Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite

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Embryonic patterning in vertebrates is dependent upon the balance of inductive signals and their specific antagonists. We show that *Noggin,* which encodes a bone morphogenetic protein (BMP) antagonist expressed in the node, notochord, and dorsal somite, is required for normal mouse development. Although *Noggin* has been implicated in neural induction, examination of null mutants in the mouse indicates that *Noggin* is not essential for this process. However, Noggin is required for subsequent growth and patterning of the neural tube. Early BMP-dependent dorsal cell fates, the roof plate and neural crest, form in the absence of Noggin. However, there is a progressive loss of early, *Sonic hedgehog* (*Shh*)-dependent ventral cell fates despite the normal expression of *Shh* in the notochord. Further, somite differentiation is deficient in both muscle and sclerotomal precursors. Addition of BMP2 or BMP4 to paraxial mesoderm explants blocks Shh-mediated induction of *Pax-1,* a sclerotomal marker, whereas addition of Noggin is sufficient to induce *Pax-1.* Noggin and Shh induce *Pax-1* synergistically. Use of protein kinase A stimulators blocks Shh-mediated induction of *Pax-1,* but not induction by Noggin, suggesting that induction is mediated by different pathways. Together these data demonstrate that inhibition of BMP signaling by axially secreted Noggin is an important requirement for normal patterning of the vertebrate neural tube and somite.

[*Key Words:* Noggin; somite; neural tube; patterning]

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Patterning of the vertebrate body axes is dependent upon signals produced by discrete organizing centers. Perhaps the best studied of these is Spemann's organizer, which encompasses the dorsal lip of the blastopore in the gastrulating amphibian embryo. Organizer signaling is implicated in dorsalization of both mesodermal and ectodermal derivatives. Dorsalization of the mesoderm leads to notochord and somite formation, whereas the dorsalized ectoderm forms neural tissue (for review, see Kessler and Melton 1994; De Robertis and Sasai 1996; Harland and Gerhart 1997). Four signals have been described that are expressed within the organizer and that have dorsalizing activity: Noggin (Smith and Harland 1992), Follistatin (Hemmati-Brivanlou et al. 1994), Chordin (Sasai et al. 1994), and Frzb (Leyns et al. 1997; Wang et al. 1997). None of these share identifiable sequence similarity, but there is evidence to suggest that the first three may act by blocking bone morphogenetic protein (BMP) signaling.

Noggin binds several BMPs with very high (picomolar) affinities, with a marked preference for BMP2 and BMP4 over BMP7. By binding tightly to BMPs, Noggin prevents BMPs from binding their receptors (Zimmerman et al. 1996). Chordin also antagonizes BMP signaling by directly binding BMP proteins, thereby preventing receptor activation (Piccolo et al. 1996). Follistatin binds to Activins, thereby preventing Activin signaling (Nakamura et al. 1990). This antagonism extends to the more distantly related BMPs with a preference for BMP7 over BMP4 (Yamashita et al. 1995; Liem et al. 1997). Thus, a key function of these peptides is to antagonize signaling by distinct members of the TGF-β superfamily.

Examination of postgastrulation stage *Xenopus* embryos indicates that some of these signaling factors are expressed at later stages. For example, *Noggin* is expressed in the notochord and dorsal neural tube, suggesting a possible role in the central nervous system (CNS) and somite patterning (Smith and Harland 1992); and in the chick, *Noggin* expression in the dorsal lip of the somite has been implicated in the control of myogenesis

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(Marcelle et al. 1997; Reshef et al. 1998). We have addressed the role of *Noggin* in mouse development. *Noggin* is not essential for neural induction but is required for normal growth and patterning of the neural tube and somite. Thus, inhibition of endogenous BMP signaling by Noggin is essential for elaboration of the vertebrate body plan.

Results

Cloning and expression of Noggin

We isolated a genomic clone that encodes the entire mouse Noggin polypeptide on a single exon (GenBank accession no. U79163). The predicted protein contains 232 amino acids (25 kD) and shares 99% and 80% amino acid identity with the human (Valenzuela et al. 1995) and *Xenopus* (Smith and Harland 1992) proteins, respectively.

Noggin expression was examined in developing mouse embryos by whole-mount and section in situ hybridization. Embryonic expression was first detected in the node at 7.5 days postcoitum (dpc; arrowed in Fig. 1A). By early somite stages, *Noggin* expression extended anteriorly along the entire length of the notochord (large arrow in Fig. 1C), a similar pattern to the notochordal marker *Brachyury* (Fig. 1D). In addition, *Noggin* was expressed in the dorsal neural tube from the caudal hindbrain to the posterior-most region of the embryo (small arrows in Fig. 1C). By the time cranial neural tube closure was completed (~9.0 dpc), *Noggin* expression was continuous along most of the dorsal midline of the neural tube (the roof plate), to its rostral termination at the base of the forebrain (Shimamura et al. 1995; small arrows in Fig. 1E). In contrast to *Brachyury* (Fig. 1G), expression in the notochord was not uniform but decreased rostrally at this stage (Fig. 1E). Expression in the neural tube and caudal notochord remained essentially unchanged during early organogenesis, from 9.5 dpc (Fig. 1H,L) to 10.5 dpc (data not shown). We also observed weak expression in the dorsal lip of the most rostral somites from 9.5 dpc (arrow in Fig. 1H,J). Expression in the somite contrasts with the chick in which *Noggin* is strongly expressed even in the most recently formed somites (Marcelle et al. 1997; Reshef et al. 1998). Finally, *Noggin* was expressed in the rostral sclerotome at 10.5 dpc (data not shown), coincident with the initial stages of cartilage condensation.

Generation of Noggin *mutants*

Experiments in a variety of vertebrates have demonstrated the importance of signaling by the node, notochord, and dorsal neural tube in patterning mesodermal

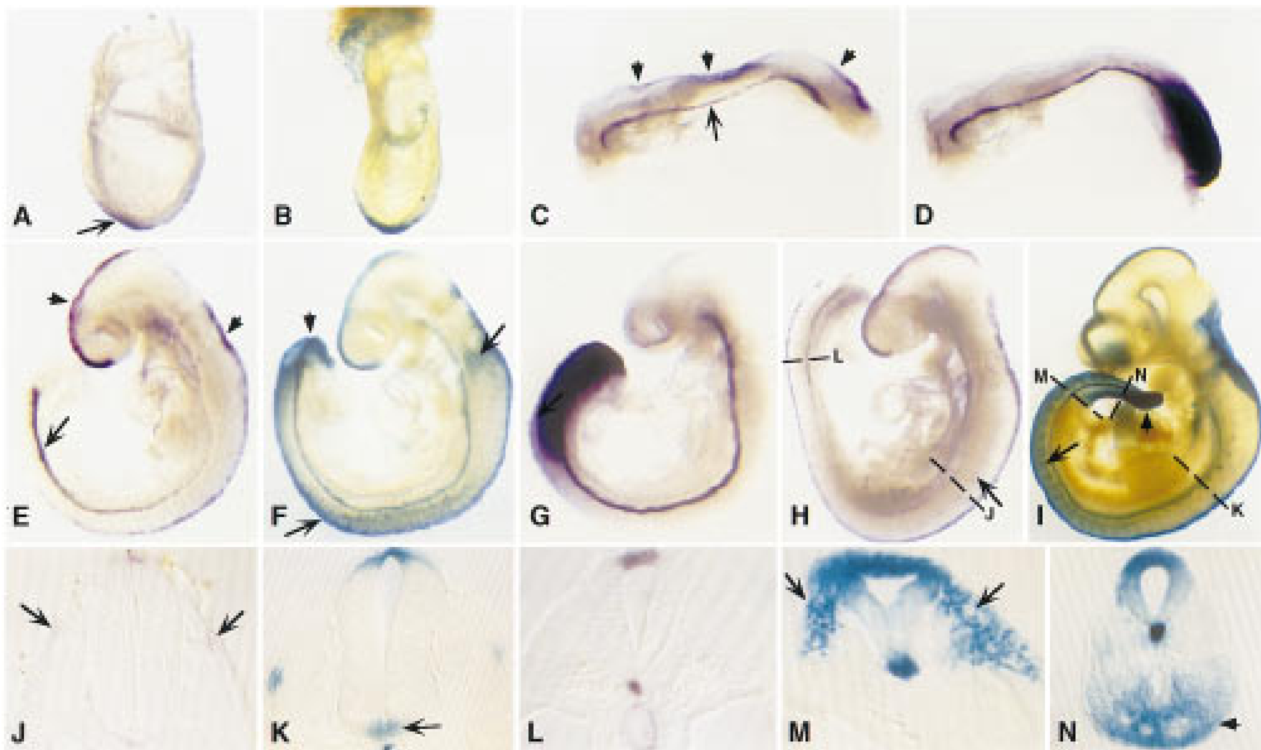

Figure 1. Expression of *Noggin* during mouse development. (*A,C,D,E,G,H,J,L*) Whole mount in situ hybridization with *Noggin* (*A,C,E,H,J,L*) and *brachyury* (*D,G*) probes; (*B,F,I,K,M,N*) β-galactosidase activity in embryos heterozygous for a targeted allele of *Noggin* in which *Noggin* coding sequences were replaced by the *lacZ* gene. (*A*) 7.75 dpc; (*B*) 7.5 dpc; (*C,D*) 10 somites (8.5 dpc); (*E–G*) 9.5 dpc; (*H,I*) 10 dpc—10.5 dpc; (*J–N*) transverse sections as indicated through embryos in *H* and *I.*

and neural tissues. To address the embryonic function of *Noggin,* we generated a null allele by fusing the first 10 amino acids of the *Noggin* coding sequence to the *lacZ* gene of *Escherichia coli* (Fig. 2A). The remainder of the coding sequence, and some 3′ flanking sequence, were deleted following gene replacement at the *Noggin* locus (Fig. 2A). A correctly targeted CJ-7 embryonic stem (ES) cell clone was introduced into the mouse germ line and the mutant allele was either outcrossed to the C57BL6/J strain or maintained on an inbred 129/Sv background.

Diagnostic Southern blot analysis with 5′ and 3′ flanking probes confirmed that the predicted targeted allele was present in *Noggin* mutants (Fig. 2B). Further, histochemical staining for β-galactosidase activity in heterozygous embryos confirmed that the *lacZ* gene was expressed in the structures predicted from in situ hybridization studies (Fig. 1B,F,I,K,M,N). We also detected *lacZ* activity transiently in migrating neural crest cells (large arrows in Fig. 1F,M), in the dorsal root ganglia (a neural crest derivative, arrow in Fig. 1I), in ventral posterior mesoderm (small arrows in Fig. 1 F,I,N), and in the rostral floor plate from 10.5 dpc (large arrows in Fig. 1K). The expression in neural crest cells most likely reflected a perduring of β-galactosidase activity in neural crest cells emerging from *Noggin* expressing regions of the dorsal neural tube. A similar observation has been made in transgenic embryos which express *lacZ* under the control of the *Wnt-1* enhancer (Echelard et al. 1994). However, expression in the ventral mesoderm and floor plate represent sites where *Noggin* transcripts are either below the level of detection by in situ hybridization, or where the *lacZ* gene was ectopically expressed. Ectopic expression could result from either the removal of 3′ flanking regions or from the influence of the PGK promoter.

General Noggin *phenotype*

Loss of *Noggin* resulted in a recessive lethal phenotype at birth. Superficial examination revealed multiple defects including a failure of neural tube closure, broad club-shaped limbs, loss of caudal vertebrae, a shortened body axis, and retention of a small vestigial tail (Fig. 3A–C). When examined on an inbred (129/Sv) or F_1 hybrid (129/Sv; C57BL6/J) background, there was a pronounced variability in cranial neural tube closure. The brain was almost always open in *Noggin* mutants on the inbred background (Fig. 3B), but most often closed in mutants on the hybrid background (Fig. 3C).

To address the role that *Noggin* may play in events regulated by midline signaling we examined over 400

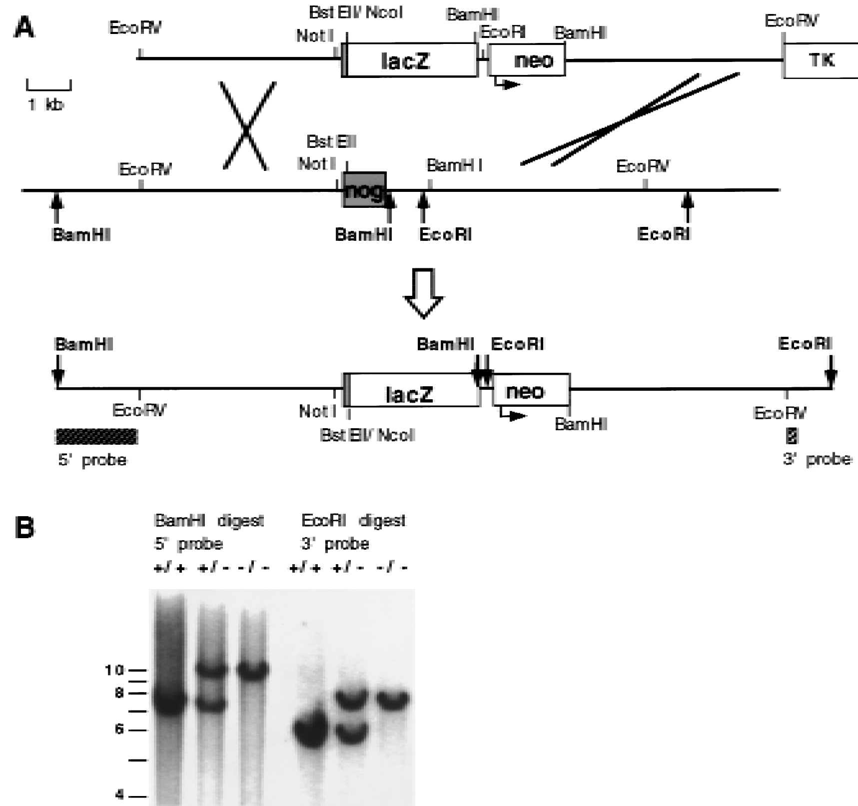

Figure 2. Gene replacement at the *Noggin* locus. (*A*) Schematic representation of gene replacement strategy in which most of the *Noggin* coding region was replaced by the *lacZ* gene of *E. coli.* (*Top*) Targeting construct that contains 5.2 kb of 5′ flanking sequence with *lacZ* fused in-frame to the first 10 amino acids of the *Noggin* coding exon (shaded), followed by a *PGKneo* cassette (*neo;* the arrow indicates the direction of transcription from the PGK promoter) and 5.0 kb of 3′ flanking homology starting at the *Bam*HI site, 1.0 kb downstream of the *Noggin* stop codon. An *MC1–HSVTK* (*TK*) cassette was included for negative selection. (*Middle*) The wild-type *Noggin* locus with the single *Noggin* coding exon (shaded). (*Bottom*) The map represents the expected targeted allele following gene replacement at the *Noggin* locus. The positions of diagnostic 5′ and 3′ flanking Southern probes are indicated (hatched boxes) and relevant restriction sites used in genotyping are shown in bold. (*B*) Analysis of *Noggin* genotypes. Southern blot analysis demonstrating the expected gene replacement at the *Noggin* locus and germ line transmission of the targeted allele. Homologous recombinants were identified by hybridizing 5′ and 3′ probes external to the targeting vector sequences to genomic DNA digested with *Bam*HI and *Eco*RI, respectively. The 5′ probe detects a 7.5-kb wild-type and 10.0-kb targeted band and the 3′ probe a 5.8-kb wild-type and 7.5-kb targeted band.

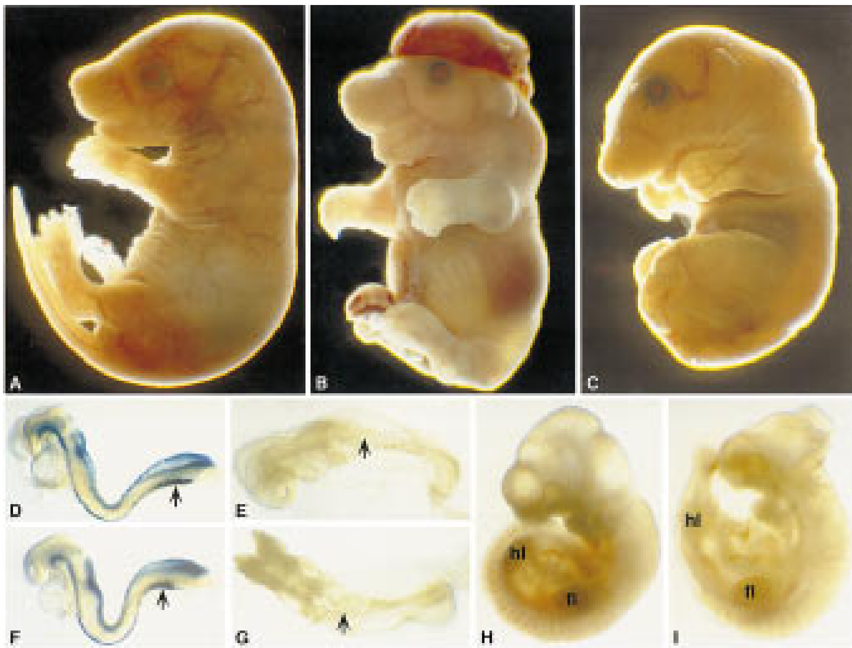

Figure 3. Superficial examination of *Noggin* mutant phenotype. (*A,D,E,H*) Wild-type; (*B,C,F,G,I*) *Noggin* mutants at 18.5 dpc (*A–C*), 8.5 dpc (7–8 somites, *D–F*), 8.75 dpc (12 somites, *E–G*), and 10.5 dpc (*H,I*). *Noggin* mutants in *C* and *F* were on a 129/SV;C57BL6/J hybrid background; all others were on an inbred 129/Sv background. Embryos in *D* and *F* were stained to visualize *lacZ* activity from the targeted allele at early somite stages. Forelimb (*fl*); hindlimb (*hl*).

homozygous mutant embryos collected between 8.0 dpc and 10.5 dpc (from predominantly the 129/Sv background), for morphology, histology, and gene expression. We report elsewhere on the function of *Noggin* at later stages in the control of cartilage morphogenesis (Brunet et al. 1998).

Staining for β-galactosidase activity at early somite stages revealed no difference in notochord or dorsal neural development between heterozygous and homozygous mutant embryos except for a slight shortening and broadening of the notochordal plate (arrowed in Fig. 3D,F). By the 8–9 somite stage, homozygous embryos on the 129/Sv background could be distinguished from wild-type or heterozygous siblings by a flattening of the elevating neural folds in the mid/hindbrain region (data not shown). Within the next few hours *Noggin* mutants on both backgrounds developed kinking of the spinal cord (arrows in Fig. 3E,G). A severe neural tube phenotype subsequently emerged. In the brain, the neural tube failed to close between the diencephalon and myelencephalon (129/Sv background) and was kinked along much of its length in presumptive spinal cord regions (both backgrounds; Fig. 3H,I). Occasionally the neural tube was open from the diencephalon to its caudal limit. The open and kinked brain perturbed eye and ear development but otherwise embryos appeared to develop fairly normally anterior to the forelimbs. In contrast, caudal to the forelimb, embryos exhibited a shortened axis, with the hindlimbs closer to the forelimbs; the tail was also short, and the somites and neural tube were considerably reduced in size (Fig. 3H,I).

Histological sections at different axial levels revealed a rostral to caudal increase in the severity of the *Noggin* phenotype. At the forelimb level, *Noggin* mutant embryos were essentially normal except for a distended dorsal neural tube (Fig. 4A,B). At lumbar levels, the spinal cord and somites were significantly reduced in size and pockets of neural crest derived cells remained at the dorsal midline (Fig. 4C,D and large arrow in Fig. 5J). Extensive apoptosis was apparent at intermediate and ventral positions within the developing spinal cord (arrows in Fig. 4F). At caudal hindlimb levels, the decrease in neural tissue was more pronounced and the dorsal medial somite, which normally undergoes a mesenchymal transformation in forming the myotome, remained epithelial (arrows in Fig. 4H). A large mass of cells, most likely originating from the neural crest, lay immediately above the epithelial somite (Fig. 4G,H). Considerable cell death was evident in the ventral neural tube (data not shown). Interestingly, at extreme caudal positions, close to the tail bud, the neural tube, notochord, and presomitic mesoderm appeared similar to that of wild-type littermates, although discrete dorsal apoptosis was observed in the dorsal neural tube of mutants (Fig. 4I,J; data not shown). At 9.0 dpc, although the neural tube was already considerably smaller, we could only detect apoptosis localized to the dorsal neural tube in thoracic regions (arrow in Fig. 4L). Thus, whereas *Noggin* does not appear to be essential for the formation of either mesodermal or neural tissue prior to 10.5 dpc, *Noggin* is required in caudal regions for normal development of both these tissues.

Noggin is required for ventralization of the posterior spinal cord

The expression of *Noggin* in the roof plate and notochord, both of which are organizing centers responsible for dorsoventral patterning of the vertebrate neural tube (Tanabe and Jessell 1996; Liem et al. 1997), suggests that *Noggin* may play some role in these events. We therefore

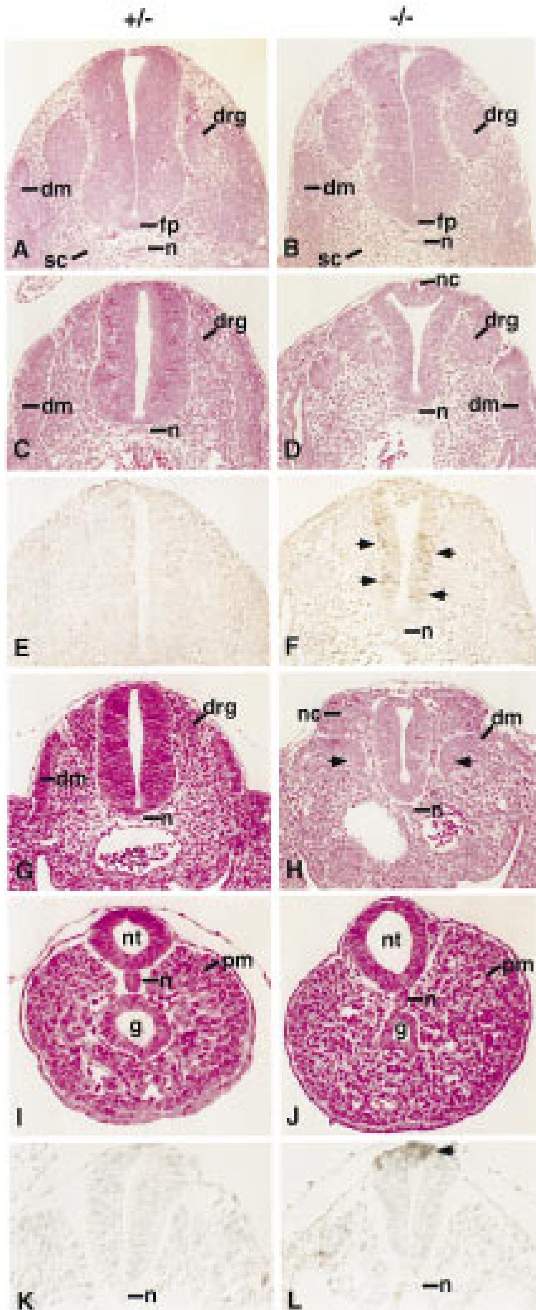

Figure 4. Histological and cell death analysis of spinal cord and somite development in *Noggin* mutants. Transverse sections through embryos heterozygous (*A,C,E,G,I,K*) or homozygous (*B,D,F,H,J,L*) for the targeted *Noggin* allele at 10.5 dpc (*A–J*) and 9.0 dpc (*K,L*). Sections were cut at the level of the forelimb (*A,B*), between the fore- and hindlimbs (*C–F,K,L*), at the caudal hindlimb level (*G,H*), and through the presomitic mesoderm just anterior to the tail bud (*I,J*). *A–D* and *G–J* are hematoxylin and eosin stained; *E,F,K,* and *L* underwent the TUNEL reaction to visualize apoptotic cell death. Pairwise comparisons were photographed at the same magnification. (dm) Dermomyotome; (drg) dorsal root ganglia; (fp) floor plate; (g) gut; (n) notochord; (nc) neural crest; (nt) neural tube; (pm) presomitic mesoderm; (sc) sclerotome.

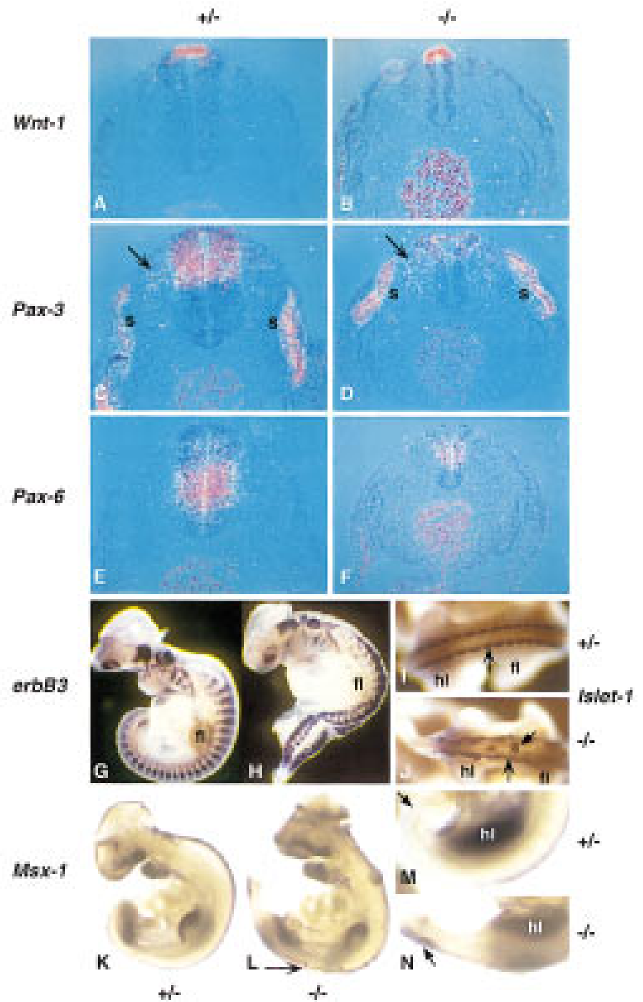

Figure 5. Analysis of dorsal neural tube patterning in *Noggin* mutants just rostral to the hindlimbs. Embryos heterozygous (*A,C,E,G,I,K,M*) or homozygous (*B,D,F,H,J,L,N*) for the targeted *Noggin* allele were examined at 10.5 dpc by section (*A–F*) or whole-mount (*G–N*) in situ hybridization for expression of *Wnt-1* (*A,B*), *Pax-3* (*C,D*), *Pax-6* (*E, F*), *erbB3* (*G, H*), *Islet-1* (*I, J*), and Msx-1 (*K–N*). The arrow in *L* indicates open neural tube in the lumbar region. (fl) Forelimb; (hl) hindlimb; (s) somite.

examined the expression of a number of molecular markers that define different dorsoventral positions in the early neural tube. To address dorsal development we examined the expression of the *Noggin–lacZ* fusion, *Wnt-1, Wnt-3a, Bmp-6,* Lmx-1a, *Msx-1,* and *Follistatin,* all of which are expressed in the roof plate; *Math1,* which is also expressed just lateral to the roof plate; *Pax-3,* which is restricted to the dorsal half of the spinal cord from the tail to the diencephalon; and *Pax-6,* which partially overlaps the ventral-most domain of *Pax-3* expression, but extends into the ventral half of the neural tube. At 10.5 dpc all of these genes were expressed in their appropriate positions in the neural tube to hindlimb levels in *Noggin* mutants (Figs. 5A–F,K,L and 8S,T, below; data not shown). Thus, although the presumptive spinal cord was clearly substantially reduced in size in the interlimb re-

gion, the earliest features of positional specification in the dorsal half of the neural tube appeared to be unaltered. Further, migrating neural crest cells, which originate from the dorsal neural tube, and one of their derivatives, the dorsal root ganglia, could be identified by expression of the *Noggin–lacZ* fusion (data not shown), *Pax-3* (arrows in Fig. 5C,D), *erbB3* (Fig. 5G,H), and *Islet-1* (large arrows in Fig. 5I,J). However, migration and differentiation of the neural crest were disrupted, most likely as a result of defective neural tube closure (Fig. 4D,F) and abnormal somite development (see below). Interestingly, *Msx-1,* unlike other roof plate markers, was ectopically activated in the most caudal neural tube of *Noggin* mutants (Fig. 5K–N). As *Msx-1* is a target of BMP4 signaling in neural tube explants in culture (Liem et al. 1995), this result suggests that Noggin may prevent the premature activation of this BMP target in the developing neural tube. In summary, although we detected increased cell death in undifferentiated regions of the dorsal neural tube, *Noggin* was not essential for most aspects of the initial steps of dorsal patterning we investigated. In contrast, the initial aspects of ventralization of the posterior neural tube were abnormal in *Noggin* mutants.

A series of interactions are responsible for the induction of ventral cell fates (reviewed in Ericson et al. 1996, 1997; Tanabe and Jessell 1996). In the first of these, high concentrations of Sonic hedgehog (Shh) produced by the notochord induce the floor plate at the ventral midline. These cells coexpress *Shh* and the transcriptional regulator *Hnf-3β.* Lower concentrations of Shh secreted by the notochord and floor plate induce the development of motor neurons ventrolaterally. Motor neurons or their progenitors express *c-RET, Islet-1, Islet-2, Sim-1,* and *Nkx2.2.* The exact pattern of marker gene expression and eventual cell fate is thought to depend on the concentration of Shh (Ericson et al. 1997). Analysis of motor neuron deficient *Islet-1* mutants suggests a second induction in which motor neurons induce *Engrailed-1* (*En-1*) expressing interneurons (Pfaff et al. 1996); however, more recent evidence argues against a direct role for motor neurons (Ericson et al. 1997).

In wild-type embryos at 10.5 dpc, *Islet-1* (small arrows in Fig. 6A,C,E), *Nkx2.2* (Fig. 6G), *Sim-1* (barbed arrow in Fig. 8Q, below), and *c-RET* (data not shown) expressing differentiating motor neurons, and *En-1* expressing ventral interneurons (arrow in Fig. 6I), extend to the hindlimb level. In contrast, in *Noggin* mutants motor neuron formation was greatly reduced between the limb buds (arrowhead in Fig. 6D) and was completely absent at hindlimb levels (Figs. 6F,H and 8R, below). Further, no *En-1* expressing interneurons were detected caudal to the forelimbs (Fig. 6J). Surprisingly, expression of *Shh* in the floor plate (large arrow in Fig. 6K,L) and notochord (small arrow in Fig. 6K,L) appeared normal where the absence of ventrolateral neuronal populations was first apparent.

The phenotype was more severe at hindlimb levels where the spinal cord was markedly reduced in size. Here, *Pax-3* was expressed throughout most of the spinal cord (data not shown), while *Pax-6* expression was lost (Fig. 7A,B). At the ventral midline, only limited floor

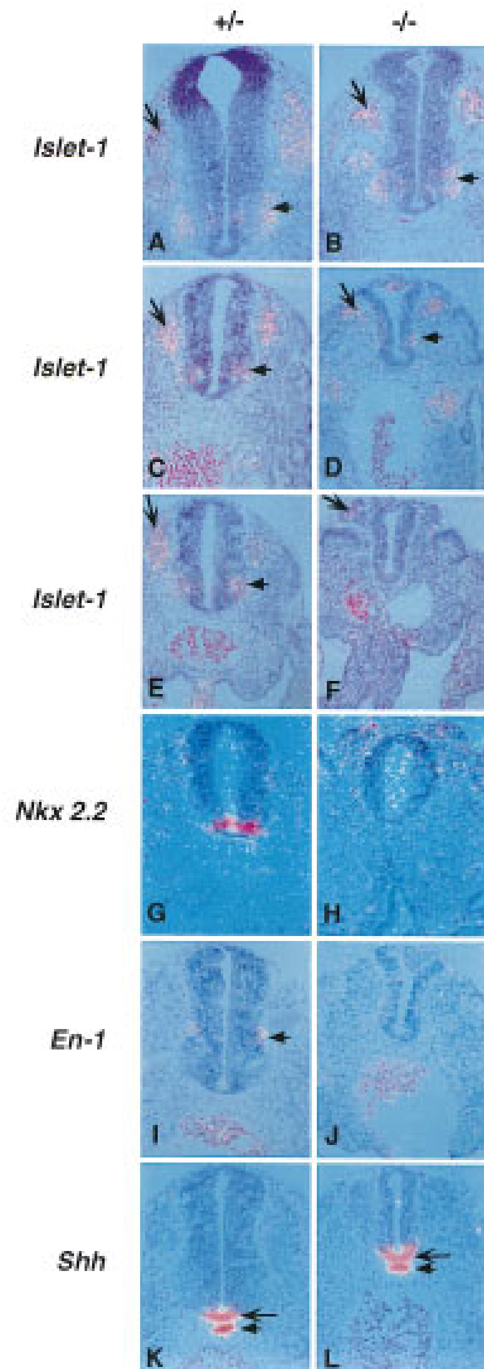

Figure 6. Failure of motor neuron and ventral interneuron development in the absence of Noggin. Section in situ hybridization at the level of the forelimb (*A,B*), between the limbs (*C,D,G,J*), and at the hindlimb (*E–H*) level in 10.5 dpc embryos heterozygous (*A,C,E,G,I,K*) or homozygous (*B,D,F,H,J,L*) for the targeted *Noggin* allele. Differentiating motor neurons or their precursors (short arrow) were detected with an *Islet-1* probe (*A–F*), which also hybridizes to transcripts in dorsal root ganglia (barbed arrow) or with an Nkx2.2 probe (*G,H*). Differentiating ventral interneurons were detected by *En-1* expression (*I,J*), and notochord and floor plate by *Shh* (*K,L*). (For abbreviations, see legend to Fig. 5.) Pairwise comparisons were photographed at the same magnification.

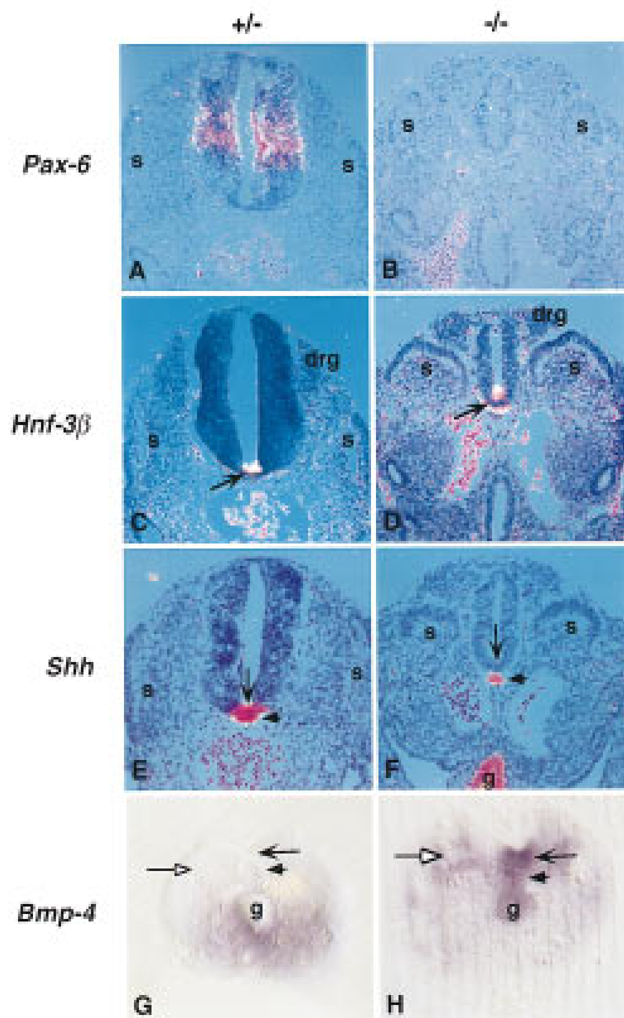

Figure 7. Development of the caudal neural tube in *Noggin* mutants. Section (*A–F*) and whole-mount (*G,H*) in situ hybridization of *Pax-6* (*A,B*), *Hnf-3B* (*C,D*), *Shh* (*E,F*), and *Bmp4* (*G,H*) probes to 10.5 dpc embryos heterozygous (*A,C,E,G*) or homozygous (*B,D,F,H*) for the targeted *Noggin* allele. (*A–F*) Hindlimb levels; (*G,H*) tail region. Pairwise comparisons were photographed at the same magnification. (For abbreviations, see Fig. 5.)

plate development was apparent despite normal *Shh* expression in the notochord (small arrow in Fig. 7E,F). *Hnf-3β* was induced (arrow in Fig. 7C,D) but no *Shh* expression was detected at the ventral midline of the neural tube (large arrow in Fig. 7E,F). These results indicate that in the absence of Noggin, Shh is not sufficient for normal patterning of the ventral neural tube. However, Shh signaling appeared to be occuring, as *Noggin* mutants showed up-regulation of the Shh receptor and transcriptional target, *Patched,* in the ventral neural tube at all axial levels (data not shown).

As Noggin is a BMP antagonist (Zimmerman et al. 1996; Liem et al. 1997), and BMPs have been implicated in dorsal neural patterning in spinal cord regions (Liem et al. 1995, 1997), we addressed the relationship between the expression of *Bmp2–Bmp7* and caudal neural tube development in *Noggin* mutants. Unlike the chick, *Bmp4* was not expressed in the dorsal neural tube but was expressed in the surface ectoderm (data not shown; Winnier et al. 1995; Dudley and Robertson 1997), coelomic epithelium underlying the neural tube and somites (data not shown), and ventral mesoderm (Fig. 7G) in the tail. Surprisingly, in the absence of Noggin we observed ectopic *Bmp4* expression in the notochord (small arrow in Fig. 7H) and at the ventral midline of the neural tube (large arrow in Fig. 7H) together with a dorsal expansion in the unsegmented mesoderm (open arrow in Fig. 7H), but only in the tail region. At more rostral positions no ectopic expression of any of the *Bmp*s investigated was observed (data not shown). This suggests a transient role for *Noggin* in preventing *Bmp4* activation in important ventralizing centers.

Noggin is required for somite development

The requirement for *Noggin* in mesodermal development was addressed by examining embryos between 8.5 dpc and 10.5 dpc of development. Although the notochord extended along the length of the axis to the tail bud at 10.5 dpc, it displayed occasional side branching and buckling in *Noggin* mutants. *Shh, Hnf-3b,* and *Brachyury* were expressed normally (Figs. 6L and 7F; data not shown), but there was a premature loss of *lacZ* activity in the rostral-most notochord of *Noggin* mutants suggesting that *Noggin* may be required for maintenance of its own expression (data not shown). Thus, *Noggin* is not required for either formation or early development of the notochord. However, after 10.5 dpc, tail development arrested and no new notochord was formed despite continued expression of the tail bud markers *Brachyury, Wnt-3a,* and *Wnt-5a* (data not shown). At this time *Bmp4* expression was observed to extend into dorsal mesoderm (open arrow in Fig. 7H; data not shown). Whether this change in *Bmp4* was responsible for the arrest of tail development is unclear. Expression of *c-RET, Bmp7,* and *Sim-1* in the mesonephric duct (arrows in Fig. 8A,B; data not shown) was similar to wild-type littermates.

In the paraxial mesoderm, *Noggin* was not essential for segmentation but was required for growth and differentiation of the somite. Recent evidence indicates that somite patterning is governed by a complex network of signals. For example, Shh signaling by the floor plate and notochord induces sclerotome formation (Fan and Tessier-Lavigne 1994; Johnson et al. 1994; Fan et al. 1995; Chiang et al. 1996). In the myotome, *Shh* and members of the *Wnt* family, which encode secreted glycoproteins, are implicated in muscle development (Johnson et al. 1994; Munsterberg et al. 1995; Currie and Ingham 1996; Hammerschmidt et al. 1996a). As well as a requirement for certain signals for somite differentiation, recent evidence suggests that inhibition of BMP4 signaling may also play an important role in myotomal development (Reshef et al. 1998). Finally, dermal development is thought to depend on contact, mediated sig-

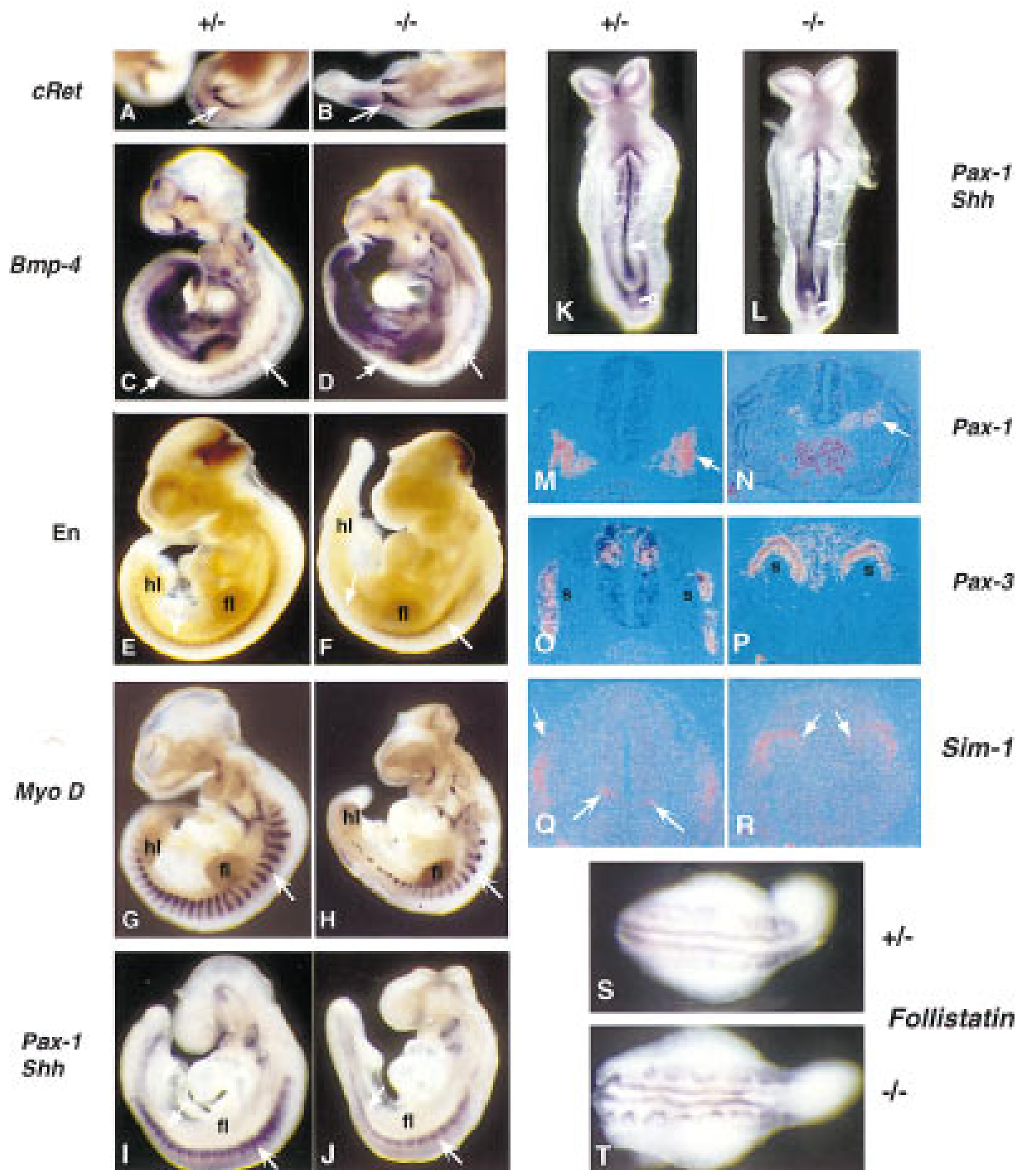

Figure 8. Somite development is disrupted in *Noggin* mutants. Somite and ventral mesoderm development was analyzed in embryos at 8.5 dpc (*K,L*), 9.5 dpc (*I,J*), and 10.5 dpc (*A–H,M–T*) by whole mount (*A–L,S,T*) or section (*M–P*) in situ hybridization using the markers indicated. Sections were probed at, or just rostral to, the level of the hindlimbs. Pairwise comparisons were photographed at the same magnification.

naling by the ectoderm (Fan and Tessier-Lavigne 1994), for which Wnt family members are strong candidates (Fan et al. 1997).

The development of somites originating rostral to the forelimb at 9.5 and 10.5 dpc appeared grossly normal. In *Noggin* mutants the expression of *Bmp4* (large arrows in Fig. 8 C,D; data not shown), En-1 (large arrows in Fig. 8E,F), *Sim-1,* and *Pax-3* (data not shown) in the dermomyotome; *myoD* (arrows in Fig. 8G,H), *myf5,* and *myogenin* (data not shown) in the myotome; and *Pax-1* (small arrows in Fig. 8I,J), *scleraxis,* and *Sox-9* in the sclerotome was similar to wild-type embryos. However, examination of early somite stages (8.5 dpc) indicated that in the absence of *Noggin,* expression of the sclerotomal marker *Pax-1* was delayed by several hours in these rostral-most somites (large arrows in Fig. 8K,L) despite normal *Shh* expression in the notochord (small arrow in Fig. 8K,L). Between the forelimbs and hindlimbs there was a more dramatic somitic phenotype; a severe reduction in both sclerotomal (small arrow in Fig. 8I,J,M,N) and myotomal, most strikingly dorsal myotomal (Fig. 8G,H), derivatives. *Pax-3* expressing limb muscle precursors were present in mutants, even at hindlimb levels (data not shown). We also observed an absence of dermomyotomal expression of *Bmp4* (small arrow in Fig. 8D) and En-1 (small arrow in Fig. 8F), indicating that dermomyotomal differentiation was arrested. Interestingly, from the hindlimb caudal, the dorsomedial somite, where dorsal

myotomal development normally initiates, remained epithelial and continued to express the predifferentiation marker, *Pax-3* (Fig. 8O,P). Furthermore, *Sim-1,* whose ventrolateral dermomyotomal expression is thought to be regulated by BMP4 signaling (Pourquie et al. 1996), extended into the dorsomedial epithelial somite at the hindlimb level (arrows in Fig. 8R). Finally, *Follistatin* (which encodes an activin and most likely BMP7 antagonist) appeared to be up-regulated in the dorsomedial somite of *Noggin* mutants (Fig. 8T). Together, the data indicate that Noggin-mediated antagonism of BMP signaling is essential for normal growth and patterning of the caudal somites.

Noggin is required for Shh-mediated induction of sclerotomal development

The substantial delay and reduction in sclerotomal and myotomal development suggests that Noggin may cooperate with Shh in patterning the somitic mesoderm. We therefore investigated whether Noggin is able to synergize with Shh in the induction of *Pax-1* in presomitic mesoderm (psm) explants (Fan et al. 1995). The presence of Noggin reduced the threshold induction of *Pax-1* by SHH-N fourfold (from 25 to 6.8 ng/ml; Fig. 9A), indicating that Noggin synergizes with Shh. Surprisingly, high concentrations of Noggin (>100 ng/ml) were sufficient to activate *Pax-1* (Fig. 9B). To determine whether the response to Noggin resulted either from potentiating low levels of Shh present in the psm, or from an early exposure to Shh signaling emanating from the notochord, we added antagonists that are known to abolish SHH-N function in this assay. Addition of a blocking antibody against SHH-N (Ericson et al. 1996), although able to inhibit SHH-N induction of *Pax-1,* did not block induction by Noggin (Fig. 9C). Furthermore, induction was not blocked by addition of IBMX and forskolin, which antagonize SHH-N signaling by elevating intracellular cAMP (Fig. 9C). Taken together, we conclude that Noggin can act alone to activate *Pax-1* expression in the psm explant.

We next tested whether purified recombinant BMP2 or BMP4 can suppress *Pax-1* induction. Both these BMPs were potent inhibitors. Ten nanograms/milliliter of either BMP completely abolished *Pax-1* induction in response to Noggin, SHH-N, or a combination of the two (Fig. 9D). These results suggest that overcoming inhibitory BMP signaling is necessary to initiate the sclerotomal program. In the embryo, inhibitory BMPs could be supplied by the paraxial mesoderm itself, or by adjacent tissue. In situ hybridization studies have demonstrated that the early somite uniformly expresses *Bmp3* (Dudley and Robertson 1997; J. McMahon et al., unpubl.). Moreover, the somite forms adjacent to the notochord that expresses *Bmp3* and *Bmp7,* above the ventral mesoderm that expresses *Bmp4,* and adjacent to the surface ectoderm that expresses *Bmp4* and *Bmp7* (Dudley and Robertson 1997; data not shown). In addition, using PCR, we explored the possibility that the psm may express low levels of *Bmp*s not detectable by in situ hybridization.

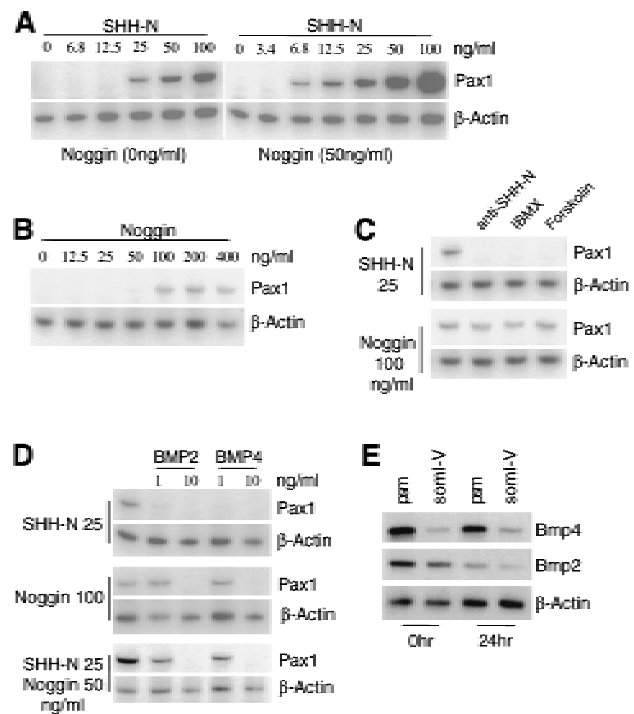

Figure 9. Noggin and Shh function synergistically to activate the expression of sclerotome marker *Pax1* in presomitic mesoderm. Mouse psm explants (9.5 dpc) were cultured in collagen gels for 24 hr in the presence or absence of purified recombinant Noggin, SHH-N (the amino-terminal 19-kD peptide), BMP2, or BMP4. The concentration of each of these is indicated. RNA samples from each culture (two pieces of psm per culture) were extracted and subjected to RT–PCR to assess marker gene expression. Expression of *β-actin* was assayed as a control for RNA recovery and cDNA synthesis (see Materials and Methods). (*A*) Psm explants were treated with a series of concentrations of SHH-N (as indicated) with (*right*) or without (*left*) 50 ng/ml Noggin. Expression of the sclerotome marker *Pax1* and the *β-actin* control was assessed by RT–PCR. (*B*) Noggin alone can also activate the sclerotomal marker *Pax1.* (*C*) Noggin can induce *Pax-1* expression in the presence of a SHH-N blocking antibody, IBMX, and forskolin, whereas all these reagents block SHH-N mediated induction of *Pax-1* in psm explants. (*D*) Purified BMP2 and BMP4 protein can inhibit *Pax-1* induction in response to SHH-N, Noggin, or a combination of each protein. (*E*) The psm and the five caudal-most somites (somI–V) were assayed immediately (0 hr) or after 24 hr of culture (24 hr) for expression of *Bmp2* and *Bmp4* (30 cycles). RT–PCR products were analyzed by Southern hybridization (see Materials and Methods). *β-actin* (13 cycles) expression was similar in all samples.

After 30 cycles we detected *Bmp2* and *Bmp4* (Fig. 9E), but failed to detect *Bmp5, Bmp6,* and *Bmp7* after 35 cycles. Although we cannot rigorously exclude the possible contamination by ventral mesoderm, these results suggest that the psm itself, or neighboring tissues, express those *Bmp*s that are known to interact with Noggin.

Discussion

Noggin *and the organizer*

Expression of *Noggin* in the organizer region of the *Xeno-*

pus embryo (Smith and Harland 1992), and the results of ectopic expression studies in the frog (Smith and Harland 1992; Lamb et al. 1993; Smith et al. 1993) and fish (Hammerschmidt et al. 1996b) are consistent with *Noggin* playing a central role in specifying dorsal cell fates in both the ectoderm (neural plate) and mesoderm (somite and notochord). The recent demonstration that Noggin is a direct antagonist of ventralizing BMP signals suggests a simple mechanism by which Noggin patterns the embryo, through the graded inhibition of ventral BMP signaling (Zimmerman et al. 1996). However, even though *Noggin* is expressed in the mouse node, a structure with similar properties to the *Xenopus* organizer (Beddington 1994), *Noggin* is not essential for formation of neural tissue, notochord, or somites. Rather, our results demonstrate that *Noggin* expression in the notochord and roof plate plays a later role in somite and neural tube development.

No phenotype was observed in *Noggin* mutants until 8.5 dpc, despite the early midline expression of *Noggin.* There only appears to be a single *Noggin* gene in vertebrates, so it is unlikely that there is redundancy between *Noggin* and a second, unidentified family member. However, at least two unrelated secreted polypeptides, Follistatin and Chordin, have similar activities. Both of these are expressed in the early mesoderm and/or node of the gastrulating mouse embryo (Albano et al. 1994; E. De Robertis, pers. comm.). *Follistatin* null mutants show no defects in organizer function (Matzuk et al. 1995). Chordin mutants have been identified in the zebrafish (Schulte-Merker et al. 1997). One of these, *dino,* has a ventralized phenotype consistent with some loss in organizer function (Hammerschmidt et al. 1996b). Consequently, it may be necessary to remove *Noggin, Chordin,* and *Follistatin* to completely uncover their putative organizer properties.

Noggin *and neural tube development*

Although *Noggin* does not play an essential role in the induction of neural tissue, it is required for subsequent development of the neural tube. In *Noggin* mutants, we observed a failure of neural tube closure in cranial and lumbar regions, a dramatic reduction in the amount of posterior neural tissue, and the progressive failure of ventral development in the posterior neural tube.

Noggin is expressed in the dorsal aspects of the neural plate, the presumptive roof plate, coincident with neural tube closure. Thus, it is possible that *Noggin* may play a direct role in regulating the cellular processes that lead to the elevation and fusion of the neural folds (for review, see Schoenwolf and Smith 1990). Examination of a large number of molecular markers expressed either in or around the closing neural tube (*Wnt-1, Wnt-3a, Bmp6, Follistatin,* Lmx-1a, *Noggin-lacZ, Math1, Pax-3*) failed to reveal any striking difference between *Noggin* mutants and wild-type siblings suggesting that the failure of neural tube closure does not appear to result from a clear defect in early patterning of the dorsal neural tube. However, we observed premature activation of a BMP4 target, *Msx-1,* in the caudal neural tube, consistent with a role for Noggin in preventing *Msx-1* activation. We also observed enhanced cell death at the dorsal midline of the neural tube in *Noggin* mutants. As *Msx* family members have been implicated in the regulation of apoptosis (e.g., Graham et al. 1994) there may be a correlation between these two observations. Furthermore, recent work suggests that regulation of cell death may play an important role in neural tube closure (Weil et al. 1997). Whether there is a link between deregulated cell death and the open neural tube remains to be explored but it seems likely that the *Noggin* mutants might provide a useful model for studying this important and poorly understood morphological process.

The other obvious CNS phenotype we observed is a reduction in posterior neural development that occurs secondarily to the formation of neural tissue. Caudal to the forelimbs there was a progressive decrease in the diameter of the neural tube that was accompanied by the appearance of extensive cell death, principally in intermediate and ventral regions of the developing spinal cord. Thus, *Noggin* appears to be required for survival, and possibly proliferation, of neural precursors in the neural tube. A reduction in the size of the neural tube is evident at both dorsal and ventral positions consistent with a role for *Noggin* in both the dorsal and ventral development. Dorsally, expression of all early regional markers investigated was appropriately positioned and extensive neural crest formation was observed. In vitro studies in the chick have demonstrated that several TGF-β family members, including BMP4 and BMP7, may contribute to the induction of dorsal neurons. Furthermore, they suggest that blocking early BMP activity may be a prerequisite for the emergence of later arising dorsal cell fates (Liem et al. 1997). Whether specific dorsal neuronal populations that emerge later in development are dependent on *Noggin* function remains to be determined.

Surprisingly, *Noggin* is clearly essential for establishment of some ventral cell fates in the developing CNS. At posterior lumbar levels, motor neurons and ventral interneurons were depleted or entirely absent. In more posterior positions, floor plate development was initiated but a mature floor plate was not formed. Finally, caudal to the hindlimbs, much of the neural tube appeared to adopt a dorsal, likely neural crest cell fate. How might these results be explained in light of our understanding of growth and patterning of the neural tube?

There is now overwhelming evidence to support the conclusion that Shh signaling plays a key role in induction of two ventral cell fates in the vertebrate CNS, motor neurons and floor plate (for review, see Tanabe and Jessell 1996; Hammerschmidt et al. 1997). In *Noggin* mutants we observed a failure of motor neuron induction, despite the normal expression of *Shh* in both the floor plate and notochord, the two signaling centers implicated in motor neuron induction (Yamada et al. 1991; Placzek et al. 1991; Yamada et al. 1993). Thus, in the embryo proper, in the absence of Noggin, Shh is not sufficient for the normal specification of motor neuron

fates. As in the *Islet-1* mutant (Pfaff et al. 1996), in the absence of motor neuron fates, *En-1* expressing ventral interneurons are also missing. The loss of *Pax-6* expression in the ventral neural tube is also consistent with these results as motor neuron and interneuron fates arise from *Pax-6* expressing neural precursors (Ericson et al. 1996, 1997). In more posterior regions, we also observed that although *Shh* expression in the notochord was sufficient to activate *Hnf-3β* at the ventral midline of the neural tube, it was not sufficient to activate *Shh* itself, a normal feature of the floor plate. Finally, in the most posterior regions, no ventral development occurred even though *Shh* was expressed in the underlying notochord. Together, these results indicate that Noggin-mediated antagonism of BMP signaling is essential for Shh-mediated ventralization of the mouse neural tube. It is unlikely that Shh signaling is lost as we observed up-regulation of *Patched* transcription in the ventral neural tube as expected (data not shown). However, Shh signaling does not result in normal ventralization of the spinal cord.

Several lines of evidence support the view that BMP-signaling might prevent Shh action. For example, implantation of *Bmp7* expressing cells adjacent to the mouse hindbrain blocks autoinduction of *Shh* in the floor plate (Arkell and Beddington 1997). Furthermore, addition of BMP4 Dorsalin-1, a TGF-β family member, to neural tube explants strongly inhibits the motor neuron inducing activity (presumably Shh) produced by ventral axial structures (Basler et al. 1993). Thus, the response to ectopic BMPs in these assays resembles the phenotype resulting from loss of *Noggin* function. A localized requirement for *Noggin* may also explain why ectopic expression of Shh throughout the neural tube of the *Xenopus* embryo (Ruiz i Altaba et al. 1995), or at the mid/hindbrain junction in the mouse (Sasaki and Hogan 1994), only results in ectopic floor plate development adjacent to the roof plate where *Noggin* is expressed.

BMPs, in particular BMP4 and BMP7, have been implicated in the induction of dorsal cell fates in presumptive spinal cord. The surface ectoderm most likely initiates dorsalization of the neural tube (Dickinson et al. 1994; Liem et al. 1995). The ectoderm expresses several *BMP*s and these appear to play a role in induction of dorsal cell fates (Basler et al. 1993, Liem et al. 1995, 1997; Dudley and Robertson 1997). Thus, in spinal cord regions, BMP signaling has an opposite function to that of Shh. Moreover, there is evidence to suggest that the neural plate is initially dorsalized, presumably in response to early acting BMP signals, and that ventral cell fate specification, which occurs later, requires the suppression of dorsal cell fates (Ericson et al. 1996). Our data show that for Shh to work effectively in the embryo, BMP signaling must be antagonized. The notochord expresses *Bmp3* and *Bmp7* (Dudley and Robertson 1997), suggesting that *Noggin* could play a direct role in antagonizing their activities. Although BMP3 binding to Noggin has not been tested, functional studies indicate that it is unlikely to have the same properties as BMP4 (K. Lyons, pers. comm.). However, Noggin does bind BMP7, albeit with considerably weaker affinity than for BMP4. Whether this has physiological significance in the embryo is difficult to assess. Follistatin, which binds BMP7 more strongly, is expressed normally in the roof plate of Noggin mutants. Alternatively, Noggin may prevent the spread of BMP4 from the ventral mesoderm, or the activity of low levels of *Bmp2* or *Bmp4* in the paraxial mesoderm. In keeping with this hypothesis, we observed the induction of *Bmp4* in the notochord and at the ventral midline of the caudal neural tube in *Noggin* mutants. Thus, in the absence of *Noggin,* BMP4 in the ventral mesoderm may be able to induce its own expression in more dorsal regions.

The loss of ventral cell fates in *Noggin* mutants does not appear to be accompanied by a concomitant expansion of dorsal cell fates. Rather, it would appear that many ventral cells die. Intriguingly, this results in a gross reduction of the neural tube that appears to be more pronounced than that of notochord, deficient embryos (van Stratten and Hekking 1991; Yamada et al. 1991). Why should the loss of a single secreted polypeptide generate a more severe phenotype than the complete loss of all notochordal activities? One possible explanation is that in the absence of Noggin, ventral cells would receive conflicting signals since Shh is still produced and cells appear to respond to Shh. These conflicting signals, BMPs (dorsalizing) and Shh (ventralizing), might cause cell death. Interestingly, in the forebrain Shh and BMPs, most likely BMP7, have been proposed to collaborate in the patterning of ventral diencephalic regions (Dale et al. 1997), suggesting that the combination of these signals might lead to the specification of diencephalic neural precurors in the spinal cord. However, we found no evidence for ectopic expression of Nkx2.1 to support such a model (data not shown).

Noggin *and somite development*

In the absence of *Noggin* there is marked reduction in both the induction and survival of sclerotomal and myotomal derivatives in the trunk. A large body of evidence indicates that the distinct cell fates are generated within the epithelial somite in response to inductive cues from surrounding tissues (for review, see Brand-Saberi et al. 1996; Cossu et al. 1996). For example, removal of the notochord results in a failure of sclerotomal induction. In vitro, the inductive properties of the notochord are mimicked by Shh (Fan et al. 1995). However, *Shh* mutants exhibit some *Pax-1* expression, indicative of limited sclerotomal development, which is followed by a secondary loss of these cells (Chiang et al. 1996). Thus, Shh is not essential for initiating all sclerotomal development. Our data suggest that *Noggin* contributes to sclerotomal induction.

First, *Noggin* is required at all axial levels for the normal induction of sclerotome. At rostral levels, induction is delayed but is otherwise normal, at thoracic levels the sclerotomal population is reduced, and at more posterior positions sclerotome is entirely absent. Second, at high concentrations Noggin induces sclerotomal development in the absence of Shh. Third, addition of Noggin to

presomitic mesoderm explants lowers the effective dose of Shh which is required to induce sclerotome. Fourth, BMP2 and BMP4 are potent inhibitors of *Pax-1* induction by Shh in culture. Moreover, grafts of BMP4-expressing cells inhibit growth and differentiation of the chick sclerotome (Monsoro-Burq et al. 1996). These findings can be explained by a simple model in which BMP signaling generally inhibits early somite differentiation. Countering this inhibition may be essential for sclerotomal induction. Binding of BMP4, and possibly other BMPs, by Noggin would provide a direct antagonism. It is unlikely that BMP signaling inhibits Hedgehog targets by activating PKA (for review, see Hammerschmidt et al. 1997), as Noggin is still able to induce *Pax-1* in the presence of PKA agonists.

The notochord and neural tube are also implicated in the induction of muscle derivatives that fail to develop when both tissues are removed (Rong et al. 1992). Shh also appears to play a role in myotome induction, most likely with dorsally expressed members of the Wnt family (Münsterberg et al. 1995; Chiang et al. 1996). The loss of myotomal derivatives in *Noggin* mutants suggests that suppression of BMP signaling is also necessary to allow effective muscle induction. The somite gives rise to distinct populations of muscle precursors in different positions (Cossu et al. 1996). Myogenic gene expression occurs first in the dorsomedial component that gives rise to the epaxial musculature, then later in the ventrolateral hypaxial precursors. BMP4 is implicated in the regulation of distinct cell fate choices within the myotome. *Bmp4* is expressed in the lateral mesoderm adjacent to the hypaxial precursors and application of BMP4 to the dorsomedial component leads to the repression of myogenic gene expression (Pourquie et al. 1995). In contrast, the formation of epaxial muscles from the dorsomedial somite requires an opposing activity, and this appears to arise from the dorsal neural tube during development (Pourquie et al. 1996). In the mouse, *Noggin* is expressed in the roof plate, then much later in development, *Noggin* is expressed rather weakly in the dorsal lip of the somite. Dorsomedial expression of *Noggin* is in general consistent with a role in antagonizing BMP signaling in the regulation of muscle patterning. In the chick, however, *Noggin* has more pronounced and earlier expression in the dorsal lip of the somite, and here expression in the somite may play a more direct role in controlling myogenesis (Reshef et al. 1998). In *Noggin* mutants we observed the loss of dorsal myogenic gene expression and a dorsal expansion of *Sim-1,* a marker of ventrolateral fates, into the dorsomedial dermomyotome at hindlimb levels. In contrast, *Noggin* is not required for formation of *Pax-3*-expressing limb muscle precursors at either the fore- or hindlimb levels (data not shown). We also observed an up-regulation of *Follistatin* in the dorsomedial somite suggesting that its expression may be positively regulated by dorsal BMP signaling. Thus, it is likely that Noggin antagonism of BMP signaling is required for both myotomal and sclerotomal development. However, the failure of dermomyotomal expression of *En-1* points to a broader inhibitory role for BMPs in the somite.

Whereas our results establish the principle of *Noggin* action, we cannot be certain as to the exact identity of the relevant targets. For example, the biochemical characterization of Noggin interactions has been restricted to a subset of BMPs, but there are several other members of the TGF-β family, notably some of the GDFs, which are also coexpressed with Noggin. Furthermore, there is no reason to believe that all family members have been identified. Of those that are known, *Bmp3* is expressed in the immature somite and is down-regulated on differentiation (Dudley and Robertson 1997; J.A. McMahon et al., unpubl.). However, BMP3 does not appear to be required for normal somite patterning and has different activities from BMP2 and BMP4, so it is unlikely to be a Noggin target (K. Lyons, pers. comm.). The most plausible candidate is *Bmp4,* which is strongly expressed in the ventral mesoderm immediately under the paraxial mesoderm and in the coelomic mesoderm underlying the developing somites. In summary, our results demonstrate that elaboration of the vertebrate body plan requires not only the positive action of a number of inductive signals, but also the specific inhibition of others. It is likely that inhibitory molecules will become increasingly important in our understanding of vertebrate development as more are identified and their functions dissected.

Materials and methods

Cloning and targeting of mouse Noggin

Initially, cDNAs partially encoding mouse *Noggin* were isolated by screening a phage λ gt10 cDNA library (gift of Brigid Hogan) with *Xenopus Noggin* using standard low stringency conditions. A mouse 129 strain λ genomic library (the generous gift of Rudolf Jaenisch, Whitehead Institute, Cambridge, MA) was screened by hybridization with a mouse *Noggin* cDNA probe and a single genomic clone was identified (Southern blotting confirmed that there is a single *Noggin* gene). The *Noggin* gene replacement construct was generated (see Fig. 2) by blunt end ligation of a 5′, 5.2-kb, *Eco*RV–*Bst*EII *Noggin* genomic fragment into an end-filled (T4 polymerase) *NcoI* site in the *lacZ* containing plasmid, pSDKlacZ (the gift of Janet Rossant). This resulted in an in-frame fusion of the first 10 amino acids of the *Noggin* coding sequence with the *E. coli lacZ* gene containing an SV40 polyadenylation sequence. For positive selection, a *PGKneo* cassette (Soriano et al. 1991) was cloned downstream of the *lacZ* gene and a 3′, 5.0-kb, *Bam*HI–*Eco*RV homology region cloned downstream of this selection cassette. This targeting construct was generated in the plasmid vector pMC1-HSVTK (Mansour and Capecchi 1988) which provides a flanking herpes virus thymidine kinase gene for negative selection against nonhomologous recombinants. The targeting construct was linearized with *Sal*I and electroporated into CJ7 ES cells (Swaitek et al. 1993). A clone with the expected recombination event was identified by Southern analysis using both 5′ and 3′ diagnostic probes (see Fig. 2) and this clone was used to generate germ-line chimeras. The mutated allele was maintained on an inbred 129/Sv background or crossed to C57BL6/J mice to generate embryos on a hybrid background.

Identification of Noggin *mutants*

Initially, *Noggin* mutants were identified by Southern blot

analysis of yolk sac DNA with a diagnostic 5′ probe (see Fig. 2). Southern hybridization to DNA digested with *Bam*HI (5′ analysis) or *Eco*RI (3′ analysis) with a coding region probe confirmed that the *Noggin* coding region was deleted from embryos that displayed a mutant phenotype. Subsequently, a PCR assay was developed in which amplification of the wild-type allele generated a 211-bp product (primers nog1 and nog2) and amplification of the mutant allele generated 160-bp product (primers nog1 and gal1). PCR samples were preheated to 93°C for 90 sec then subjected to 35 cycles of amplification alternating between a 30-sec 93°C denaturation and 45-sec 72°C extension step (McMahon et al. 1992).

PCR primers: nog1, 5′-GCATGGAGCGCTGCCCCAGC-3′; nog2, 5′-GAGCAGCGAGCGCAGCAGCG-3′; gal1, 5′-AAGG-GCGATCGGTGCGGGCC-3′.

Histology, in situ hybridization, and cell death

For routine histological analysis, embryos were fixed in Bouin's solution, dehydrated, paraffin embedded, sectioned at 6 µm, dewaxed, and either hematoxylin and eosin or toluidine blue counterstained prior to mounting. β-Galactosidase staining and in situ hybridization were essentially as described (Wilkinson et al. 1987; Whiting et al. 1991; Wilkinson 1992). Apoptotic cell death was visualized using the TUNEL procedure with a kit from Boehringer Mannheim.

Explant tissue culture and RT–PCR

Mouse psm was dissected at 9.5 dpc, embedded in collagen gels, and cultured for 24 hr in serum-free medium; OPTI-MEM/F12/DME (50:25:25) supplemented with 5 ng/ml FGF to promote survival in serum-free conditions (Fan and Tessier-Lavigne 1994). Forskolin was added at 90 µM and IBMX at 100 µM (Fan et al. 1995). Anti-SHH-N blocking antibody, 5E1 (Ericson et al. 1996) was added at 3.5 µg/ml. *Xenopus* Noggin protein was purified from CHOB3 conditioned medium as described (Lamb et al. 1993) and was the gift of José de Jesus. SHH-N protein was the gift of Phil Beachy (Johns Hopkins Medical School, Baltimore, MD) and BMP2 and BMP4 were generously supplied by Genetics Institute. The RNA sample of each explant culture (containing two pieces of psm) was purified and ⅓ of each sample was used for RT–PCR reactions in the presence of [^{32}P]dCTP (Amersham) as described previously (Fan et al. 1995). The resulting radioactive PCR products were resolved on 6% polyacrylamide gels, dried, and exposed to X-ray films (Kodak) for 2 hr. The oligonucleotide primers used to detect *Pax1* and *β-actin* were described by Fan et al. (1995). To detect *Bmp2* and *Bmp4* transcripts in psm and somites, two pieces of psm and two strips of somite I–V (caudal-most five somites) were used for each culture. One half of each RNA sample was used for RT–PCR for 30 cycles (94°C–60°C–72°C cycle). The PCR products were resolved on 2% agarose gels, transferred to Gene Screen filters (NEN), hybridized with [^{32}P]dCTP *Bmp2* and *Bmp4* cDNA probes, washed under the standard high stringency condition (Sambrook et al. 1989), and exposed to X-ray films for 1 hr. The primers used were 5′-CGGAGACTCTCTCAATGGAC-3′ and 5′-GTTCCTCCACGGCTTCTAGT-3′ for *Bmp2* which generates a 436-nucleotide product; and 5′-CTCCCAAGAATC-ATGGACTG-3′ and 5′-AAAGCAGAGCTCTCACTGGT-3′ for *Bmp4,* which generates a 468-nucleotide product. *Bmp2* and *Bmp4* gene sequences were described by Feng et al. (1994 and 1995, respectively).

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