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Endogenous and Ectopic Expression of *noggin* Suggests a Conserved Mechanism for Regulation of BMP Function during Limb and Somite Patterning

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The gene *noggin*, originally cloned in *Xenopus*, encodes a secreted factor expressed in the Spemann organizer, where it functions to oppose the ventralizing influence of bone morphogenetic proteins (BMPs). Noggin protein acts by binding directly to BMPs, thereby preventing them from interacting with their receptors. Here we describe the pattern of expression of the chicken *noggin* gene during somite and limb development, two tissues in which BMPs have been postulated to play essential patterning roles. We find that *noggin* is expressed in dynamic restricted patterns consistent with an important role in the modulation of BMP signaling. Using a replication competent retrovirus we have ectopically expressed *noggin* in developing somitic and limb bud mesoderm and observed phenotypes consistent with complete block of BMP activity. This includes suppression of lateral somite differentiation and, in the limb, complete inhibition of chondrogenesis and local suppression of programmed cell death. In addition, we find that ectopic *noggin* expression in the limb has no effect on anteroposterior limb pattern, suggesting that BMPs are unlikely to play a significant role in this process. Taken together, our results indicate that *noggin* is a key regulator of vertebrate limb and somite patterning and suggest that the antagonistic Noggin–BMP interaction is a widely used mechanism to modulate BMP signaling during multiple inductive events in vertebrate embryogenesis.

INTRODUCTION

The development of complex multicellular organisms requires the precise execution of a detailed set of instructions deeply imbedded within the molecular fabric of the fertilized zygote. Included among the many processes that these instructions encode for are the generation of signaling centers and the specification of cells which respond to them. Indeed, cell–cell interactions and inductive signaling mechanisms are the means by which pattern is specified in a broad range of developing tissues (Slack, 1991). Recently, the molecular nature of several key signaling molecules has begun to be defined. An initial surprise was that many of these factors are evolutionary ancient signaling molecules which interact with equally ancient signaling pathways. In retrospect, the finding that these signaling pathways are used during multiple inductive interactions is, perhaps, not so surprising given their antiquarian origins.

One such evolutionarily conserved pathway is the *Bone Morphogenetic Protein/decapentaplegic* pathway (*BMP/dpp*), which operates in many tissues in vertebrates and flies (Hogan, 1996; Holley and Ferguson, 1997). BMPs, which are members of the transforming growth factor-β (TGF-β) superfamily of secreted factors, were originally identified by their ability to induce ectopic bone formation in mammals (Urist *et al.*, 1979; Wozney *et al.*, 1988), but since then it has become evident that BMPs are involved in many developmental decisions in multiple organisms (reviewed by Hogan, 1996). Included in the *BMP/dpp* pathway are molecules that act as dominant inhibitors of BMP and Dpp activities, namely, Noggin and Chordin in vertebrates (Smith and Har-

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land, 1992; Sasai *et al.*, 1994) and, in flies, the product encoded by the *short gastrulation* (*sog*) gene, the *Drosophila* homolog of *chordin* (Francois *et al.*, 1994; Biehs *et al.*, 1996). Sog has been shown to antagonize Dpp activity during neurogenesis in the embryonic ectoderm (Biehs *et al.*, 1996) and during wing vein patterning (Yu *et al.*, 1996). To this date, Noggin and Chordin activities in vertebrates have only been described in the context of dorsal mesoderm specification and neural induction in *Xenopus*. Noggin is able to mimic the effect of the Nieuwkoop center in the induction of the organizer, and also mimics the activity of the Spemann organizer in the dorsalization of lateral and ventral mesoderm and in the induction of neural tissue (Smith and Harland, 1992; Lamb *et al.*, 1993; Smith *et al.*, 1993). *Noggin* is expressed during gastrulation in the dorsal mesoderm, accumulating in the presumptive notochord. During neurulation it expands into the prechordal mesoderm, and at the tailbud stages the expression persists in the notochord and extends to the roof plate, head mesoderm, and some neural crest derivatives (Smith and Harland, 1992). The activities of the Spemann organizer in mesoderm dorsalization and induction of neural tissue are antagonized in *Xenopus* by BMPs such as BMP-4, which are able to induce epidermis and ventral mesoderm (Dale *et al.*, 1992; Jones *et al.*, 1992; Fainsod *et al.*, 1994; Wilson and Hemmati-Brivanlou, 1995). Noggin and BMP-4 had been previously shown to compete to control the extent of the dorsalization within the marginal zone of the embryo (Re'em-Kalma *et al.*, 1995). Recently, an explanation for the opposing activities of Noggin and BMP-4 has been found: Noggin protein has been shown to bind BMPs, preventing the interaction of these factors with their receptors, thus blocking BMP signaling (Zimmerman *et al.*, 1996). *Xenopus* Noggin also binds Dpp when expressed in *Drosophila* embryos (Holley *et al.*, 1996), but no *Drosophila noggin* gene has been reported yet. Chordin (Sasai *et al.*, 1995; Piccolo *et al.*, 1996) also antagonizes BMPs by a similar mechanism (reviewed by Thomsen, 1997). Follistatin is another protein which antagonizes BMP signals, and it has been reported that it can interact directly with the BMP-4 protein *in vitro* (Fainsod *et al.*, 1997).

We reasoned that, if Noggin/BMP (Dpp) interactions were present in the common ancestor between flies and vertebrates, they might also function in a similar manner to pattern new derived structures which developed during the chordate radiation. Two such structures which have received considerable attention as models for pattern formation in vertebrate embryos are the limbs and somites. BMPs have been shown to be involved in the control of mediolateral patterning in the somites, and in the development of cartilage condensations and the control of cell death and cell proliferation in the limbs.

In vertebrates, the unsegmented paraxial mesoderm condenses into somites, metamerically arranged units which are composed of epithelial cells, and of mesenchymal cells located within the somitocoele (Huang *et al.*, 1994). The epithelial somite initially lacks any obvious polarity but, as development proceeds, morphological differences are revealed along the dorsoventral and mediolateral axes of each somite. In the mediolateral axis, the somites are subdivided into a medial domain, which will give rise to the axial muscles and the skeleton, and a lateral domain, which will generate the muscles of the body wall and the limbs (Ordahl and Le Douarin, 1992). The signaling molecule BMP-4, expressed in the lateral plate mesoderm, has been proposed to act as a diffusible lateralizing signal which controls the expression of the lateral marker *cSim-1* and antagonizes an unidentified diffusible medializing signal provided by the neural tube (Pourquie *et al.*, 1996; Tonegawa *et al.*, 1997). In the case of the vertebrate limb, BMPs are expressed in restricted patterns which have been associated with the control of cell proliferation, cell death, and the development of cartilage condensations (Lyons *et al.*, 1990; Jones *et al.*, 1991; Francis *et al.*, 1994; Francis-West *et al.*, 1995). In chicken embryos, experiments involving different methods of administration of BMPs (Francis *et al.*, 1994; Duprez *et al.*, 1996a,b; Gañan *et al.*, 1996; Macías *et al.*, 1997) and of different forms of dominant negative BMP receptors (Kawakami *et al.*, 1996; Yokouchi *et al.*, 1996; Zou and Niswander, 1996), have confirmed the involvement of BMPs in these three processes, although the functions of BMPs on an individual or collective basis remain uncertain.

In this paper, we describe the expression of chicken *noggin* and, through gain of function experiments, we address its potential role in the regulation of BMP activities in limb and somite patterning. Our results indicate that *noggin* is a key integrator and modulator of multiple BMP pathways which operate in the limbs and somites.

MATERIALS AND METHODS

Cloning of chicken noggin. The *Xenopus noggin* cDNA (Smith and Harland, 1992) was used as a probe to isolate a chicken genomic clone, using standard procedures for library screening (Sambrook *et al.*, 1989). After using the genomic sequence to screen a stage 10–15 chicken cDNA library, several cDNAs were isolated. One of these cDNAs (MS3), contained the entire chicken *noggin* ORF, which was used for our studies (see also Connolly *et al.*, 1997). Chicken Noggin protein has 83% amino acid identity to mouse Noggin and 86% identity to *Xenopus* Noggin.

Construction of RCAS viruses and viral infections. A cDNA fragment containing the entire coding region of the chicken *noggin* was cloned into the *SLAX-13* vector (Riddle *et al.*, 1993), and then subcloned into *RCAS(BP)A* (Hughes *et al.*, 1987). Viral supernatants were obtained essentially as described in Riddle *et al.* (1993), with minor modifications. Infection of the somites was achieved by injecting the *RCAS–noggin* virus into the presomitic mesoderm of the right side of Hamburger–Hamilton (1951) stage 10 embryos (according to Johnson *et al.*, 1994). The extent of the infection was monitored by whole-mount *in situ* using a probe to detect the viral message (Riddle *et al.*, 1993). The *RCAS–Shh* virus was described by Riddle *et al.* (1993)

In situ hybridizations. After viral infection, embryos were fixed in 4% paraformaldehyde in PBS overnight at 4°C and processed for

whole-mount *in situ* at the appropriate stage as described in Riddle *et al.* (1993). Antisense RNA probe for *noggin* was synthesized from a plasmid containing the MS3 cDNA clone. The probe for *cSim-1* was synthesized from a plasmid containing the entire ORF of the chicken *Sim-1* gene (kindly provided by C. M. Fan; Pourquie *et al.*, 1996). The probe for *BMP-4* was described in Roberts *et al.* (1995), and the *RCAS* and *HoxD13* probes were described in Riddle *et al.* (1993). The stained embryos were viewed under a Nikon microscope and photographed using Kodak EB 100 film. Selected stained embryos were dehydrated in 30% sucrose in PBS and embedded in a mixture of 1.5% agar and 5% sucrose in PBS. The blocks were frozen at −20°C and 50-μm sections were cut using a Leitz cryomicrotome. Sections were rehydrated in PBS and mounted in Aquamount. Pictures were taken in a Zeiss Axiophot microscope using Nomarsky optics and a Kodak Ektachrome 64T film.

Alcian blue staining of cartilage. To reveal the cartilage pattern resultant from the ectopic expression of *noggin* in the limbs, embryos infected with the *RCAS–noggin* virus at stage 10 (presomitic mesoderm, right side) or 17–20 (right forelimb, at different locations) were processed for Alcian blue staining. Injected embryos at day 9 or 10 of development were fixed in 4% paraformaldehyde in PBS overnight at 4°C. Embryos were then eviscerated, rinsed in PBS several times, and stained for 6–8 h with Alcian blue solution (0.02% Alcian blue 8GX, dissolved in a mixture of 70% ethanol and 30% glacial acetic acid). Embryos were then rinsed through ethanol series (100, 95, 70, 40, 15%), 1 h each, and washed with distilled water. Finally, embryos were run through 0.5% potassium hydroxide:glycerol series (3:1, 1:1, 1:3) for 2 h each step and stored in pure glycerol. Pictures of the dissected limbs were taken using a Nikon microscope and Kodak EB 100 film.

Cell implants and ZPA grafts. Chicken embryonic fibroblasts (CEFs) were transfected with the *RCAS–Shh* construct, and cell pellets were prepared to be implanted in the anterior margin of forelimbs, as described by Riddle *et al.* (1993). For ZPA grafts, posterior mesenchyme from stage 18 forelimb buds was dissected using tungsten needles and grafted to the anterior margin of *RCAS–noggin*-infected limb buds. The operated limbs were examined regularly to make sure that the grafts stayed in place.

Detection of apoptotic cells in the limb. We followed exactly the protocol described by Conlon *et al.* (1995) and Conlon and Rossant (1992), for detection of apoptotic cells in whole mounts by the TUNEL procedure

RESULTS

Expression of noggin in Midline Axial Tissues during Early Somite Development

noggin transcripts are detected in axial tissues slightly before the head process stage (stage 5 according to Hamburger and Hamilton, 1951), in Hensen's node and the anterior notochord (Fig. 1A, hn indicates Hensen's node; corresponding transverse section in Fig. 1E, see also Connolly *et al.*, 1997). *Noggin* continues to be expressed in the notochord as Hensen's node regresses (Figs. 1B and 1C, transverse section corresponding to Fig. 1C is shown in Fig. 1F). Around stage 8+ (or 5-somite stage), *noggin* starts to be expressed faintly in the somitic tissue. From that point on, *noggin* is expressed in the lateral part of the 4–5 most recently formed somites (Fig. 1D, arrowhead), and its expression shifts dorsomedially as somites mature (a transverse section of the embryo shown in Fig. 1D is shown in Fig. 1G). *noggin* is also expressed in the dorsal neural tube at the level of the unsegmented mesoderm (asterisk in Fig. 1D indicates that level) and the most newly formed somites (arrowhead in Fig. 1D), and also in the intermediate mesoderm at the level of the segmental plate (not shown).

Involvement of noggin in the Mediolateral Patterning of the Somites

The pattern of expression of *noggin* in the dorsomedial somite and midline axial structures such as the notochord (Fig. 1G), suggests that *noggin* may play an important role in mediolateral somite patterning. Consistent with this idea are observations which support a role for *BMP-4* in specification of lateral somite fates. *BMP-4* is expressed in the lateral plate mesoderm during mediolateral axis specification of somites (Pourquie *et al.*, 1996; Tonegawa *et al.*, 1997) and implants of cells expressing BMP-4 are able to induce ectopically the lateral marker *cSim-1* in the medial domain of the somites (Pourquie *et al.*, 1996; Tonegawa *et al.*, 1997). An attractive possibility is that midline expression of *noggin* prevents BMP-4 from converting medial somite tissues into lateral tissues. To gain support for this model and to test for a requirement of BMP-4 activity in lateralization of somitic mesoderm, we followed a well-established procedure to ectopically express *noggin* in somitic mesoderm. We constructed a replication-competent retroviral vector (Hughes *et al.*, 1987) which expressed the chicken *noggin* gene (*RCAS–noggin*) and we injected the virus into the right side of the unsegmented mesoderm of stage 10 embryos (according to Johnson *et al.*, 1994; see also Fig. 2D).

We find that ectopic expression of *noggin* in the somite completely represses the expression of *cSim-1* in the lateral somite, without significantly affecting its expression in structures not targeted by our infection protocol, such as the neural tube. Figure 2A shows the expression of *cSim-1* in the control side, restricted to the lateral somite, and Fig. 2B shows *cSim-1* expression in the injected side of the same embryo, where the arrowheads demarcate the region where repression of *cSim-1* is complete. Twenty embryos were examined. Note that Fig. 2A has been flipped in order to better compare it with Fig. 2B. The transverse section of the same embryo is shown in Fig. 2C. The asterisk indicates repression of *cSim-1* in the injected side. ms indicates reduced *cSim-1* expression in the mesonephros in this specimen (in 10% of the injected embryos we do detect severe down-regulation of *cSim-1* in the mesonephros). The expression in the ventral neural tube is unaffected. When delivered through retroviral infection, *noggin*'s effect on the development of the somitic mesoderm is quite specific insofar as we have not detected any obvious alteration in other aspects of somite development in *RCAS–noggin*-infected embryos (data not shown).

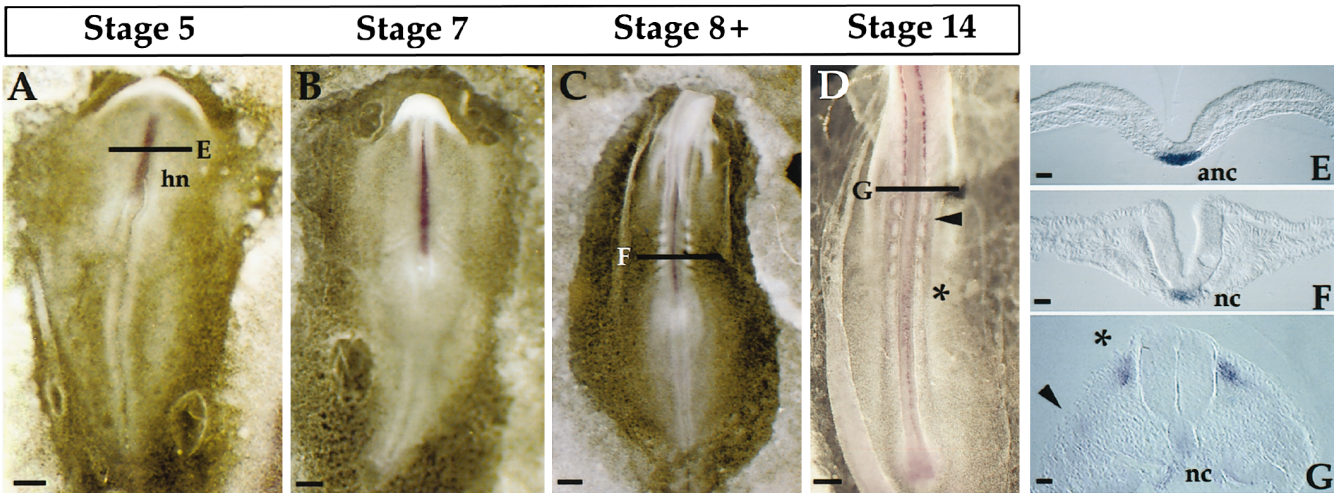

FIG. 1. Expression of *noggin* in early chicken embryos. Whole-mount *in situ* hybridizations of head process stage, or stage 5 (A, expression is detected in the anterior notochord and Hensen's node), stage 7 (B), stage 8+ (C), and stage 14 (D, detail) embryos. *noggin* is expressed in the notochord in all these stages. D shows that *noggin* is expressed in the lateral part of the four or five most recently formed somites (arrowhead), but the expression becomes dorsomedial in more mature somites (at the level indicated by G). There is weak, spotty expression of *noggin* in the dorsal neural tube, which is a little bit stronger at the level of the unsegmented mesoderm (asterisk), which also shows a very weak staining in the intermediate mesoderm. Scale bars, 250 μm. (E–G) Transverse sections of the embryos, at the levels indicated by the corresponding bars in A–D. Hensen's node is indicated by hn in (A); anc indicates the anterior notochord in E, and nc indicates the notochord in F and G. The arrowhead in G points to residual expression of *noggin* in the lateral part of the somite, at the level where strong expression has already shifted dorsomedially (asterisk in G). Scale bars, 25 μm.

Following the viral injection protocol described above, we typically obtain a very reproducible retroviral infection of the right side of the somitic tissue, although stronger infection is always seen dorsally (see Fig. 2E and also Johnson *et al.*, 1994). In this particular experiment we injected 20 embryos and confirmed the satisfactory extent of viral infection in 5 of them by performing whole-mount *in situ* hybridization to detect either the viral message (Fig. 2E) or the *noggin* message (not shown).

From these experiments, we conclude that the expression of *noggin* contributes to restrict the lateralizing influence of BMP-4 on the somitic tissue, as monitored by the expression of the lateral marker *cSim-1*.

noggin Is Expressed in a Dynamic Pattern during Limb Development

In the early limb bud, *noggin* is first detected at stages 16–17 in a stripe of proximoventral mesenchymal cells, and this pattern persists until around stage 21 (Figs. 3A–eq). Later the pattern becomes more complicated, with additional mesenchymal domains that express *noggin* (Figs. 3E and 3G). Around stage 26–27, expression of *noggin* in the limbs is clearly associated with the mesenchymal cells that condense to form cartilage (indicated by the white dots in Figs. 3F and 3H), and it is also expressed in discrete subpopulations of mesenchymal cells in the anterior and posterior margins of the limbs. From that moment on, expression of *noggin* is restricted to the condensing cartilage of the digits (Figs. 3I–3K), displaying a pattern complementary to the late pattern of some *BMPs*, which at those stages are expressed in the interdigital spaces (Lyons *et al.*, 1990; Francis *et al.*, 1994; Luo *et al.*, 1995). Expression of *BMPs* in interdigital areas has been proposed to play a role in the control of programmed cell death (Gañan *et al.*, 1996; Yokouchi *et al.*, 1996; Zou and Niswander, 1996). The pattern of *BMP-4* at similar stages is shown for comparison (Fig. 3L, LL, M).

Ectopic noggin Affects Proliferation and Bone Development in the Limb

The complex and dynamic expression of *noggin* in limb bud mesenchyme suggests its involvement in multiple events during early limb patterning. Given *noggin*'s demonstrated ability to inhibit BMP signaling, we postulate that *noggin*'s function in the limb is linked to the control of BMP activity. BMPs have been suggested to play a significant role in three processes during limb development: anteroposterior axis specification, initiation of chondrogenesis, and promotion of cell death (Lyons *et al.*, 1990; Jones *et al.*, 1991; Francis *et al.*, 1994; Francis-West *et al.*, 1995; Duprez *et al.*, 1996a,b; Gañan *et al.*, 1996; Kawakami *et al.*, 1996; Yokouchi *et al.*, 1996; Zou and Niswander, 1996; Macías *et*

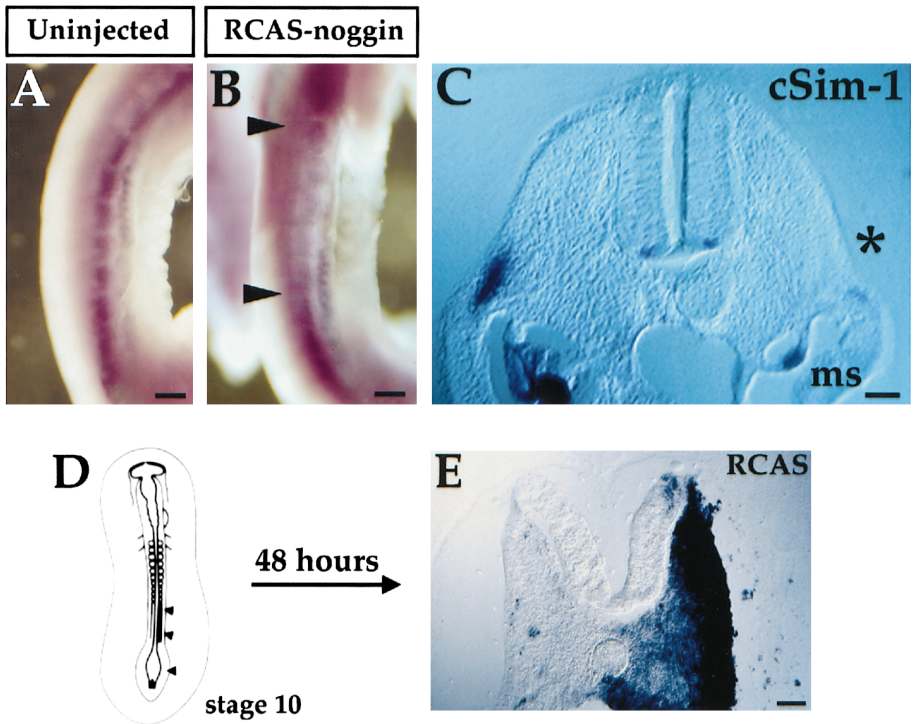

FIG. 2. Consequences of ectopic expression of *noggin* in the somites. An embryo injected with the *RCAS–noggin* virus at the right flank at stage 10 was fixed 2 days later for whole-mount *in situ* hybridization with a *cSim-1* probe. Expression of *cSim-1* in the noninjected flank, showing the wild-type repetitive pattern in the lateral part of each somite (A), and in the injected flank of the same embryo, where *cSim-1* has been completely repressed in the somite (B, especially in the region between the arrowheads, which typically corresponds to the flank region which is reproducibly infected according our injection procedure). Scale bars, 100 μm. A has been flipped to be better compared to B. Transverse section of the same embryo (C), showing repression of *cSim-1* in the injected side (asterisk). The expression of *cSim-1* in the mesonephros (ms in C) is reduced in this embryo, and the expression in the neural tube is not altered. Scale bar, 50μm. A scheme of the sites of injection in the stage 10 embryos is shown in D. Two days later, the extent of infection is monitored by whole-mount *in situ* hybridization using a probe that detects the viral message. A section of a representative embryo, where the somitic tissue is completely infected in the injected side, is shown in E. Scale bar, 50 μm.

al., 1997). To determine whether ectopic expression of *noggin* can influence these presumed BMP-dependent processes, we examined the phenotype of *RCAS–noggin*-injected limbs.

We used two types of injections. First, we were able to achieve complete infection of the forelimb by injecting the *RCAS–noggin* virus into the presumptive forelimb region of stage 10 embryos (site 1 shown in Fig. 4A, detection of viral message by *in situ* hybridization, 3 days later, shown in Fig. 4B). Second, the local injection of the virus either in the anterior or the posterior margin of stage 17–20 forelimbs (Fig. 4C) resulted in restricted infection of only the anterior or the posterior half of the limb, respectively, as shown in Figs. 4D and 4E, which illustrates the result of an anterior injection at the site indicated by 2 in Fig. 4C. Thus, when we inject the anterior margin of stage 17–20 forelimbs, before mesenchymal condensations are developed, the resultant limbs lack anterior cartilage structures when analyzed 6–7 days after the injection (Fig. 4F, where humerus, radius and digit II are absent; $n = 20$). Similarly, posterior cartilage structures are always missing when the posterior mesenchyme of the forelimb is infected (Fig. 4G, where the ulna and digits III and IV are absent; $n = 17$). In both cases, we observe that the infected limbs are consistently shorter. The phenotypes described are observed in 100% of injected embryos ($n = 37$ in total). When, as mentioned above, the entire limb is infected by injecting presumptive forelimb mesenchyme at stage 10 (Figs. 4A and 4B), a more severe phenotype is observed. In this case, small limbs develop which contain only a single cartilage element (Fig. 4H; $n = 12$). We can also obtain this phenotype by injecting the mesenchyme of stage 17–18 forelimbs with *RCAS–noggin* virus at three or four different locations (anterior and posterior margin, plus one or two intermediate locations), which also results in the complete infection of the forelimb (not shown, but it is identical to

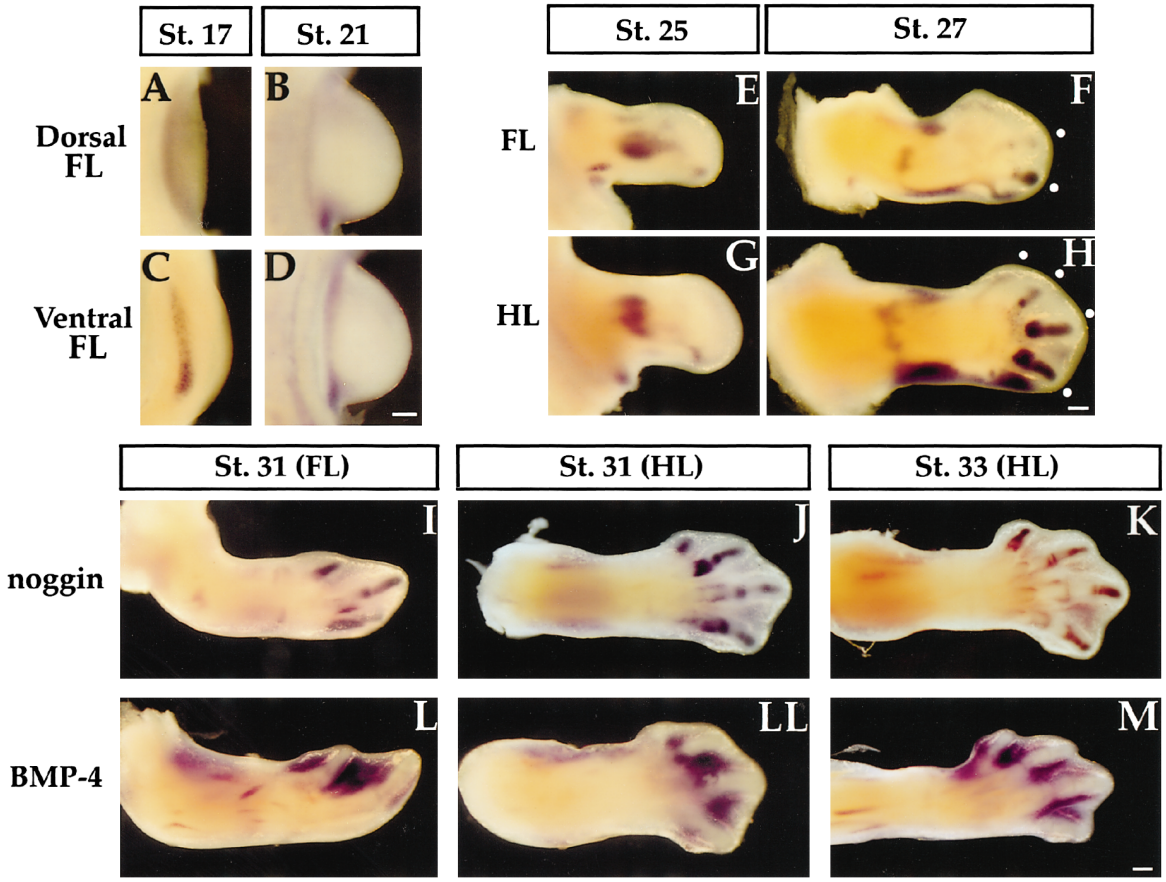

FIG. 3. Expression of *noggin* during limb development. Whole-mount *in situ* hybridizations of stage 17 (A, dorsal view; C, ventral) and stage 21 (B, dorsal view; D, ventral) forelimbs (FL), demonstrating *noggin* expression in the proximoventral mesenchyme. Later, a complicated pattern of mesenchymal expression is observed at stage 25 (E, forelimb; G, hindlimb, HL), which resolves at stage 27 (F, forelimb; H, hindlimb) into strong expression of *noggin* in the condensing mesenchyme which will give rise to the cartilage condensations (white dots in F and H). *noggin* is also expressed in other regions of the limbs at these stages. *noggin* continues to be expressed in the condensing cartilage in older limbs (I–K), and the pattern of expression of *BMP-4* (which is mostly complementary to that of *noggin*) is shown in limbs of similar ages for comparison (L–M). Stage 31 forelimbs (I, L), stage 31 hindlimbs (J, LL), and stage 33 hindlimbs (K, M). In this and all subsequent figures, limbs are oriented in the pictures with the anterior margin up and the posterior margin down, unless otherwise indicated. Scale bars, 250 μm.

Fig. 4B). Importantly, we note that the phenotypes observed are not due to nonspecific cytotoxic effects of the virus since control limbs infected with the *RCAS* virus alone have no morphological abnormalities. Also, when *RCAS–noggin*-infected limbs are stained after several days to detect the expression of a variety of molecular markers (see below), we detect up-regulation of some markers and down-regulation of other markers, indicating that the response of different genes to *noggin* overexpression is specific. Thus, we conclude that the effects of inhibition of chondrogenesis and additional localized proliferation (perhaps due to an inhibition of programmed cell death, see below) are consistent with blockage of BMP signaling within the limb caused by ectopic *noggin*.

Ectopic noggin Does Not Interfere with Polarizing Activity

One possible explanation for the truncations, or arrested development, that we observe in *RCAS–noggin*-infected limbs, is that anteroposterior polarity and/or polarizing activity is affected. Continued proliferation and distal outgrowth of the limb is dependent on signaling from the apical ectodermal ridge (AER) to the underlying mesenchyme (reviewed by Johnson and Tabin, 1997). The AER in turn is dependent on the presence of the zone of polarizing activity (ZPA) in the posterior mesenchyme. When either AER function or polarizing activity is lost, limb truncations result which at least superficially resemble what we observe in *RCAS–noggin*-injected limbs (Summerbell, 1974; Pagan *et*

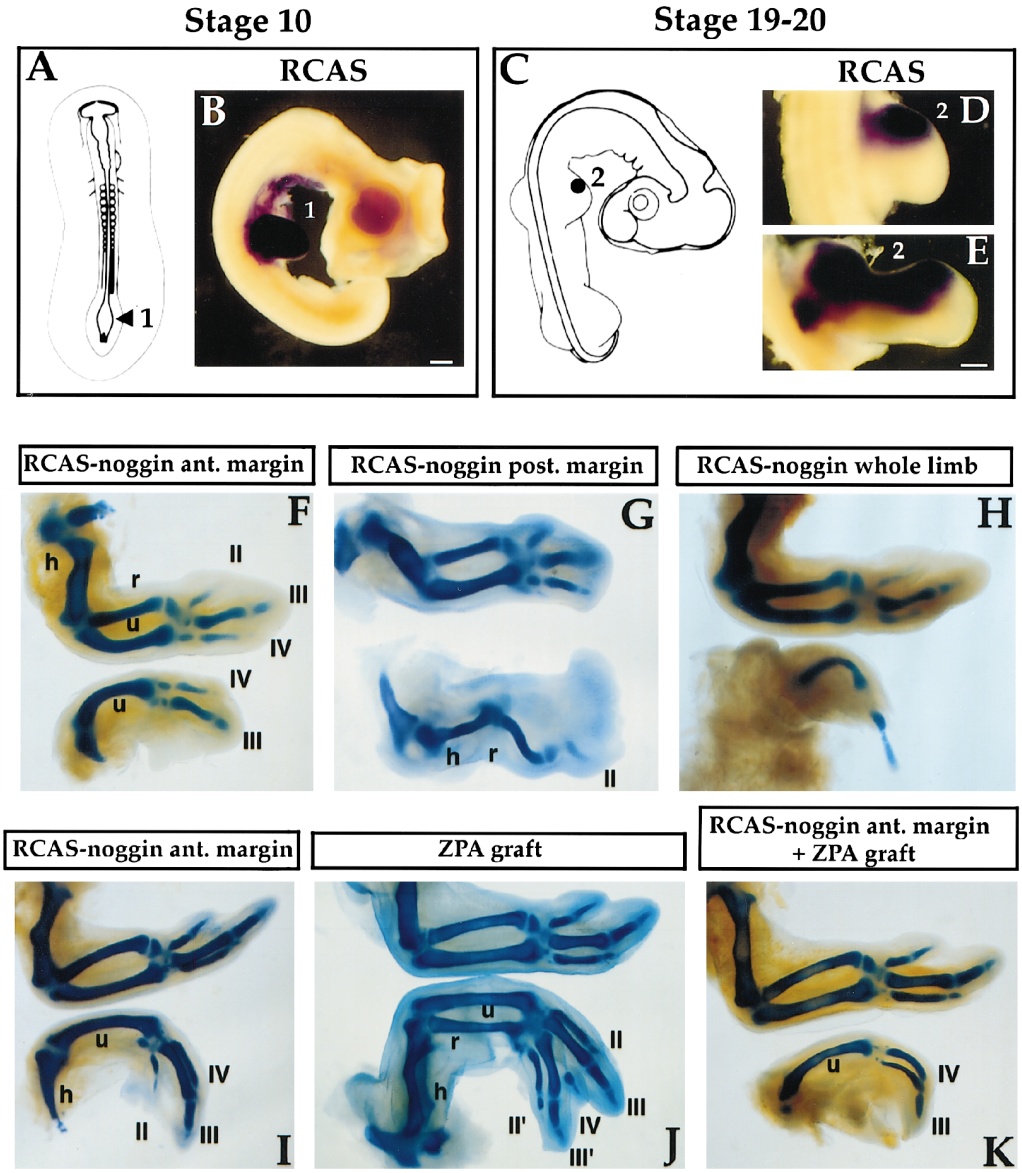

FIG. 4. Consequences of ectopic expression of *noggin* in the limbs. Injection in the right presumptive forelimb region of stage 10 embryos (indicated by the arrowhead 1) is schematized in A, and it results in complete infection of the right forelimb (indicated by 1 in B). Injection in the anterior margin of the forelimb is indicated by 2 in C, and it results in the infection of the anterior half of the limb (indicated by 2 at two subsequent stages of limb development, in D and E). Injection in the posterior margin of the forelimb gives a complementary pattern of infection (not shown). The extent of viral infection is determined by whole-mount i*n situ* hybridization to detect the viral message. Scale bars, 250 μm. Right forelimbs injected with the *RCAS–noggin* virus at stage 17 at the anterior margin alone (F), or at the posterior margin alone (G), and right forelimbs resultant from injection in the presumptive forelimb region at stage 10 (H), were processed for Alcian blue staining to reveal the pattern of cartilage elements at day 9 or 10 of development. In this and all the subsequent panels, the control (uninjected) limb is shown up and the corresponding injected limb from the same embryo is shown down, in a symmetrical disposition. In F, as a key for all the figures, II, III, and IV indicate the digits, from anterior to posterior, h indicates the humerus, r indicates the radius, and u indicates the ulna. In the anteriorly injected limb (F), the anterior cartilage elements (digit II and radius), plus the humerus, are missing. In the posteriorly injected limb (G), the posterior cartilage elements (digits III and for and ulna) are missing.

ZPA grafts do not rescue the alterations produced by *noggin* overexpression. In normal limbs, ZPA grafts into the anterior margin of stage 20 limbs give rise to mirror-image duplications of the cartilage elements (shown in J). But if the limb is infected with *RCAS–noggin* in the anterior margin at stage 17, anterior ZPA grafts at stage 20 are not able to rescue either the overproliferation or the suppression of cartilage elements (K), and the limbs are therefore similar, if not identical, to those that have been infected with *RCAS–noggin* in the anterior margin at stage 17 and let develop without further manipulation (I).

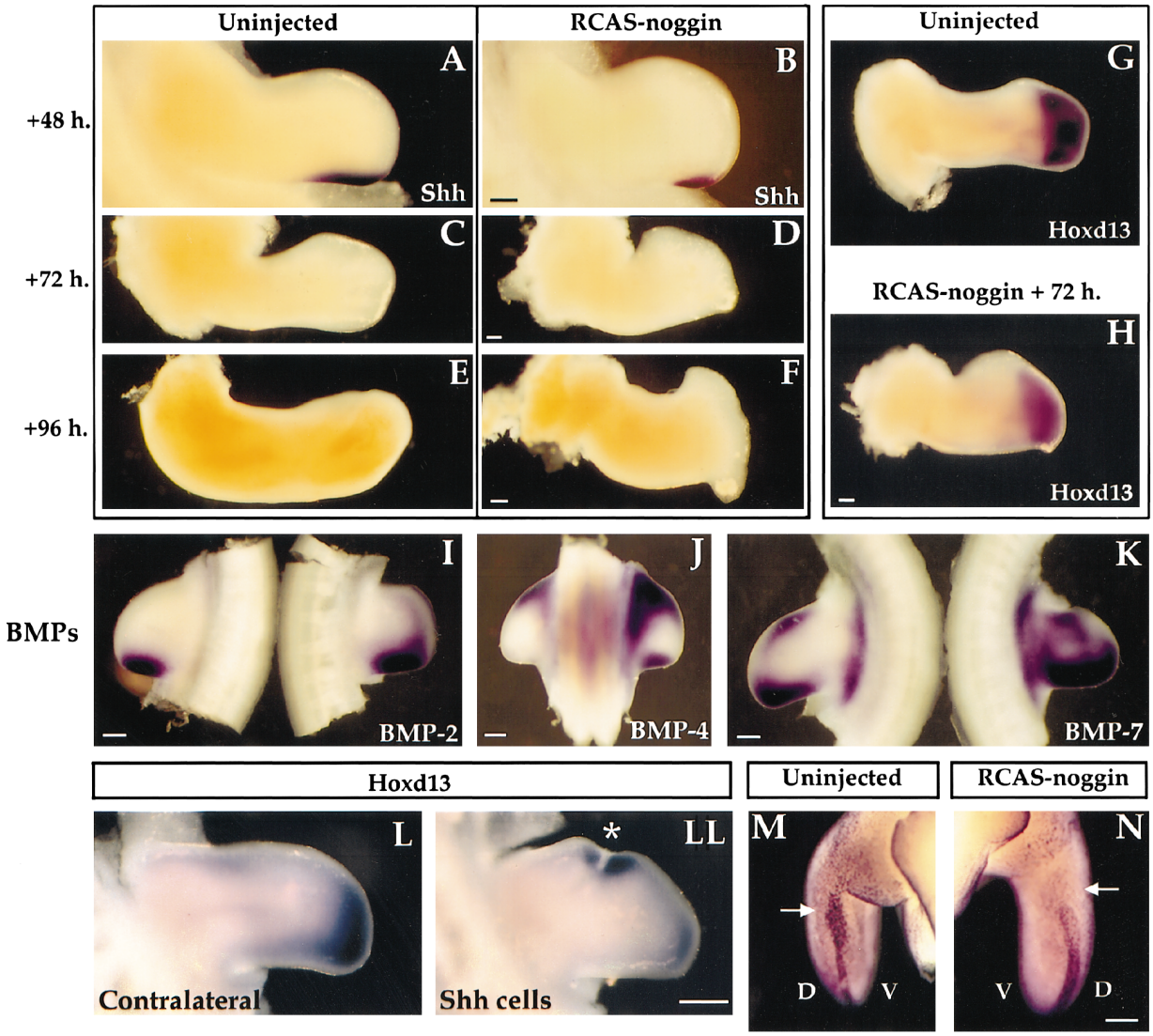

FIG. 5. Expression of molecular markers in *RCAS–noggin*-infected limbs. The expression of *Shh* and the morphology of forelimbs injected with the *RCAS–noggin* virus were monitored 2 days (A, B), 3 days (C, D), and 4 days (E, F) after the infection of the right presumptive forelimb region at stage 10 embryos, resulting in complete infection of the right forelimb. The control (uninjected) forelimbs are A, C, and E, and the injected limbs of the same embryo are B, D, and F. Two days after the injection, the limbs look shorter and *Shh* is still expressed in the limb, although it starts fading (B). Three days after the injection, the limbs are shorter and more symmetrical (D) than those of control (C). No *Shh* is detectable in either control or injected limbs at this stage. Four days after the injection, the injected limb (F) is half the length of the control limb (E), and the distal part shows an anteroposterior symmetrical shape, with no sign of cartilage condensations. These forelimbs usually develop into single-boned structures similar to the injected limb shown in Fig. 4H. Expression of *HoxD13* is shown in an uninjected limb (G), and in the limb of the same embryo resultant from the injection of *RCAS–noggin* at stage 10 in the presumptive forelimb region (H, note the shape of the limb and the repression of *HoxD13*, only observed 3 days after the injection).

BMP expression is up-regulated in *RCAS–noggin*-infected limbs. Expression of *BMP-2* (I), *BMP-4* (J), and *BMP-7* (K) in control limbs (to the left in all the pictures) and limbs resultant from the injection of *RCAS–noggin* at stage 10 in the presumptive forelimb region of the same embryo (to the right in all the pictures). Expression of all *BMPs* in the injected limbs is reinforced, basically in their normal domains. In J, expression of *BMP-4* in the posterior margin of the control limb is weak.

noggin overexpression does not interfere with the activation of *HoxD13* by *Shh*. Expression of *HoxD13* in the contralateral forelimb (L) and in the corresponding experimental forelimb of the same embryo (RCAS–*noggin*-injected at stage 10) where a pellet of CEFs infected with an *RCAS–Shh* virus was implanted in the anterior margin (asterisk), 36 h before the whole-mount staining procedure (LL). *HoxD13* is induced in the surrounding tissue by the *Shh*-expressing CEFs (indicated by the asterisk), which in our hands always induce local overgrowth around the operated area.

al., 1996). To test whether polarizing activity is altered by ectopic *noggin* expression in the limb, we examined the expression of *Sonic hedgehog* (*Shh*), a marker for the polarizing region, and also the expression of posterior mesenchyme markers (*HoxD13* and *BMP-2*) which are known to be responsive to *Shh* and reflective of proper polarizing region function (Riddle *et al.*, 1993; Laufer *et al.*, 1994). Until 48 h postinfection, *Shh* is still expressed in the injected limb ($n = 6$; Figs. 5A and 5B), and only later it starts fading, suggesting that the truncations we observe are not due to an early loss of *Shh* transcription. Figures 5C–5F show the control and injected limbs at two time points after the injection. For this and all subsequent *in situ*'s shown in Fig. 5, more than 7 experimental embryos were stained with each probe, and Figs. 5A, 5C, 5E, and 5G were flipped.

Similarly, *HoxD13* is normal after *RCAS–noggin* infection, and only 60–72 h postinfection its expression is reduced ($n = 5$; Figs. 5G and 5H). In contrast, the transcription of three different *BMPs* is enhanced in response to ectopic *noggin* ($n = 15$ in total; Figs. 5I–5K). This could reflect a mechanism of compensation, where the blocking of BMP pathways by ectopic Noggin at the level of the interaction of BMPs with their receptors is compensated by the overexpression of *BMPs*, basically in their normal domains. Despite this up-regulation of ligand expression, we interpret that BMP signaling in the limb is strongly attenuated or abolished by ectopic Noggin expression, since we do not observe any cartilage condensation in the areas infected with the *RCAS–noggin* virus.

To further examine whether inhibition of BMP signaling might disrupt ZPA function, we compared polarizing activity and activation of *HoxD13* by *Shh* in the context of *RCAS–noggin*-infected versus uninfected limbs. In uninfected limbs, ZPA grafts readily afford complete duplications ($n = 6$; Saunders and Gasseling, 1968; Tickle *et al.*, 1975; Fig. 4J). In contrast, ZPA grafts into *RCAS–noggin*-infected limbs in the anterior margin do not result in duplications ($n = 8$; Fig. 4K, very similar to a *RCAS–noggin*-infected limb shown in Fig. 4I), although the ZPA grafts induce *HoxD13* expression (not shown). Moreover, when we graft chicken embryonic fibroblasts (CEFs) expressing *Shh* in the anterior margin of a *RCAS–noggin*-infected limb, we see induction of *HoxD13* ($n = 6$; asterisk in Fig. 5LL, compare with the normal expression of *HoxD13* in the control limb in Fig. 5L), although these limbs never develop pattern duplications (not shown). Figure 5L has been flipped. CEFs–*Shh* anterior grafts have been previously shown to induce mirror-image pattern duplications (Riddle *et al.*, 1993).

We interpret these results to indicate that the early function of the ZPA, namely specification of anteroposterior pattern, is not affected by ectopic *noggin* expression. However, subsequent limb outgrowth is blocked. These results are consistent with our finding that both anterior and posterior local injection of *RCAS–noggin* virus yields equivalent results with respect to limb truncation and inhibition of chondrogenesis. Hence we conclude that *noggin* and, hence, *BMPs* do not participate in early ZPA function.

Local Repression of Programmed Cell Death by Ectopic noggin

Finally, we decided to investigate a possible role of *noggin* in the control of programmed cell death in the limb, a process where BMPs have been previously shown to be involved (Gañan *et al.*, 1996; Kawakami *et al.*, 1996; Yokouchi *et al.*, 1996; Zou and Niswander, 1996; Macías *et al.*, 1997). In our study, we focused on the analysis of the anterior necrotic zone (ANZ) of the forelimb, a mass of necrotic cells in the superficial mesoderm of the anterior edge of the wing bud that can be detected from stage 21 on (see Saunders *et al.*, 1962, for a description of the necrotic zones in the limb bud). Taking into account that the pattern of expression of *noggin* in the limb is very dynamic (see Fig. 3), we observe that at the stage analyzed in this experiment (stage 25), *noggin* is not expressed in the region that corresponds to the ANZ (Fig. 3E). The ANZ forms a well defined spot at the time of our study. Figure 5M shows a detail of the anterior edge of the control limb, the arrow indicating the ANZ as revealed by TUNEL staining. Ectopic expression of *noggin* in the anterior margin of the forelimb (following the protocol depicted in Fig. 4C) completely represses programmed cell death in the cells that normally constitute the ANZ ($n = 9$; Fig. 5N shows the injected limb of the same embryo shown in Fig. 5M; the arrow points the region where ANZ should be detected). Necrotic cells in and around the AER seem to be unaffected.

We conclude that ectopic *noggin* in the limb is able to locally repress programmed cell death, as demonstrated by the repression of the ANZ. The analysis of the effect of ectopic *noggin* on other necrotic zones in the limb is in progress in our laboratory.

DISCUSSION

In this paper, we have described the pattern of expression of a chicken *noggin* cDNA, and we have analyzed its ability

Ectopic *noggin* represses programmed cell death in the limb. ANZ as revealed by TUNEL staining of a stage 25 control forelimb (arrow in M), and repression of ANZ in the forelimb anteriorly injected with *RCAS-noggin* (arrow in N points to the region normally occupied by the ANZ). D and V indicate the dorsal and the ventral aspects of the limbs, respectively. A, C, E, G, and L have been flipped in order to be better compared to the contralateral limbs. Scale bars, 250 μm.

to affect pattern and cell-type differentiation in limbs and somites. Our results point to a key role of *noggin* in modulating *BMP* function during limb and somite development, and demonstrate that delivery of *noggin* via a replication competent retrovirus is an effective means by which to inhibit BMP signaling activities in a variety of tissues.

noggin and the Mediolateral Pattern of the Somites

noggin is expressed in the notochord and in the somites in a way that suggests its involvement in mediolateral patterning of the somites, where *BMP-4*, expressed in the lateral plate mesoderm, has been shown to control the expression of *cSim-1* in the lateral somite (Pourquie *et al.*, 1996; Tonegawa *et al.*, 1997). During somitogenesis, *noggin* is expressed in the lateral part of the four or five most recently formed somites, and its expression shifts dorsomedially as somites mature (Figs. 1D and 1G). If *noggin* is blocking BMP activity medially, then its ectopic expression in the whole somite should interfere with the control of *cSim-1* by BMP-4, and this is indeed the case. Ectopic expression of *noggin* in the whole somite, driven by the *RCAS–noggin* virus, completely represses *cSim-1* (Figs. 2B and 2C). Thus, *noggin* fulfills the requirement to be a signal which represses the extension of lateral fates to the medial compartment of the somites, although more experiments are needed in order to determine whether *noggin* is solely responsible for this activity. The observed lateral to medial shift in the expression of *noggin* in the somite could be a mechanism to fine-tune the timing and level of *cSim-1* expression.

BMPs, noggin, and the Control of Cell Proliferation, Programmed Cell Death, and Chondrogenesis in the Limb

The analysis of the role of BMPs in limb patterning is complicated by at least two factors. First, several BMPs (reviewed by Hogan, 1996) and receptors (Rosen *et al.*, 1996; Kawakami *et al.*, 1996) are expressed in the limb with different but overlapping patterns, which suggests that functional redundancies may exist. Thus, experiments involving the inactivation of a single *BMP* (for example, single *BMP* knock-outs in mice) may not produce an informative phenotype. For example, *BMP-4* and *BMP-2* null mutant mice die too early to analyze any possible limb alterations (Winnier *et al.*, 1995; Zhang and Bradley, 1996). *BMP-7* null mutants display a phenotype of hindlimb polydactyly (Dudley *et al.*, 1995; Luo *et al.*, 1995; Hofmann *et al.*, 1996); however, other important roles of *BMP-7* may be masked by the presence of additional *BMPs* (Lyons *et al.*, 1995; Dudley *et al.*, 1997). Second, experiments involving ectopic expression of BMPs in the limbs have given different results depending on the method and/or the time of BMP delivery. Heparin beads soaked in purified BMP-2 or BMP-7 protein solutions induce cell death when implanted in the chick limb bud (Macías *et al.*, 1997), but it has also been proposed that BMP-2 mediates some aspects of polarizing activity in that *Fgf-4* and *HoxD* genes are induced when cells expressing BMP-2 are implanted in the anterior margin of the limb bud (Duprez *et al.*, 1996b). BMPs have also been shown to be potent stimulators of chondrogenesis *in vivo* and *in vitro* (Urist *et al.*, 1979; Wozney *et al.*, 1988). These different effects of BMPs on limb development may reflect multiple roles of BMPs in limb patterning and differentiation or may be a consequence of the specific methods used for BMP delivery. In any event, these experiments highlight the complexity of BMP signaling in limb development.

Ectopic expression of *noggin* allows us to circumvent the complexity of BMP ligand and receptor expression by using a single reagent to effectively block BMP signaling at the level of the interaction of the ligands with their receptors. Although we cannot say for sure that ectopic *noggin* is blocking all BMP activities in the limb, our results can be favorably compared to experiments in which dominant negative receptors have been used to attenuate BMP signaling in the limb (Kawakami *et al.*, 1996; Yokouchi *et al.*, 1996; Zou and Niswander, 1996). In these reports, some dominant negative BMP receptors inhibit chondrogenesis, albeit only in distal regions of the limb, while others affect patterns of cell death in the autopod. In contrast, in our experiments involving ectopic expression of *noggin* in the whole limb, we observe complete lack of cartilage formation, giving rise to severely truncated limbs which only retain the most proximal cartilage structures (Fig. 4H). We speculate that the increased severity of our cartilage differentiation phenotype relative to earlier reports using dominant negative receptors reflects a more complete inhibition of BMP signaling at earlier stages.

Another proposed function of BMPs in the limb has been to mediate the polarizing activity of *Shh*. *Shh* is thought to act upstream of *BMPs* in limb patterning, since ectopic *Shh* is able to activate the expression of *BMPs* (Laufer *et al.*, 1994), while ectopic BMPs do not affect *Shh* (Duprez *et al.*, 1996b). Consistent with this view, we demonstrate that the strong effect of *noggin* on cartilage development is not likely to be caused by an early effect on *Shh* expression, since the transcription of *Shh* and *Shh* targets in the *RCAS–noggin*-injected limbs is not significantly affected until at least 2 days after infection, when the injected limbs are already considerably affected, as judged by the fact that they are much shorter than the control ones and display altered shapes (Figs. 5B, 5D, and 5F). Thus, *Shh* does not seem to be an immediate target for *noggin*, nor does *noggin* infection affect the ability of ectopic *Shh* to activate some of its well know targets (*HoxD13*, Fig. 5LL). We interpret that *noggin* is affecting limb patterning downstream of *Shh* and of *BMPs*, namely, at the level of the interaction of BMPs with their receptors. We do not think that *BMPs* play a direct role in the specification of anteroposterior limb patterning, since ectopic *noggin* does not seem to specifically affect anteroposterior polarity.

If the primary activity of *noggin* is to inhibit BMP signal-

ing, then we speculate that endogenous *noggin* functions to modulate BMP activities during limb development and that the expression pattern of *noggin* gives clues as to what those functions might be. *noggin* is transiently expressed in tissues just prior to or during initial chondrogenic blastemal formation. In distal regions, *noggin* expression is maintained within the condensations and then is restricted to interphalangeal spaces. One possibility is that *noggin* may delay or otherwise modulate an early BMP activity involved in initial condensation events. Correlation of *noggin* expression with early markers of chondrocytic development (e.g., *Sox-9* or TGF-βs) may help to evaluate this proposed function for *noggin*.

We also demonstrate that ectopic *noggin* is able to repress programmed cell death in specific regions of the limb. *noggin*, which is not expressed in the ANZ, is able to repress the ANZ when ectopically expressed in the anterior margin of the limb. Thus, the expression of *noggin* in the limb may reflect important roles in protecting some tissues from induced cell death mediated by *BMP* expression, in interdigital mesenchyme as in other necrotic zones. We have not yet assayed for effects on interdigital cell death, but modification of our infection protocol should make these later events accessible, by examining the effect of local *RCAS–noggin* injections in the interdigital regions, preferably after cartilage condensation has already been initiated. All these potential roles for *noggin* are best addressed by analysis of mice which lack functional Noggin protein. However, it must be cautioned that functional redundancy may exist in the form of Chordin which possesses similar activities to Noggin in attenuating BMP activity. Hence, a *noggin* null mouse may not accurately depict deregulated BMP activity in the limb or other tissues.

Taken together, our results indicate that *noggin* expression is widely used to modulate BMP signaling. We envision three scenarios in which *noggin* might function to regulate *BMP* activity. First, Noggin and BMPs may be expressed in nonoverlapping cells, as is the case in mediolateral somite patterning or in the autopod of the limb. In this case Noggin opposes long-range effects of BMPs. Second, Noggin and BMPs may be coexpressed in the same cells, as is the case for early limb condensations and during vertebral condensation (data not shown). Hence, Noggin may operate to attenuate, but not eliminate, BMP activity. Finally, Noggin and BMP expression may initially overlap spatiotemporally, but Noggin or BMP expression fade at a predetermined time, as seen during early limb bud development. This arrangement would lead to a tight temporal control of BMP activity. In this manner, the selective deployment of activators and inhibitors of signaling pathways can achieve tight modulation of signaling activities, at both spatial and temporal levels. The recent identification of dominant secreted inhibitors of the *wingless (wg)/Wnt* pathway (reviewed by Moon *et al.*, 1997) and the existence of inhibitors of *activin* and TGF-β signaling mechanisms indicates that this strategy for tight control of signaling pathways may be widespread in a variety of developmental processes.

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