGFβ1 or TGFβ2 in the interdigital regions blocks interdigital cell death and promotes the formation of ectopic extra digits (Gañan *et al.*, 1996). In this study the appearance of these interdigital cartilages was preceded by the induction of an ectopic domain of *bmpR-1b* gene expression (n = 16), first identifiable 10–15 h after the implantation of the bead (10–14 h prior to the identification of the cartilage with Alcian green). This induced expression of *bmpR-1b* followed a precise temporo–spatial pattern of distribution (Figs. 1E–1G). Initial expression of *bmpR-1b* was in the mesenchyme immediately proximal to the bead (Fig. 1E). In the course of development, expression of *bmpR-1b* shifted distally (Fig. 1F) taking by 24–30 h a characteristically elongated shape reminiscent of the distal phalanx of a digit (Fig. 1G) suggesting that progress zone cells are recruited into this domain of expression as they lose the influence of the AER. To discard the possibility that this pattern of expression was related with an irregular diffusion of the growth factor from the bead we compared the expression of *bmpR-1b* with that of *ck-erg*, a gene related with the early events of chondrogenesis also induced by TGFβs (Gañan *et al.*, 1996). As can be seen in Fig. 1H, *ck-erg* expression exhibited a precise concentric distribution around the bead which contrasted with the elongated pattern of *bmpR-1b* expression at the same interval after implantation of the bead (n = 5).

FGFs constitute the distal signal of the limb bud responsible for the maintenance of the progress zone. Previously, we reported that implantation of FGF beads delayed interdigital cell death and inhibited the chondrogenic effect of TGFβs (Macias *et al.*, 1996; Gañan *et al.*, 1996). In this study implantation of FGF beads in the interdigital regions (n = 9) did not cause modifications in the normal pattern of expression of BMP-receptor genes in spite of its inhibitory effect on apoptosis. In contrast, when an FGF bead was implanted at the tip of the growing digit (n = 18) *bmpR-1b* expression was severely inhibited (Fig. 1I) and chondrogenesis was delayed (not shown). The inhibitory effect of FGFs on chondrogenesis and *bmpR-1b* expression was additionally confirmed by performing side-by-side implantation of one FGF bead and one TGFβ bead in the same interdigit (n = 6). In this experiment interdigital induction of *bmpR-1b* by TGFβ beads was inhibited (Fig. 1J).

In previous studies we observed that implantation of beads bearing BMP-2, -4, or -7 in the limb bud was followed by a dramatic enlargement of the cartilages or by apoptotic cell death, depending on the stage of mesoderm in the zone of implantation of the beads (Gañan *et al.*, 1996; Macias *et al.*, 1997). Our present observations showed that when BMP beads were implanted at the tip of the growing digits expression of *bmpR-1b* was upregulated around the bead except in the

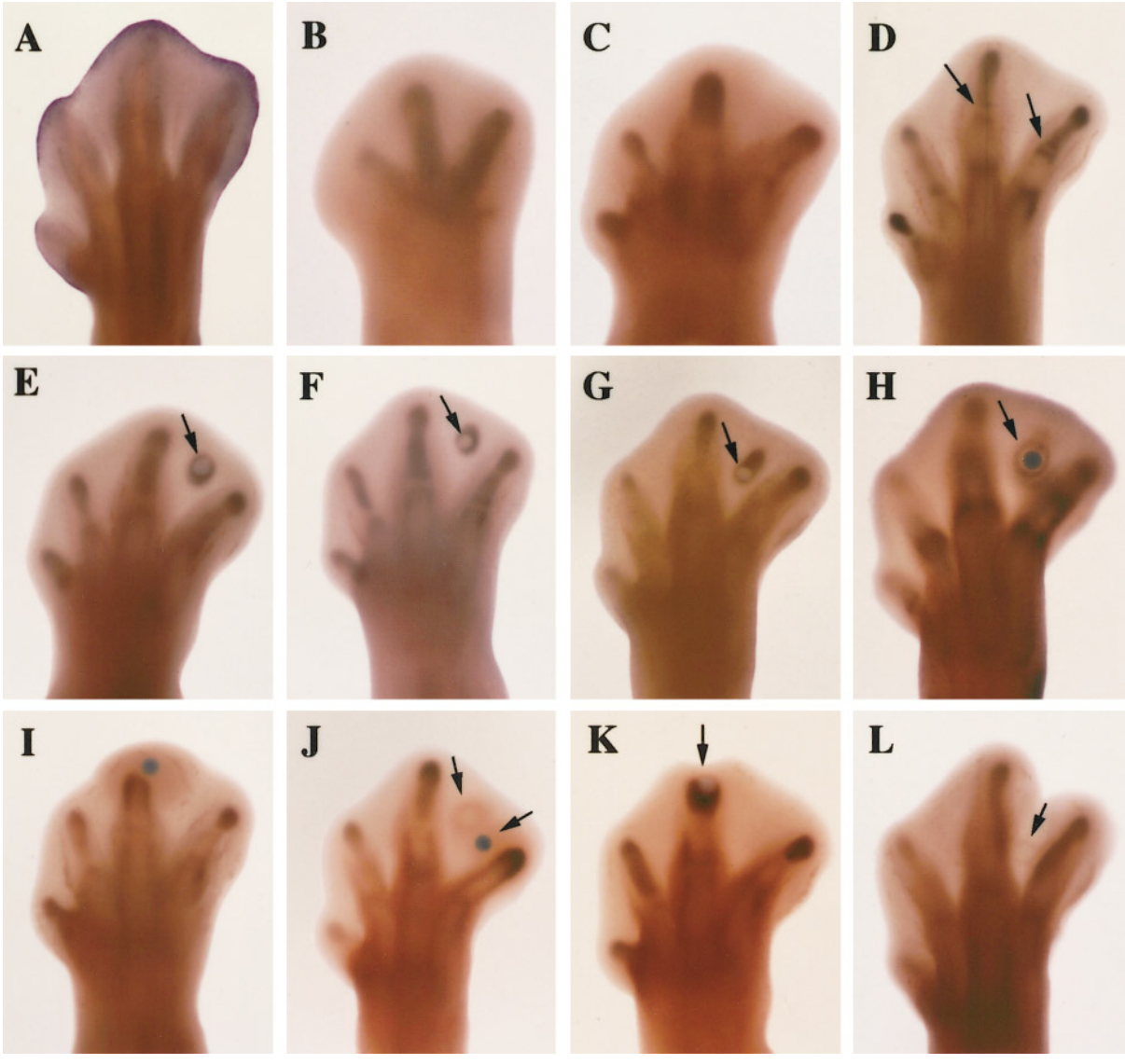

FIG. 1. (A) *In situ* hybridization showing the expression of *bmpR-1a* at stage 30. (B–D) *In situ* hybridizations showing the expression of *bmpR-1b* in the developing autopod at stages 27 (B), 28 (C), and 30 (D). Note the precise domains of *bmpR-1b* in the digital blastemas except at the zones of joint formation (arrows) and the low expression of *bmpR-1a* in the subridge mesoderm and undifferentiated mesenchyme. (E–G) Ectopic interdigital expression of *bmpR-1b* gene 15 (E), 20 (F), and 24 h (G) after implantation of a TGFβ bead (arrow) in the third interdigit at stage 28. Note the progressive distal displacement of the induced domain of *bmpR-1b* gene expression in the course of development. (H) *In situ* hybridization with *ck-erg* in a leg bud autopod 24 h after implantation of a TGFβ bead in the third interdigit. Note the precise concentric distribution around the bead of this gene (arrow) in contrast to the distribution of *bmpR-1b* (G) at the same interval after implantation of the TGFβ bead. (I) Expression of the *bmpR-1b* gene 24 h after implantation of a FGF bead at the tip of digit III. Note that the distal distribution of this gene is inhibited by the FGF bead (compare with D). (J) Expression of the *bmpR-1b* gene 24 h after implantation in the third interdigit of one TGFβ bead (white bead, arrow) and a FGF bead (blue bead, arrow). Note that under this condition TGFβ beads fail to induce the expression of *bmpR-1b* in comparison with the large ectopic expression domain of this gene illustrated in G. (K) Expression of *bmpR-1b* 18 h after the implantation of a BMP bead at the tip of digit III. Note the intense upregulation of this gene by the bead and the absence of gene expression in the mesenchyme distal to the bead (arrow). (L) *bmpR-1b* expression 24 h after the implantation of a BMP bead in the third interdigit at stage 29 (arrow). Note that there is not ectopic interdigital expression of *bmpR-1b* around the bead, and gene expression in the adjacent digits appears displaced from the axis of the phalanges toward their interdigital margin lacking the joint interruptions observed in normal limbs (compare with D).

undifferentiated distal mesenchyme which underwent cell death (Fig. 1K; $n = 12$). In agreement with previous results, BMP beads implanted in the interdigits accelerated programmed cell death but neither *bmpR-1a* nor *bmpR-1b* was induced in the interdigital mesenchyme (Fig. 1L; $n = 16$). However, there was a significant modification in the expression of the *bmpR-1b* gene in the adjacent digits, with the spatial distribution appearing displaced toward the margin of the digits adjacent to the bead (Fig. 1L).

2. Tgfβ2 Gene Is Expressed in the Digital Rays and Is Regulated by TGFβs, FGFs, and BMPs

In view of the ability of TGFβs to induce ectopic extradigits, we analyzed the expression of *tgfβ2* and *tgfβ3* genes in the course of digit morphogenesis. *Tgfβ2* was the only one of these genes that exhibited a pattern of expression compatible with a role in digit morphogenesis. Transcripts of this gene were associated with the digital rays from stage 26. As shown in Figs. 2A–2C, the *tgfβ2* gene was first expressed in the mesenchyme running dorsally and ventrally to the condensing digital blastemas and by stages 28–29, *tgfβ2* transcripts were distributed in the anlage of the tendons, the zones of interphalangeal joint formation, and in the distal tip of the growing digits (Figs. 2C and 3). *Tgfβ-3* was only expressed in the developing tendons (not shown). Implantation of TGFβ beads in the interdigital region was followed by upregulation of the *tgfβ2* gene in a temporal and spatial pattern similar to that of *bmpR-1b* (Figs. 2D–2F; $n = 16$).

As observed in the case of the *bmpR-1b* gene, *tgfβ2* is upregulated at the tip of digits after treatments with BMP beads ($n = 9$) and the expanded domain also excludes the most distal mesenchyme (Fig. 2G) which undergoes cell death after this treatment (Gañan *et al.*, 1996). The phenotype of the digits following this BMP treatment (Fig. 2H) was fully consistent with the new pattern of *tgfβ2* (Fig. 2G) and *bmpR-1b* (Fig. 1K) gene expression.

Also as observed for *bmpR-1b*, FGFs implanted at the tip of the digits delayed the distal progress of *tgfβ2* expression at the tip of the digits (Fig. 2I).

3. Noggin Is Expressed in the Developing Autopod and Blocks Digit Growth and Interdigital Cell Death

Implantation of noggin beads at the tip of the growing digits caused digit truncation (Fig. 4A). During the first 20–40 h after the implantation of the beads the progress zone cells accumulated at the tip of the digit without differentiating (Fig. 4B). By 48 h after the implantation of the bead these cells underwent massive cell death by apoptosis (Figs. 4C and 4D). In correlation with this antichondrogenic effect of noggin, *bmpR-1b* expression at the tip of the treated digit was downregulated 20 h after the implantation of the noggin bead (Fig. 4E). We then addressed the question of whether noggin beads were able to block ectopic TGFβ-induced chondrogenesis. To this end, one TGFβ bead and one noggin bead were implanted in the same interdigit. Under these conditions ectopic chondrogenesis was not blocked but significantly impaired as deduced by the absence of formation of extra digits and by the reduced size of the ectopic cartilages (not shown). Implantation of noggin beads in the interdigital regions did not cause modifications in the pattern of BMP-receptor genes but was followed by an intense temporal inhibition of interdigital cell death (Figs. 4F and 4H–4K). This inhibitory effect was transient and cell death was restored 40 h after the implantation of the beads (Fig. 4G) when cell death was no longer present in the control limbs (Fig. 4D).

In view of the effects of exogenous noggin in digit morphogenesis we analyzed its expression in the course of digit development (Figs. 5A–5E). *Noggin* gene exhibited a precise pattern of distribution in the developing limb from stage 25 to 26 coincidentally with the formation of the digits. At these stages *noggin* expression was intense in the condensing digital rays and in the anterior and posterior margins of the zeugopod (Fig. 5A). A small domain of *noggin* expression which was maintained up to stage 30 was also detected in the posterior margin of the autopod (Figs. 5A and 5B). From stage 29 to 30, *noggin* transcripts localized through the whole extension of the cartilage elements of the autopod except at the joint-forming regions (Figs. 5B and 5C). Later in development (from stage 32 to 33) *noggin* expression concentrated at the prehypertrophic cartilage of the developing diaphyses and in the interzone mesenchyme of the differentiating joints (Figs. 5D and 5E). At these stages *noggin* was also expressed in the differentiating muscles (not shown).

4. FGF and BMPs Regulate noggin Expression

Possible interactions between noggin and the other growth factors present in the autopod were studied by analyzing the influence of beads bearing FGF, TGFβs, or BMPs on the pattern of *noggin* expression. FGF beads implanted at the tip of the growing digits inhibited the expression of *noggin* (Fig. 5F; $n = 5$). BMP beads implanted at the tip of the digits caused a prominent expansion of the domain of *noggin* expression identifiable 12–14 h after implantation of the beads (Fig. 5G; $n = 9$). By contrast, BMP beads were unable to induce an ectopic expression of *noggin* when they were implanted in the interdigital regions (not shown). When TGFβ beads were implanted in the interdigital regions, *noggin* was detected 30 h after treatment, when the ectopic digit was formed, but not earlier (Fig. 4H; $n = 8$), in contrast with the early induction of *bmpR-1b* which was evidenced as soon as 10 h after implantation of TGFβ beads.

DISCUSSION

The formation of limb skeleton constitutes a paradigmatic model for analyzing the molecular basis of morpho-

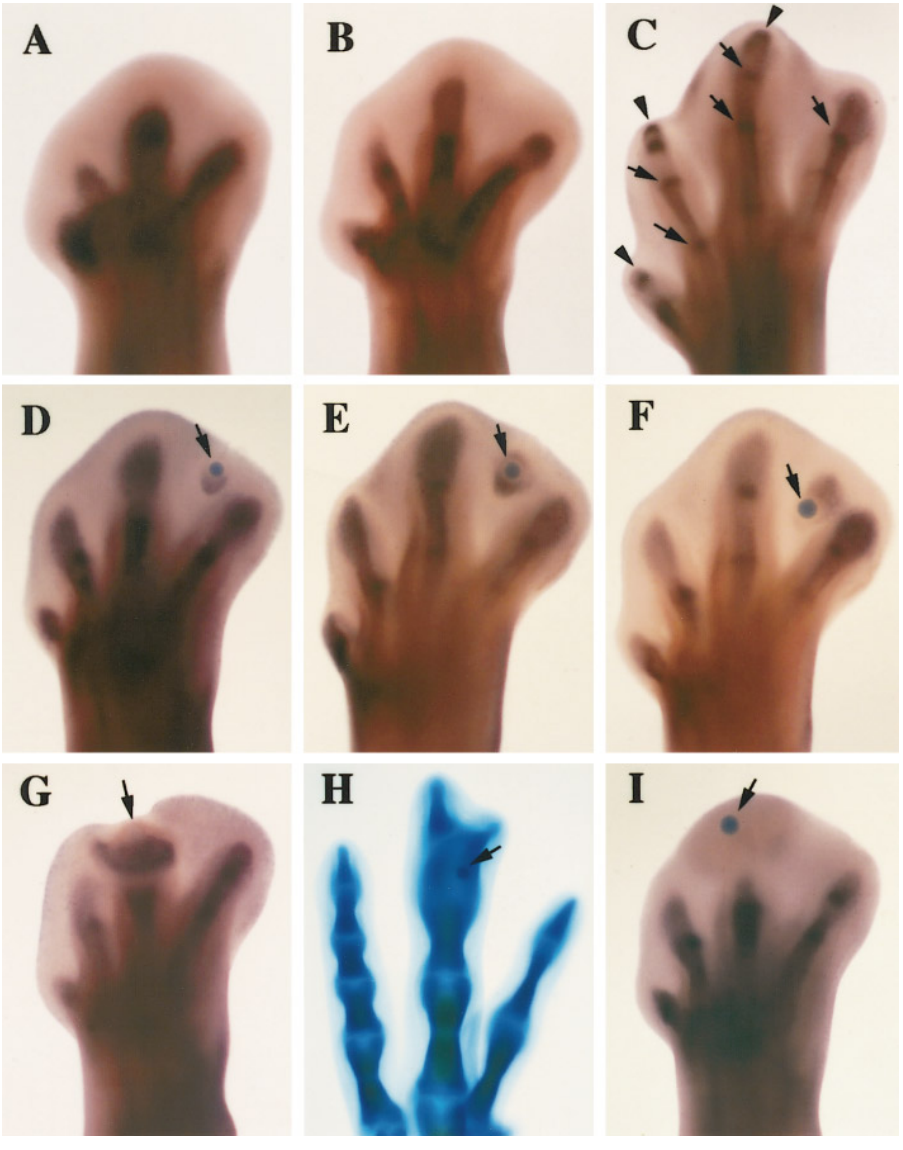

FIG. 2. (A–C) *In situ* hybridizations showing the expression of the *tgfβ2* gene in the developing leg bud at stages 28 (A), 30 (B), and 31 (C). All the specimens are viewed from the dorsal surface. Positivity is observed in the developing tendons, joints (arrows), and in the distal tip of the growing digits (arrowheads). (D–F) Ectopic interdigital expression of the *tgfβ2* gene 16 (D), 20 (E), and 24 h (F) after the implantation of a TGF*β* bead in the third interdigit (arrow) at stage 28. Note that the ectopic distribution of this gene is similar to that of *bmpR-1b* (Figs. 1E–1G). (G) Expression of the *tgfβ2* gene 24 h after implantation of a BMP bead at the tip of digit III. Note the intense upregulation induced by the bead which shows a pattern similar to that observed for *bmpR-1b* following the same treatment (Fig. 1K). (H) Whole-mount cartilage-stained leg bud autopod showing the morphology of digit III after implantation at stage 28 of a BMP bead (arrow) at its distal tip. Note the enlargement of the phalanx around the bead and the distal bifurcation of the digit. (I) Expression of the *tgfβ2* gene 22 h after implantation of a FGF bead at the tip of digit III. Note the intense inhibition of *tgfβ2* gene expression in the treated digit (compare with B).

genesis in vertebrates. Information accumulated over the past decade provides detailed knowledge of the molecular basis for the establishment of spatial coordinates within the early limb bud which precedes and controls the subsequent appearance of the skeleton (see review by Tickle and Eichele, 1994). How these early signals are later translated into particular skeletal morphologies remains largely unknown, but diffusible signals regulating proliferation, differentiation, and cell death are the most likely candidate molecules for this function. In this study we show that in the autopodial plate of the developing limb bud there are well-defined domains of *tgfβ2* and *noggin* in the digital rays,

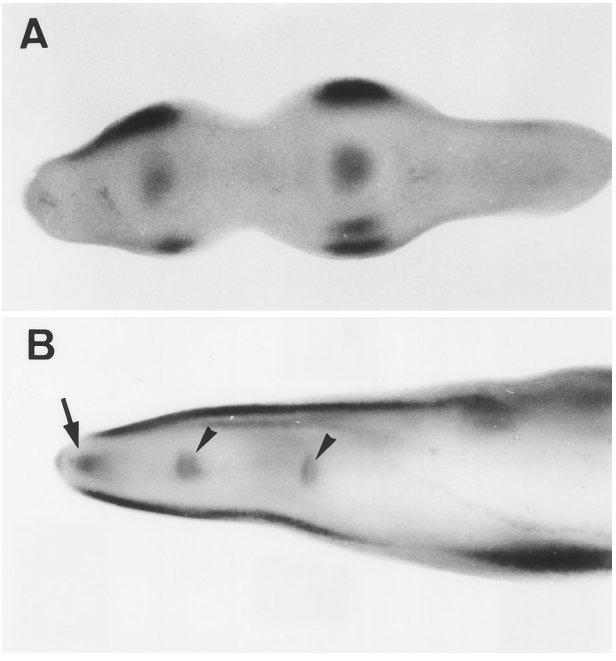

FIG. 3. *tgfβ2* gene expression in transverse (A) and longitudinal (B) sections of leg bud autopods at stages 30 (A) and 31 (B). Positive staining is observed at the distal tip of the developing digits (arrow) and developing joints (arrowheads) as well as in the developing tendons.

in addition to the well-characterized distribution of FGFs in the AER (Mahmood *et al.,* 1995; Vogel *et al.,* 1996) and BMPs in the progress zone and interdigital regions (Francis *et al.,* 1994; Laufer *et al.,* 1997). Furthermore, we provide evidence showing that the formation of the digital rays is controlled by interactions among all these diffusible signals. These interactions regulate the spatial distribution of *bmpR-1b* gene expression in the digit-forming mesenchyme, which prefigures the morphology of the future digit (summarized in Fig. 6). In contrast, expression of *bmpR-1a* is not modified by the growth factors affecting the establishment of the digital rays. This indicates that the chondrogenic influence of this receptor occurs at later stages of cartilage maturation in agreement with Zou *et al.* (1997).

The involvement of *bmpR-1b* in digit chondrogenesis has been recently proposed on the basis of its pattern of expression in the limb bud and by functional studies using retroviruses carrying dominant negative or dominant active constructs of this gene (Kawakami *et al.,* 1996; Zou *et al.,* 1997). Our present observations show that the effects on chondrogenesis induced by exogenous administration of FGFs, TGFβs, and BMPs are preceded by changes in the expression of *bmpR-1b* consistent with a role of this receptor in the onset of chondrogenesis. In addition, digit formation is blocked by exogenous administration of noggin, a BMP antagonist that inhibits the binding of BMP-2, -4, and -7 to their receptors (Zimmerman *et al.,* 1996). All these findings indicate that *bmpR-1b* is a physiological transducer for the formation of digital cartilages, rather than a circumstantial molecular marker of chondrogenesis.

A basic requirement for the maintenance of limb outgrowth is that the distal mesoderm remains in an undifferentiated and proliferating state. There is a great deal of evidence indicating that FGFs are the AER signals which serve these functions (Niswander *et al.,* 1993; Fallon *et al.,* 1994; Mahmood *et al.,* 1995; Vogel *et al.,* 1996). Accordingly, exogenous administration of FGFs inhibits ectopic TGFβ-induced chondrogenesis (Gañan *et al.,* 1996). Our present findings showing that FGFs downregulate *bmpR-1b* at the tip of the digit and block its ectopic induction by TGFβs provide mechanistic evidence for the above-mentioned effect of FGFs. In addition, FGF beads inhibit the expression of *noggin* and *tgfβ2* at the tip of the digits. The inhibitory effect of FGFs on the expression of genes associated with digit-forming regions is compatible with a role of this factor in the induction of *msx* genes (Kostakopoulou *et al.,* 1997) which are transcriptional repressors characteristically expressed in the progress zone and interdigital mesoderm.

TGFβs are able to induce ectopic extra digits when they are administered to the interdigital mesoderm (Gañan *et al.,* 1996) suggesting that members of this family may serve as chondrogenic proximal signals opposite to FGFs responsible for the establishment of the digital rays. In this study we show that *tgfβ2* exhibits a pattern of expression in the growing digits compatible with a function in the control of digit chondrogenesis and joint and tendon formation. In accordance with this finding, mice deficient in TGFβ2 exhibit limb skeletal abnormalities (Sanford *et al.,* 1997) and targeted disruption of TGFβ signaling causes severe joint alterations (Serra *et al.,* 1997). However, gross digital alterations are not present in these mutant mice. This could be explained by a functional redundancy between different TGFβs, since several TGFβ isoforms are expressed during the early stages of chondrocyte differentiation in the mouse and human (Sandberg *et al.,* 1988; Gatherer *et al.,* 1990; Pelton *et al.,* 1990; Millan *et al.,* 1991). Our study also shows that ectopic chondrogenesis induced by TGFβ beads is preceded by the induction of *bmpR-1b* suggesting that chondrogenesis by TGFβs is mediated through BMP signaling. In accordance, noggin, which inhibits BMPs but not TGFβs (Zimmerman *et al.,* 1996), significantly reduces the size of cartilages induced by TGFβ beads.

In addition to the observation showing the influence of FGFs and TGFβs through *bmpR-1b* in the establishment of the digital rays as discussed above, we have found evidence for an interactive feedback loop between noggin and BMPs which controls digit skeletogenesis. The *noggin* gene is coexpressed with *bmpR-1b* in the condensing region of the growing digits and exogenous noggin downregulates *bmpR-1b* and blocks digit formation. Since all data available show that noggin functions by interacting with and inhibiting BMPs, the observed downregulation of *bmpR-1b* by noggin indicates that stimulation of BMPR-1b receptor

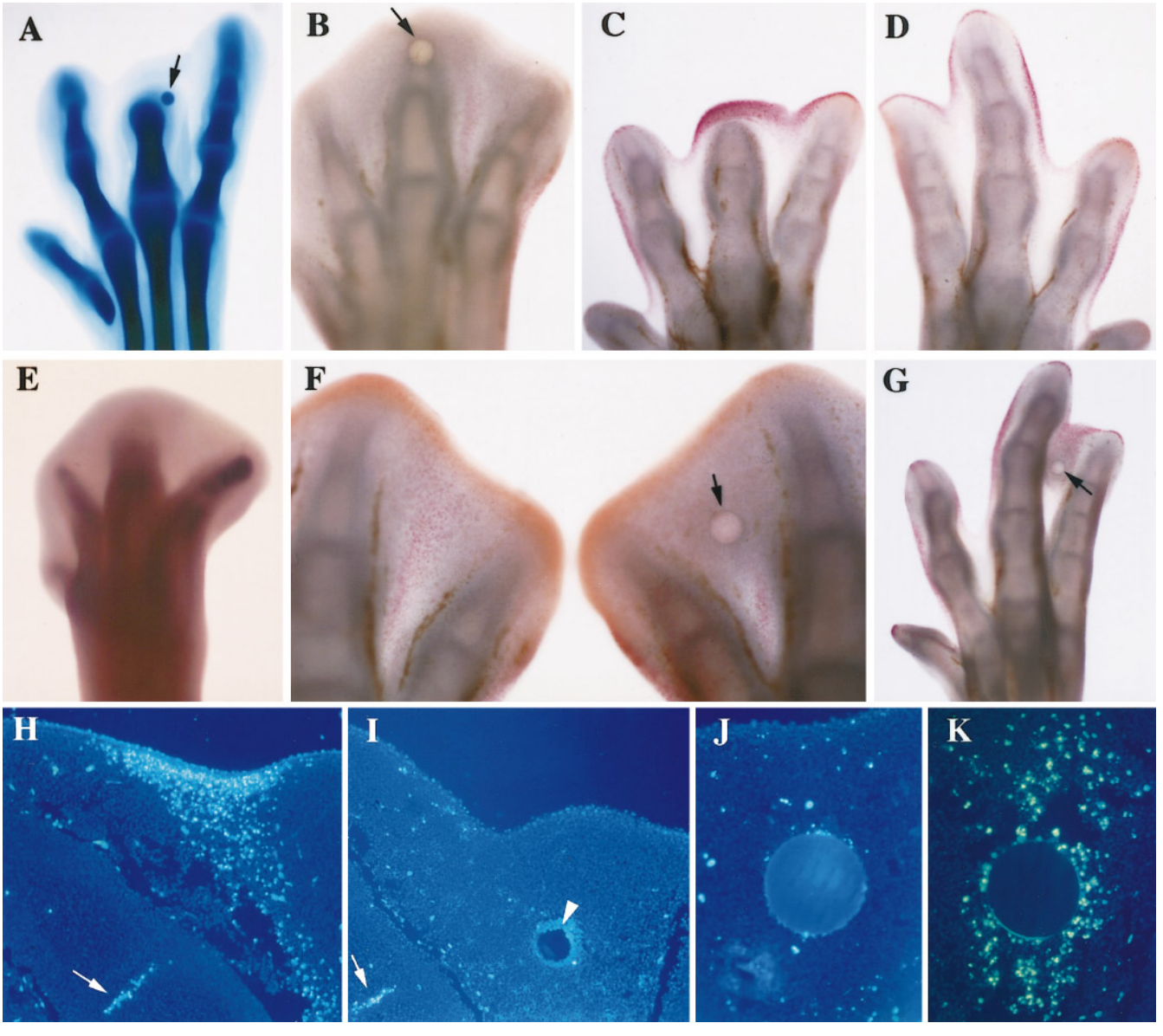

FIG. 4. (A) Whole-mount cartilage-stained leg bud autopod illustrating the phenotype of the autopod after the implantation at stage 28 of a noggin bead at the tip of digit III. Note the severe truncation of the treated digit. Arrow shows the position of the bead. (B) Leg bud autopod 24 h after the implantation of a noggin bead at the tip of digit III (arrow) vital stained with neutral red. Note that mesodermal cells around the bead remain undifferentiated and healthy. (C, D) Experimental (C) and control (D) autopods 50 h after the implantation of a noggin bead at the tip of digit III, vital stained with neutral red. Note the area of cell death at the tip of the treated digit. (E) Expression of *bmpR-1b* in an experimental autopod 22 h after implantation of noggin beads at the tip of digit III. (F) Detailed view of control and experimental autopods showing the inhibition of interdigital cell death by noggin. The limbs were stained with neutral red 20 h after implantation of a noggin bead (arrow) in the third interdigit at stage 30. (G) Experimental leg bud 48 h after the implantation of a noggin bead in the third interdigit (arrow) vital stained with neutral red. Note that cell death is now restored in the interdigital mesenchyme (compare with D, which is a control limb of an equivalent stage). (H–K) Illustrations showing the inhibitory effect of noggin on interdigital cell death by means of TUNEL assay. H and I are control (H) and experimental (I) interdigits of the same embryo 18 h after implantation of a noggin bead (arrowhead) at stage 30. Arrows show the areas of physiological cell death in the developing joints of digit III. J and K are detailed views showing the pattern of interdigital apoptosis 20 h after the implantation of a noggin bead (J) and a control bead soaked in PBS (K). Note the intense inhibition of apoptosis around the bead in J in comparison with the normal pattern of cell death observed in K.

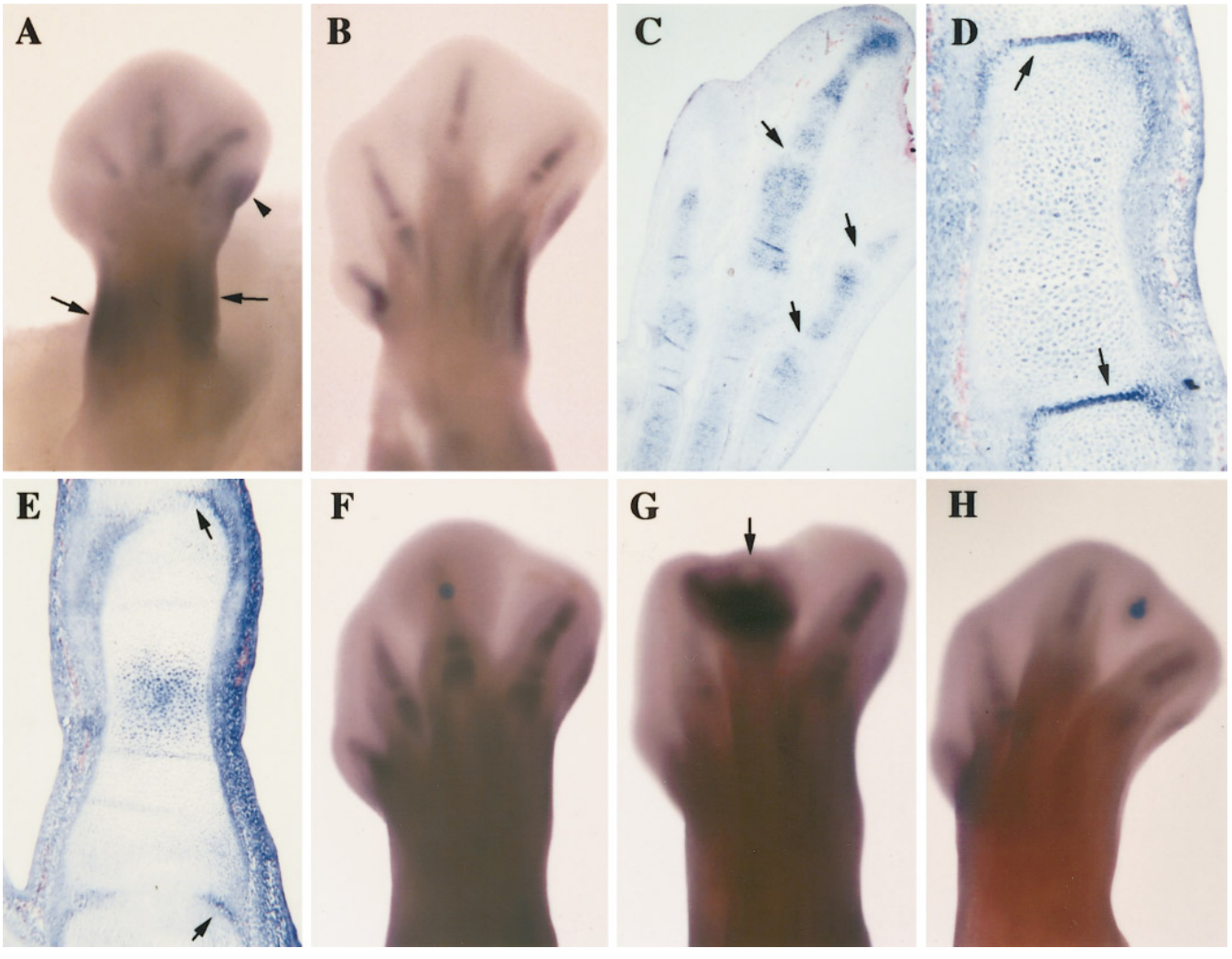

FIG. 5. (A, B) Whole-mount *in situ* hybridizations showing the expression of *noggin* in the leg bud autopod at stages 26 (A) and 30 (B). Note that the expression of this gene in the developing digital rays is coincident with that of *bmpR-1b* (compare B with Fig. 1B). (C) Expression of *noggin* in a tissue section of the leg bud autopod at stage 31. Note that expression is high through the cartilaginous elements of the digital rays and absent from the zones of joint formation (arrows). (D) Longitudinal section of digit IV at stage 32 showing the appearance of *noggin* transcripts in the differentiating interphalangeal joints (arrows) at this stage. (E) Longitudinal section of the proximal phalanx of digit II at stage 34 showing the accumulation of *noggin* transcripts in the cartilage in the course of hypertrophic differentiation at the central region of the diaphysis and in the differentiating joint tissues (arrows). (F, G) Regulation of *noggin* expression by FGF-2 (F) and BMP-2 (G) 20 h after the implantation of beads at the tip of digit III. Note the intense downregulation caused by the FGF bead (F) and the upregulation caused by the BMP bead (G). Note that as observed for *bmpR-1b*, upregulation of *noggin* is not observed in the mesenchyme distal to the BMP bead (G, arrow). (H) Expression of *noggin* 24 h after the implantation of a TGFβ bead in the third interdigit. Note that *noggin* is not yet expressed in association with the bead, although the ectopic cartilage induced by the bead is already present.

upregulates its own gene expression. The observed upregulation of *bmpR-1b* following treatment with BMPs also supports this interpretation. However, BMPs also upregulate the *noggin* gene, thus forming an interactive loop which may control the size and shape of the developing cartilages. Such a mechanism would avoid the formation of abnormally large cartilages, which happens in experiments when the local concentration of BMPs is increased in the developing cartilages (Duprez *et al.*, 1996; Macias *et al.*, 1997). All these findings provide a molecular basis for the mechanisms regulating chondrogenesis in the autopod which is summarized in Fig. 6.

The inhibition of cell death observed here following implantation of noggin beads in the interdigital regions supports previous studies implicating BMPs in this function (Zou and Niswander, 1996; Gañan *et al.*, 1996; Yoko-

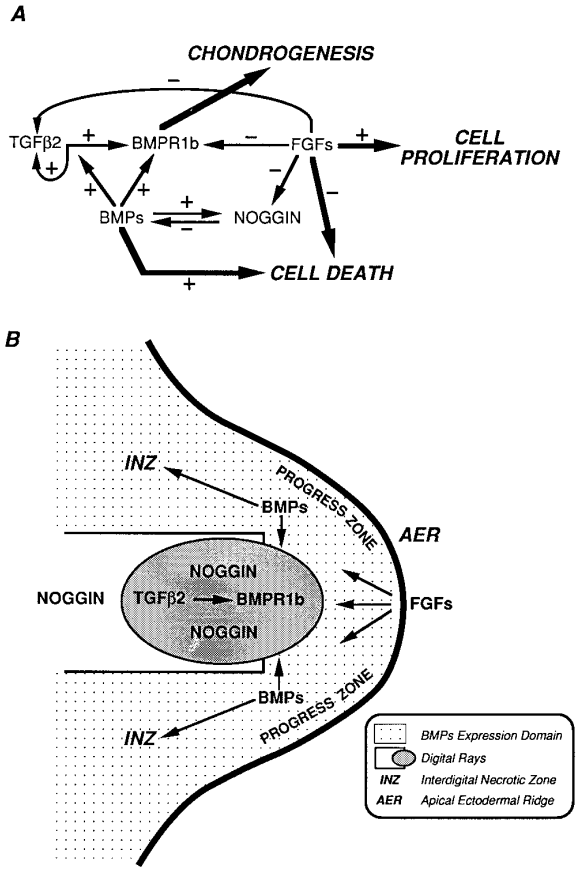

FIG. 6. (A) Model of interactions between the secreted signals present in the autopod in the course of digit formation. (B) Schematic representation of the tip of a growing digit and its adjacent regions showing the topographical distribution of the signals indicated in A.

uchi *et al.*, 1996; Kawakami *et al.*, 1996; Macias *et al.*, 1997). Previous experiments overexpressing modified type I BMP receptors in the limb bud provided contradictory results concerning the BMP receptor involved in cell death (Zou and Niswander, 1996; Yokouchi *et al.*, 1996). Zou *et al.* (1997) recently reported that infection of early limb buds with retroviruses carrying a dominant active *bmpR-1b* gene is followed by massive cell death suggesting a role for this receptor in the physiological control of cell death. In accordance with the absence of expression of *bmpR-1b* in the interdigital regions, our findings argue against that interpretation. As discussed above, inhibition of interdigital cell death by TGFβs was accompanied by the formation of an ectopic domain of *bmpR-1b*. In addition, upregulation of *bmpR-1b* after the implantation of BMP beads at the tip of the digits characteristically excludes the area of cell death induced at the tip of the digit by this treatment (Macias *et al.*, 1997). Thus, cell death caused by overexpression of the dominant active *bmpR-1b* may not be a physiological response of the undifferentiated limb mesoderm. However, we have not found evidence supporting the alternative implication of *bmpR-1a* in cell death (Kawakami *et al.*, 1996; Yokouchi *et al.*, 1996). None of the treatments causing modifications in the pattern of cell death were accompanied by changes in the expression of this gene, including inhibition of cell death by FGFs or by TGFβs or induction of cell death by BMPs. In this case, our observations are in agreement with the lack of cell death observed in experiments of overexpression of a dominant active *bmpR-1a* (Zou *et al.*, 1997). A tempting hypothesis which would explain these contradictory findings is the existence of a still unidentified BMP receptor containing the characteristic death domain present in other proapoptotic receptors (see Wallach, 1997) which may be responsible for this BMP function, as has been observed for the neurotrophins (see Dechant and Barde, 1997).

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REFERENCES

Chang, C., Wilson, P. A., Mathews, L. S., and Hemmati-Brivanlou, A. (1997). A Xenopus type I activin receptor mediates mesodermal but not neural specification during embryogenesis. *Development* **124,** 827–837.

Crossley, P. H., Minowada, G., MacArthur, C. A., and Martin, G. R. (1996). Roles for FGF-8 in the induction, initiation, and maintenance of chick limb development. *Cell* **84,** 127–136.

Dechant, G., and Barde, Y. A. (1997). Signaling through the neurotrophin receptor p75 (NTR). *Curr. Opin. Neurobiol.* **73,** 413–418.

Duprez, D. M., Bell, E. J., Richardson, M., Archer, C. W., Wolpert, L., Brickell, P. M., and Francis-West, P. H. (1996). Overexpression of BMP-2 and BMP-4 alters the size and shape of developing skeletal elements in the chick limb. *Mech. Dev.* **57,** 145–157.

Enomoto-Iwamoto, M., Iwamoto, M., Mukudai, Y., Kawakami, Y., Nohno, T., Higuchi, Y., Takemoto, S., Ohuchi, H., Noji, S., and Kurisu, K. (1998). Bone morphogenetic protein signaling is required for maintenance of differentiation phenotype, control of proliferation, and hypertrophy in chondrocytes. *J. Cell Biol.* **140,** 409–418.

Fainsod, A., Deißler, K., Yelin, R., Marom, K., Epstein, M., Pillemer, G., Steinbeisser, H., and Blum, M. (1997). The dorsalizing and neural inducing gene follistatin is an antagonist of BMP-4. *Mech. Dev.* **63,** 39–50.

Fallon, J. F., Lopez, A., Ros, M. A., Savage, M. P., Olwin, B. B., and Simandl, B. K. (1994). FGF-2, apical ridge growth signal for chick limb development. *Science* **264,** 104–107.

Feijen, A., Goumans, M. J., and Van den Eijnden-van Raaij, A. J. M. (1994). Expression of activin subunits, activin receptors and follistatin in postimplantation mouse embryos suggests specific developmental functions for different activins. *Development* **120,** 3621–3637.

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

Gañan, Y., Macias, D., Duterque-Coquillaud, M., Ros, M. A., and Hurle, J. M. (1996). Role of TGFβs and BMPs as signals controlling the position of the digits and the areas of interdigital cell death in the developing chick limb autopod. *Development* **122,** 2349–2357.

Gañan, Y., Macias, D., Basco, R. D., Merino, R., and Hurle, J. M. (1998). Morphological diversity of the avian foot is related with the pattern of *msx* gene expression in the developing autopod. *Dev. Biol.,* **196,** 33–41.

Gatherer, D., Ten Dijke, P., Baird, D. T., and Akhurst, R. J. (1990). Expression of TGF-β isoforms during first trimester human embryogenesis. *Development* **110,** 445–460.

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

Kawakami, Y., Ishikawa, T., Shimabara, M., Tanda, N., Enomoto-Iwamoto, M., Iwamoto, M., Kuwana, T., Ueki, A., Noji, S., and Nohno, T. (1996). BMP signaling during bone pattern determination in the developing limb. *Development* **122,** 3557–3566.

Kostakopoulou, K., Vogel, A., Brickell, P., and Tickle, C. (1996). ''Regeneration'' of wing bud stumps of chick embryos and reactivation of Msx-1 and Shh expression in response to FGF-4 and ridge signals. *Mech. Dev.* **55,** 119–131.

Kulyk, W. M., Rodgers, B. J., Greer, K., and Kosher, R. A. (1989). Promotion of embryonic chick limb cartilage differentiation by transforming growth factor-β. *Dev. Biol.* **135,** 424–430.

Laufer, E., Pizette, S., Zou, H., Orozco, O. E., and Niswander, L. (1997). BMP expression in duck interdigital webbing: A reanalysis. *Science* **278,** 305.

Leonard, C. M., Fuld, H. M., Frenz, D. A., Downie, S. A., Massague, J., and Newman, S. A. (1991). Role of transforming growth factor-β in chondrogenic pattern formation in the embryonic limb: Stimulation of mesenchymal condensation and fibronectin gene expression by exogenous TGF-β and evidence for endogenous TGF-β-like activity. *Dev. Biol.* **145,** 99–109.

Lyons, K. M., Hogan, B. L. M., and Robertson, E. J. (1995). Colocalization of BMP7 and BMP2 RNAs suggests that these factors cooperatively mediate tissue interactions during murine development. *Mech. Dev.* **50,** 71–83.

Macias, D, Gañan, Y., Ros, M. A., and Hurle, J. M. (1996). *In vivo* inhibition of programmed cell death by local administration of FGF-2 and FGF-4 in the interdigital areas of the embryonic leg bud. *Anat. Embryol.* **193,** 533–541.

Macias, D., Gañan, Y., Sampath, T. K., Piedra, M. E., Ros, M. A., and Hurle, J. M. (1997). Role of BMP-2 and OP-1 (BMP-7) in programmed cell death and skeletogenesis during chick limb development. *Development* **124,** 1109–1117.

Mahmood, R., Bresnick, J., Hornbruch, A., Mahomy, C., Morton, N., Colguhoum, K., Martin, P., Lumsden, A., Dickson, C., and Mason, I. (1995). A role for FGF-8 in the initiation and maintenance of vertebrate limb outgrowth. *Curr. Biol.* **5,** 797–806.

Millan, F. A., Denhez, F., Kondaiah, P., and Akhurst, R. J. (1991). Embryonic gene expression patterns of TGFβ1, β2 and β3 suggest different developmental functions *in vivo*. *Development* **111,** 131–144.

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

Pelton, R. W., Dickinson, M. E., Moses, H. L., and Hogan, B. L. M. (1990). *In situ* hybridization analysis of TGF-β3 RNA expression during mouse development: Comparative studies with TGF-β1 and -β2. *Development* **110,** 600–620.

Piccolo, S., Sasai, Y., Lu, B., and De Robertis, E. M. (1996). Dorsoventral patterning in *Xenopus:* Inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell* **86,** 589–598.

Sandberg, M., Vuorio, T., Hirrovan, H., Alitalo, K., and Vuorio, E. (1988). Enhanced expression of TGF-β and c-fos mRNAs in the growth plates of developing human long bones. *Development* **102,** 461–470.

Sanford, L. P., Ormsby, I., Gittenberger-De Groot, A. C., Sariola, H., Friedman, R., Boivin, G. P., Cardell, E. M., and Doetschman, T. (1997). TGFβ2 knockout mice have multiple developmental defects that are non-overlapping with other TGFβ knockout phenotypes. *Development* **124,** 2659–2670.

Schoefiel, J. N., and Wolpert, L. (1990). Effect of TGF-β1, TGF-β2, and bFGF on chick cartilage and muscle cell differentiation. *Exp. Cell Res.* **191,** 144–148.

Serra, R., Johnson, M., Filvaroff, E. H., LaBorde, J., Sheehan, Derynck, R., and Moses, H. L. (1997). Expression of a truncated, kinase-defective TGF-β type II receptor in mouse skeletal tissue promotes terminal chondrocyte differentiation and osteoarthritis. *J. Cell Biol.* **139,** 541–552.

Tickle, C., and Eichele, G. (1994). Vertebrate limb development. *Annu. Rev. Cell Biol.* **10,** 121–152.

Vogel, A., Rodriguez, C., and Izpisua-Belmonte, J. C. (1996). Involvement of FGF-8 in initiation, outgrowth and patterning of the vertebrate limb. *Development* **122,** 1737–1750.

Wallach, D. (1997). Cell death induction by TNF a matter of self control. *TIBS* **22,** 107–109.

Yamashita, H., ten Dijke, P., Huylebroeck, D., Sampath, T. K., Andries, M., Smith, J. C., Heldin, C. H., and Miyazono, K. (1995). Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. *J. Cell Biol.* **130,** 217–226.

Yokouchi, Y., Sakiyama, J., Kameda, T., Iba, H., Suzuki, A., Ueno, N., and Kuroiwa, A. (1996). BMP-2/-4 mediate programmed cell death in chicken limb buds. *Development* **122,** 3725–3734.

Zimmerman, L. B., De Jesús-Escobar, J. M., and Harland, R. M. (1996). The Speman organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* **86,** 599–606.

Zou, H., and Niswander, L. (1996). Requirement for BMP signaling in interdigital apoptosis and scale formation. *Science* **272,** 738–741.

Zou, H., Wieser, R., Massagué, J., and Niswander, L. (1997). Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. *Genes Dev.* **11,** 2191–2203.

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