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Neural Induction and Patterning in the Mouse in the Absence of the Node and Its Derivatives

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The signals which induce vertebrate neural tissue and pattern it along the anterior–posterior (A-P) axis have been proposed to emanate from Spemann's organizer, which in mammals is a structure termed the node. However, mouse embryos mutant for *HNF3β* lack a morphological node and node derivatives yet undergo neural induction. Gene expression domains occur at their normal A-P axial positions along the mutant neural tubes in an apparently normal temporal manner, including the most anterior and posterior markers. This neural patterning occurs in the absence of expression of known organizer genes, including the neural inducers *chordin* and *noggin.* Other potential signaling centers in gastrulating mutant embryos appear to express their normal constellation of putative secreted factors, consistent with the possibility that neural-inducing and -patterning signals emanate from elsewhere or at an earlier time. Nevertheless, we find that the node and the anterior primitive streak, from which the node derives, are direct sources of neural-inducing signals, as judged by expression of the early midbrain marker *Engrailed,* in explant–recombination experiments. Similar experiments showed the neural-inducing activity in *HNF3β* mutants to be diffusely distributed. Our results indicate that the mammalian organizer is capable of neural induction and patterning of the neural plate, but that maintenance of an organizer-like signaling center is not necessary for either process. © 1999 Academic Press

Key Words: **neural induction; organizer; node; mouse; *HNF3β*.**

INTRODUCTION

Classic experiments by Hans Spemann and colleagues led to the identification of the dorsal lip of the blastopore of the early amphibian gastrula as capable of inducing an organized ectopic neural axis; hence this tissue was called Spemann's organizer or simply the organizer (Harland and Gerhart, 1997). Subsequent work has shown that the organizer and its derivatives, including the notochord, are also a source of signals which confer axial polarity to the developing neural plate. Throughout its length, the notochord is a strong ventralizing influence on the overlying neural plate (Placzek, 1995). The organizer also confers anterioposterior (A-P) positional cues (Saxen, 1989). The early organizer, and the most anterior portion of the notochord (which emerges from the organizer earliest), can induce anterior markers in competent ectoderm. Older organizers and posterior notochord induce posterior markers. Such observations suggested to Spemann the existence of a "head organizer" and a "trunk organizer," which initially coincide in the dorsal lip but separate in space as gastrulation proceeds.

Recent molecular evidence has suggested that the activity of the organizer in promoting neural induction and patterning is largely brought about by its production of factors that block the action of signals that normally promote ectodermal cell fate (Hemmati-Brivanlou and Melton, 1997). Noggin, Chordin, and Follistatin are all antagonists of BMP signaling and are expressed specifically in the organizer. This action prevents BMP from promoting ecto-

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dermal fate and leads to expression of the "default" neural pathway. Organizer activity cannot be entirely explained by this pathway, and increasing evidence suggests that there may be separate signals for anterior and more posterior neural induction, consistent with the concept of a head and trunk organizer. A *Xenopus* gene, *cerberus,* is expressed in the endoderm subjacent to the organizer and induces ectopic heads (Bouwmeester *et al.,* 1996). Biochemically, Cerberus has been shown to be capable of blocking both BMP and Wnt signaling (Piccolo *et al.,* 1999; Glinka *et al.,* 1997). Another molecule with some head organizer activity is Dickkopf, and it too has Wnt-antagonizing properties (Glinka *et al.,* 1998). A general model can begin to be built, in which anterior induction requires both BMP and Wnt antagonism, while the trunk organizer activity needs only BMP antagonism. Full elaboration of the pattern of the posterior elongating axis involves the action of non-organizer-associated factors, including FGFs, Wnts, and retinoic acid (Doniach, 1995; Sasai and DeRobertis, 1997).

The murine organizer is the node, as defined by gene expression, lineage, and functional criteria (Tam and Behringer, 1997). The node develops at the anterior end of the primitive streak as the streak reaches the distal end of the embryo. Grafts of this region can induce a partial ectopic axis in host embryos (Beddington, 1994; Tam *et al.,* 1997) and the node expresses the organizer-localized BMP antagonists, *noggin* and *chordin* (McMahon *et al.,* 1998; Tam and Behringer, 1997). However, a mouse homolog of *cerberus, Cer1,* is not expressed in the node but in a region of the anterior visceral endoderm (AVE) away from the developing node; indeed, a variety of embryological, genetic, and molecular studies suggest that the AVE is important for anterior embryonic development in the mouse (reviewed by Beddington and Robertson, 1998). Thus in both mouse and frogs, there may be a distinct head organizer composed of primitive endodermal cells, independent of a trunk organizer. In frogs, these two sources initially colocalize to the dorsal lip of the blastopore (Bouwmeester *et al.,* 1996), whereas in mouse they appear to be spatially separated (Beddington and Robertson, 1998). In this paper, the term "organizer" refers to Spemann's organizer, which in all species includes at least the trunk organizer.

The actual requirement for Spemann's organizer in the intact embryo is unclear, despite all its remarkable inductive properties in ectopic grafts. Complicating matters is that surgical ablation of the organizer has little effect on the body plan; however, in at least the chick ablations, the organizer reconstitutes quickly (see Yuan *et al.,* 1995; Psychoyos and Stern, 1996; and references therein). The mouse system has offered a unique insight into organizer function because null mutations in the *HNF3β* gene prevent organizer development (Ang and Rossant, 1994; Weinstein *et al.,* 1994). Expression of *HNF3β*, encoding a winged-helix transcription factor, marks the anterior end of the primitive streak, the node, and ultimately the derivatives of the node as gastrulation proceeds (Sasaki and Hogan, 1993; Monaghan *et al.,* 1993; Ang *et al.,* 1993). Embryos homozygous for targeted null alleles of *HNF3β* completely lack the node and its derivatives; nevertheless, such embryos develop a neural tube, which displays A-P pattern yet lacks dorsal–ventral pattern (Ang and Rossant, 1994; Weinstein *et al.,* 1994).

In this paper, we assess further the role of the organizer in mammalian neural induction using embryological and molecular studies of wild-type and *HNF3β* mutant embryos. We investigate the nature of the A-P patterning which occurs in the node's absence and determine the expression of genes encoding secreted factors produced by the node and by other signaling centers in the absence of a definitive node. We also examine the neural-inducing capacity of different regions of wild-type and *HNF3β* mutant embryos. We find that secreted neural-inducing factors produced by the node are absent or only transiently expressed in *HNF3β* mutant embryos and that neural-inducing capacity is present but is diffuse and weak. Our results suggest that the maintenance of the node and its derivatives is not required for the induction and broad A-P patterning of the nervous system, but is more involved in refinement of pattern by organizing the alignment of other signaling sources.

MATERIALS AND METHODS

Mouse Strains, Crosses, and Genotyping

A colony of outbred mice heterozygous for a null allele of *HNF3β* (Ang and Rossant, 1994) was maintained for these studies. Male heterozygotes were crossed to CD1 (Charles River) or ICR (Harlan) random-outbred females to generate stock for timed matings. Noon of the day on which the copulation plug was detected was taken as 0.5 days postcoitum (dpc). Genotyping was performed on genomic DNA using the PCR genotyping primers and protocol previously described for this allele (Ang and Rossant, 1994). In the case of living mice, DNA was prepared from 0.5-cm tail biopsies collected at weaning (21–28 days after birth). Embryos were genotyped using yolk sac or other extraembryonic tissues as a DNA source, with each sample collected with clean instruments after extensive rinsing through fresh PBS to remove any adherent maternal tissue. Genomic DNA was prepared as described by Moens *et al.* (1992).

For the detection of retinoid signaling response or *noggin* expression in *HNF3β* mutants, marker transgenes were crossed into the *HNF3β* mutant strain. Males homozygous for the *RARE-lacZ* transgene (Rossant *et al.,* 1991) were crossed to *HNF3β* female heterozygotes. Double heterozygotes were crossed back to the parental transgenic strain, and males who were homozygous for the transgene and heterozygous for *HNF3β* were identified. These were crossed to female *HNF3β* heterozygotes for timed matings. Males heterozygous for *Nog*^{9e}, which bears a *lacZ* within the *noggin* locus (McMahon *et al.,* 1998), were obtained from Drs. A. McMahon and R. Harland.

Additional strains used were as follows. Wild-type embryos for *in situ* hybridization and explant studies were derived from timed matings between CD1 or ICR males and females. Embryos for the detection of *lacZ* expression under the control of *Engrailed2 (En2)* or *Cordon-bleu (Cobl)* were obtained by crossing ICR females to homozygous males for *En2-lacZ* transgenic line *Tg5* (Logan *et al.,* 1993) or the gene trap *C101,* an insertion of a *lacZ* expression vector into *Cobl* (Gasca *et al.,* 1995).

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Visualization of Gene Expression

Staining of embryos for *lacZ* activity was performed as detailed by Logan *et al.* (1993). The details specific to our use of the procedure were that whole embryos were dissected and fixed in 0.2% glutaraldehyde for 10 min and that staining occurred overnight at 37°C. In some cases embryos were sectioned after staining. Such embryos were postfixed in 3.7% formaldehyde overnight, embedded in wax, and sectioned at 4 μm. Serial sections were mounted on slides, dewaxed, and counterstained with eosin.

Two different protocols were used for whole-mount *in situ* hybridization. At least four mutant embryos were studied for each probe. Wild-type embryos were always hybridized in parallel. All probes were hybridized using the conditions described by Conlon and Rossant (1992), except that the sodium borohydride incubation step was omitted. Expression of many genes in the mutant embryos was also investigated using the protocol of Henrique *et al.* (1995). In addition, an RNase digestion step was added after incubation with the probe. RNase T1 (100 U/ml; Boehringer Mannheim) was added in RNase incubation buffer (Conlon and Rossant, 1992) for 30 min at 37°C. All whole-mount *in situ* hybridizations were performed with digoxygenin-labeled RNA antisense probes, made according to the protocol of Conlon and Rossant (1992), using reagents from Boehringer Mannheim.

In situ hybridization on sectioned wild-type and mutant embryos was performed in the case of *Wnt5b,* because we were unable to obtain good quality whole-mount hybridization. Preparation of radiolabeled probe, preparation of sections, and hybridization were performed as described by Guillemot and Joyner (1993).

The probes used in this study were as follows: *BF1* (Tao and Lai, 1992), *BMP4* (Winnier *et al.,* 1995), *BMP7* (Arkell and Beddington, 1997), *Cer1* (Pearce *et al.,* 1999), *Chd* (Bachiller *et al.,* submitted for publication), *En2* (Davis *et al.,* 1988), *Fgf3* (Wilkinson *et al.,* 1988), *Fgf4* (Niswander and Martin, 1992), *Fgf5* (Hebert *et al.,* 1991), *Fgf8* (Crossley and Martin, 1995), *Foll* (Albano *et al.,* 1994), *Hoxb1* (Wilkinson *et al.,* 1989a), *Hoxb5* (Frohman *et al.,* 1990), *Hoxb9* (Hunt *et al.,* 1991), *Krox20* (Wilkinson *et al.,* 1989b), *Nog* (McMahon *et al.,* 1998), *Nodal* (Conlon *et al.,* 1994), *Otx2* (Ang *et al.,* 1994), *Pax6* (Walter and Gruss, 1991), *Shh* (Echelard *et al.,* 1993), *Six3* (Oliver *et al.,* 1995), *Wnt1* (Wilkinson *et al.,* 1987), and *Wnt3a, Wnt5a,* and *Wnt5b* (Takada *et al.,* 1994).

Explant Culture and Recombination

Explants were prepared from wild-type random outbred, *En-lacZ/+,* or *HNF3β* mutant embryos from prestreak to headfold stages, depending on the experiment. In all cases explants were dissected from embryos with glass needles in PBS, then transferred directly to culture medium (Dulbecco's modified Eagle medium with streptomycin and penicillin, plus 15% fetal calf serum) or first treated with enzymes to separate tissue layers. Explants were transferred to 500 μl of 2.5% pancreatin (Sigma), 0.5% trypsin (Sigma) and incubated 15 min at 4°C. They were then removed to PBS, and tissue layers were manually dissociated with glass needles. Desired fragments were rinsed in culture medium. All explants were cultured in individual drops of medium under mineral oil on plastic petri plates (Fisher) in a 37°C humidified incubator with 5% CO_2. For recombination experiments, explants were placed together in a small depression made in the plastic dish with a blunt needle. Explants which failed to recombine were discarded. Explants were collected after 2 or 3 days (2 days for headfold explants, 3 days for streak-stage explants), washed several times in PBT, and stained for *lacZ* activity as described above.

TABLE 1
Anteroposterior Markers of the Neuraxis Expressed in Wild-Type and *HNF3β* Mutant Embryos

Gene	Conserved expression domain
Six3	FB/extreme ant.
Bf1	FB/extreme ant.
Pax6 (ant.)	FB/extreme ant.
Otx2	FB, MB
Fgf8	MB
En2	MB/HB junction
Wnt1	MB/HB junction
Krox20	HB: rhomb. 3 and 5
Hoxb1 (ant.)	HB: rhomb. 4
Fgf3	HB: rhomb.
Pax6 (post.)	HB (post.)/SC (ant.)
Hoxb5	SC: middle
Hoxb9	SC: post. 2/3
Hoxb1 (post.)	SC: post. end

Note. The left column indicates genes for which antisense probes were hybridized to E8.5–9.5 *HNF3β* mutant embryos. Genes are listed in the order of their expression domain from anterior to posterior. Some of these genes exhibit two A-P domains and thus appear twice. The right column indicates the location of the domain in wild-type embryos and the corresponding location of that domain in mutant embryos. Abbreviations: FB, forebrain; ant., anterior; MB, midbrain; HB, hindbrain; rhomb., rhombomere; SC, spinal cord; post., posterior.

RESULTS

Anteroposterior Patterning of the Neural Tube in *HNF3β* Mutant Embryos

We have examined expression of a large number of A-P restricted genes in *HNF3β* homozygous embryos to understand in detail the nature of the A-P patterning which occurs in their neural tubes. Thirteen genes marking restricted axial domains from the most anterior to the most posterior aspects of the neural tube were compared in wild-type and mutant embryos at 8.5–9.0 dpc (Table 1). All genes were expressed in the mutants and their expression domains occurred at approximately their normal axial position. Precise localization was difficult to determine in many cases because of the absence or distortion of morphological landmarks. For example, the putative transcription factor *Six3* is expressed in the anterior forebrain rudiments (Oliver *et al.,* 1995) and is seen in the anterior extreme of the wild-type embryo (Fig. 1A, left) and in the mutant embryo (Fig. 1A, right). The most posterior marker examined was *Hoxb9* (Hunt *et al.,* 1991). We detected *Hoxb9* expression in very similar domains in wild-type (Fig. 1B, left) and *HNF3β* embryos (Fig. 1B, right). In both wild-type and mutant embryos, a posterior domain of expression of *Hoxb9* was seen in the neural tube and in the somites, with maintenance of the offset anterior boundary of expression in the two tissues.

Although the order of expression of A-P markers along the

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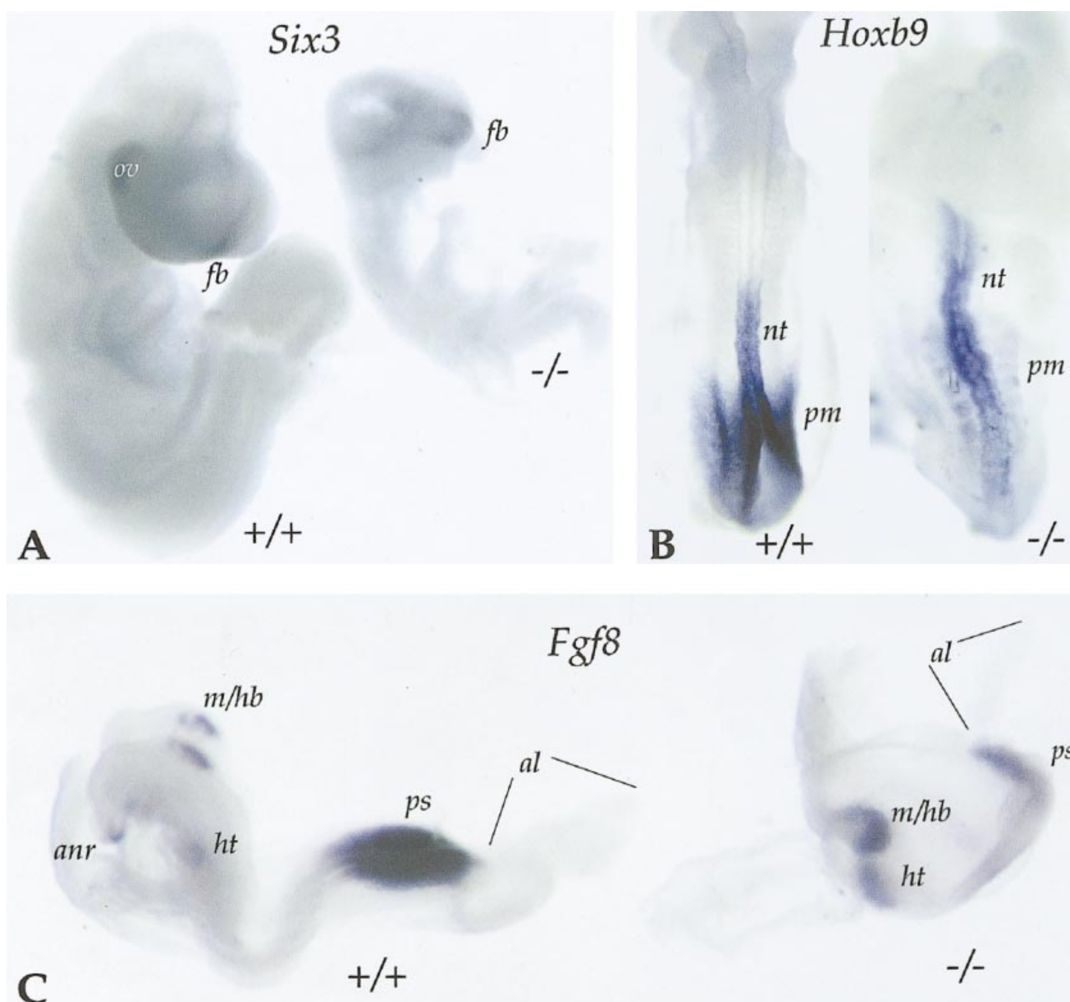

FIG. 1. Anteroposterior patterning is essentially correct in the absence of the node. Whole-mount *in situ* hybridizations in pairs of wild-type (left) and *HNF3β* homozygous (right) sibling embryos. (A) *Six3* gene expression at E9.0. *Six3* expression is restricted to the most anterior portion of the central nervous system in the forebrain (fb) and is also strong laterally in the optic vesicles (ov). In the mutant embryo, *Six3* expression occurs at the anterior extreme in the neural tube, i.e., the forebrain region. Lateral anterior expression is absent, however. Some mutant embryos do not express *Six3.* (B) *Hoxb9* expression at E8.5 (dorsal view). Transcripts occur in the most posterior portion of the neural tube (nt) in both wild-type and mutant embryos. It is also observed in a more posteriorly restricted domain of the paraxial mesoderm (pm). In the mutants, this domain may extend a bit more anteriorly. (C) *Fgf8* expression at E8.0 (anterior to the left). Expression in wildtype is seen at the rostral limit of the neural tube (anterior neural ridge; anr), at the midbrain/hindbrain junction (m/hb), and at the primitive streak (ps), up to the allantois (al). Additional expression occurs in the presumptive heart region (ht). In the mutant embryo at right, all of these domains of expression occur except that of the anterior neural ridge. The embryo is truncated anteriorly, with relatively less tissue anterior to the midbrain/hindbrain junction, accounting for the lack of this domain.

neural tube is correct in *HNF3β* embryos, the degree of normal morphological anterior development varies among mutants. An example of an embryo with a truncation of the anterior neural tube is shown in Fig. 1C, at the right. Relative to wildtype (Fig. 1C, left), the midbrain/hindbrain expression domain of *Fgf8* in the developing brain (Crossley and Martin, 1995) occurs closer to the anterior terminus of the embryo, and the anterior *Fgf8* expression domain, which marks the rostral limit of the neuraxis, is missing. The extent of anterior development in *HNF3β* mutants is typically less than is observed when the extraembryonic endoderm is composed of wild-type cells (Dufort *et al.,* 1998).

Expression of Putative Organizer Signaling Factors in HNF3β Mutant Embryos

A possible basis for the neural induction and A-P pattern we see in *HNF3β* mutants is that organizer gene expression

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TABLE 2
Expression of Genes Encoding Putative Secreted Neural Patterning Factors in Headfold-Stage Wild-Type and *HNF3β* Embryos

Gene	Axial expression	Exp'd in 3β?	Other exp sites	Other exp in 3β?
Shh	Late node, HP	−		
Nog	Node, HP	−	Amnion	+
Chd	Ant. streak, node	−	6.5: ant. streak	+
Foll	Entire streak	+		
Cer1	AVE	+		
nodal	Perinodal	−	6.5: prox. streak	+
BMP7	Ant. streak, node	−	Extraemb.	+
BMP4	Post. streak	+	Extraemb.	+
Fgf5	Epiblast	+		
Ffg3	Entire streak	+	Extraemb.	+
Fgf4	Ant. 3/4 streak	+		
Fgf8	Entire streak	+	Headfolds	+
Wnt3a	Entire streak	+		
Wnt5a	Post. streak	+		
Wnt5b	Post. streak	+		

Note. Genes for which antisense probes were used to detect expression in E7.5 *HNF3β* mutant embryos are indicated in the leftmost column, followed by their wild-type expression domains at E7.5. Presence or absence of detectable expression in mutant embryos is indicated by a plus (+) or minus (−) sign, respectively. Other domains of expression at E7.5 or E6.5 (6.5) are indicated at the right, along with their presence or absence in mutant embryos. Abbreviations: HP, head process; ant. streak, anterior streak; AVE, anterior visceral endoderm; prox. streak, proximal streak; extraemb., extraembryonic tissue; post. streak, posterior streak.

persists in these mutant embryos, even though the morphological structure known as the node is absent. We examined the expression of putative secreted factors normally produced by the node in mutant embryos around 7.5 dpc (Table 2). We compared expression of each probe in three or more mutant embryos to that in wild-type littermates. Consistent with observations made at later stages of development in *HNF3β* mutant embryos (Ang and Rossant, 1994; Weinstein *et al.*, 1994; Dufort *et al.*, 1998), we did not detect any *sonic hedgehog (Shh)* expression in its earlier midline domain (Figs. 2A and 2B) (Echelard *et al.*, 1993; Roelink *et al.*, 1994). Similarly, we did not detect expression in the distal tip of mutant embryos of the node marker *BMP7* (Fig. 2D), which is normally seen in the anterior primitive streak and then the node and head process (Fig. 2C; Lyons *et al.*, 1995; Arkell and Beddington, 1997). However, extraembryonic expression of *BMP7* was observed (Fig. 2D, Table 2) as in wildtype. The *nodal* gene is expressed in a horseshoe-shaped domain just lateral to the node (Zhou *et al.*, 1993; Conlon *et al.*, 1994). This perinodal domain of *nodal* expression was not observed in homozygous embryos (Table 2). The lack of expression of *nodal* in distal portions of *HNF3β* mutant embryos has been confirmed by analysis of a *nodal-lacZ* transgene; however, the earlier domain of *nodal* expression in the nascent primitive streak does occur in *HNF3β* homozygotes (J. Collignon and E. Robertson, personal communication).

Of greatest interest was expression of the secreted factors implicated in neural induction by the organizer in amphibians—particularly *noggin* and *chordin,* which are also expressed in the mouse organizer (McMahon *et al.*, 1998; Tam and Behringer, 1997). We did not detect expression of a murine homolog of *Nog,* either by *in situ* or by visualizing expression of a *lacZ* transgene insertion (McMahon *et al.*, 1998), in the distal portion of mutant embryos (Figs. 2E and 2G). However, a few scattered cells in the most proximal portion of mutant embryos expressed the *lacZ* reporter (Figs. 2F and 2H). These cells seem to represent remnants of the normal amniotic domain of *Nog* expression (Fig. 2G). In addition, expression was observed in ectopic membrane material often attached to the distal end of *HNF3β* mutant embryos (Fig. 2F). Expression of *Nog* is not observed in the embryo proper prior to late-streak stages (R. Stottmann and J.K., unpublished observations). Therefore, *Nog* expression was not examined in earlier mutant embryos.

A murine homolog of *chordin (Chd)* is expressed in the anterior primitive streak and in the node and head process, persisting until late headfold stages (Fig. 2I) (D. Bachiller, J. Klingensmith, J. Rossant, and E. DeRobertis, manuscript in preparation). This expression pattern is very similar to the axial expression of *Nog.* However, *Chd* is expressed earlier than *Nog, Shh, Cobl* (Gasca *et al.*, 1995), and other markers of the node per se. Expression appears by midstreak stages at the anterior tip of the primitive streak in a pattern very similar to *goosecoid (Gsc)* and *HNF3β* (Blum *et al.*, 1992; Sasaki and Hogan, 1993; Monaghan *et al.*, 1993; Ang *et al.*, 1993) (Fig. 2K). No expression of *Chd* was detected anywhere in *HNF3β* mutant embryos analyzed at 7.5 dpc (Fig. 2J), when littermates were at late-streak to headfold stages. Some expression was occasionally detected in the membrane material ectopically attached to the distal tip of the embryo, in the same pattern as shown above for *Nog.* However, at 6.5 dpc, reduced but significant *Chd* transcription was detected in mutant embryos in a position very similar to that of wild-type midstreak littermates (Fig. 2L). This is similar to the pattern of expression of *Gsc* in *HNF3β* mutants, as previously reported (Ang and Rossant, 1994).

In summary, we did not detect expression of node-signaling genes in the distal tips of *HNF3β* mutant embryos. However, *Chd* was transiently expressed in a domain presumably corresponding to the precursor tissue of the node, the anterior primitive streak of midstreak stage embryos. In addition to *Chd* and *Nog,* a third neural-inducing molecule expressed in the *Xenopus* organizer, Follistatin (Hemmati-Brivanlou *et al.*, 1994), is also expressed in mouse gastrulae. However, mouse *follistatin (Foll)* is expressed throughout the primitive streak (Fig. 3E), but is absent from the node (Albano *et al.*, 1994). In *HNF3β* mutants, a domain of *Foll* expression is seen in the posterior proximal portion of the embryo (Fig. 3F).

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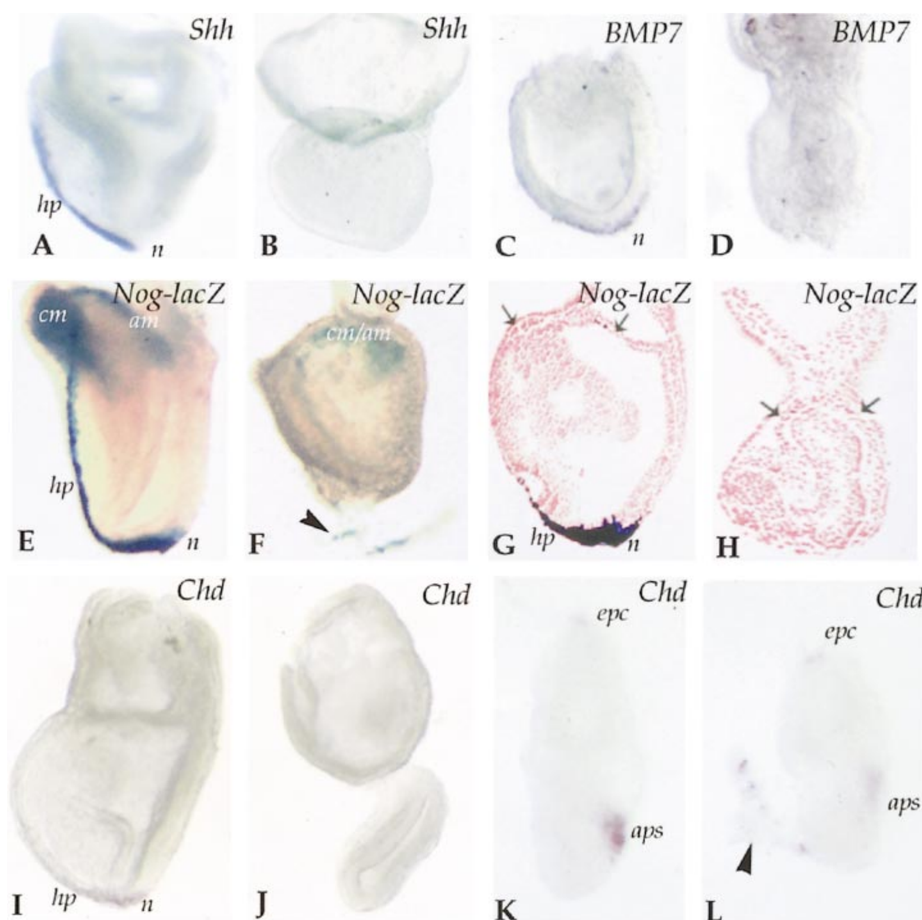

FIG. 2. Expression of genes encoding putative secreted factors of the organizer and axial midline in *HNF3β* mutant embryos. Wild-type and *HNF3β* mutant embryos dissected at E7.5 and subjected to whole-mount *in situ* hybridization are depicted. The embryos in K and L were dissected at E6.5. All embryos are shown in lateral view, with anterior to the left and proximal at top. (A) *Shh* expression in wild-type embryos marks the head process (hp) and extends posteriorly to the node (n). (B) *Shh* expression in an *HNF3β* mutant embryo—no hybridization was detected in mutants. (C) *BMP7* in wildtype occurs in the node and later the head process. (D) *BMP7* expression in mutant embryos was not detected, although some extraembryonic expression was observed. (E) *Noggin-lacZ (Nog-lacZ)* expression in wild-type embryos is detected in the node, head process, cephalic mesenchyme (cm), and amnion (am). (F) *Nog-lacZ* expression in *HNF3β* mutant embryos is detected in a proximal domain just below the extraembryonic junction, which corresponds to the amnion and/or cephalic mesoderm expression (cm/am). Expression is also detected in the mass of ectopic extraembryonic membrane (arrowhead) which is sometimes attached to the distal end of the embryo. (G) Histological section of a wild-type embryo stained for *Nog-lacZ* activity. Expression in the node and head process is very strong. Much weaker expression is seen in scattered cells of the amnion and cephalic mesoderm (arrows). (H) Histological section of *HNF3β* mutant embryo stained for *Nog-lacZ* activity. No axial midline expression of *Nog* is detected, but a few scattered cells in the proximal region of the embryo are stained (arrows). These cells are clearly in the mesodermal layer. (I) *Chordin (Chd)* expression in a wild-type embryo is restricted to the node and the emergent head process. (J) *Chd* expression is not detected anywhere in a *HNF3β* mutant embryo at E7.5. (K) An E6.5 embryo at early- to midstreak stage shows *Chd* expression in the anterior primitive streak (aps) and in the ectoplacental cone (epc) at the limit of the extraembryonic tissue. (L) *Chd* expression in a mutant embryo at E6.5 is observed in the anterior primitive streak, the ectoplacental cone, and the mass of ectopic extraembryonic membrane (arrowhead), which is often ectopically attached to the distal end of the embryo.

Putative Posterior Patterning Signals Persist in HNF3β Mutant Embryos

There is considerable evidence from *Xenopus* that posterior nonorganizer tissues, such as paraxial mesoderm and somites, can also provide important signals to pattern the embryo, particularly in more posterior domains (Bang *et al.*, 1999, and references therein). Three major classes of candidate posteriorizing signals have been proposed: FGFs, Wnts, and retinoic acid (RA) (Doniach, 1995). Mutations in certain *Wnt* and *Fgf* genes expressed in the postnodal primitive streak reveal a role in posterior development in the mouse (Takada *et al.*, 1994; Liu *et al.*, 1999; Sun *et al.*, 1999). We

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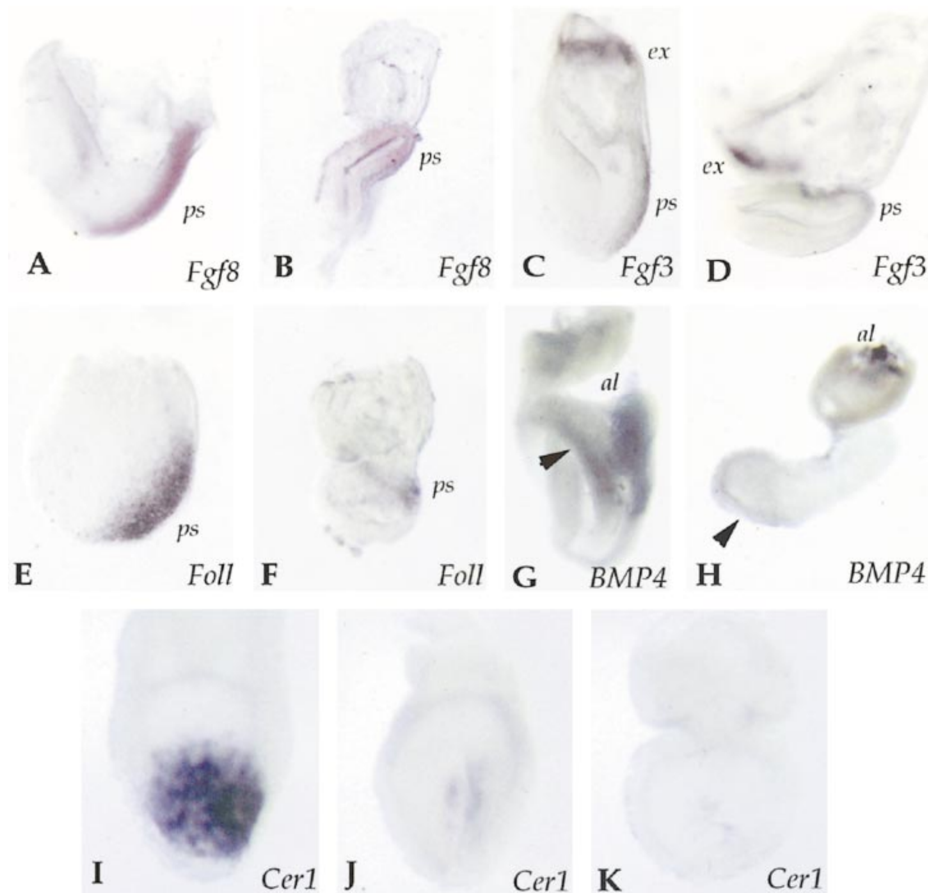

FIG. 3. Expression of genes encoding putative secreted factors of the primitive streak and anterior visceral endoderm in *HNF3β* mutant embryos. Wild-type and *HNF3β* mutant embryos dissected at E7.5 and subjected to whole-mount *in situ* hybridization are depicted. In A–H, views are lateral with anterior to the left and proximal at top. In I–K, the view is anterior, with proximal at top. (A) *Fgf8* expression in a wild-type embryo. Transcripts occur throughout the primitive streak (ps). (B) *Fgf8* expression in a mutant embryo. Expression marks the truncated primitive streak, which fails to elongate distally. (C) *Fgf3* expression in wildtype occurs in a restricted region of proximal extraembryonic tissue (ex) and throughout the primitive streak. (D) In an *HNF3β* mutant embryo, *Fgf3* expression is seen in both the extraembryonic and the primitive streak domain, although both domains are malformed. (E) *Follistatin (Foll)* expression in a wild-type embryo occurs throughout the primitive streak. (F) *Foll* expression in a mutant embryo is present in the truncated, proximally restricted primitive streak. (G) *BMP4* is expressed in wild-type embryos in the posterior primitive streak and up into the allantois (al) and in the amnion. Low-level expression extends from the anterior (arrowhead). (H) *BMP4* expression in a *HNF3β* mutant embryo which is relatively more advanced than the other mutant embryos shown. Expression is seen in the allantois and extending anteriorly along the top of the embryo, presumably reflecting the amnion and anterior domains (arrowhead). (I and J) *Cer1* expression at E7.5 in wild-type embryos varies from broad to narrow anterior domains, in a highly dynamic manner dependent on the precise developmental stage of the embryo. (K) In *HNF3β* mutant embryos at E7.5, *Cer1* expression is usually detected in a small, diffuse anterior domain. Because of the impossibility of staging the mutant embryos precisely, it is not possible to know which wild-type pattern is the more relevant comparison.

investigated whether such potential posteriorizing signals were still present in *HNF3β* mutant embryos.

We examined expression of four FGFs reported to be expressed in gastrulating embryos. Each persisted in *HNF3β* mutants. *Fgf8* is expressed throughout the primitive streak (Crossley and Martin, 1995), as shown in Fig. 3A. In *HNF3β* embryos, a relatively diffuse domain of *Fgf8* expression occurred immediately adjacent to the extraembryonic tissue (Fig. 3B). Expression also marks the precardiac mesoderm of wild-type headfold stage embryos (arrowhead in Fig. 3A). This domain was not clearly identifiable in homozygous littermates. *Fgf3* is also expressed throughout the primitive streak of wild-type gastrulating embryos (Fig. 3C), as well as in a domain of extraembryonic tissue (Wilkinson *et al.,* 1988). In *HNF3β* mutants, we detected a patch of expression of *Fgf3* in the extraembryonic membranes (Fig. 3D). We also saw embryonic expression immediately adjacent to the embryonic/extraembryonic junction, at one side

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of the narrow constriction formed at the junction (Fig. 3D). The embryonic expression pattern seen with *Fgf3* probes was very similar to that observed for *Fgf4* (Table 2), which is expressed in all but the most posterior extreme of the primitive streak (Niswander and Martin, 1992). *Fgf5* is expressed throughout the epiblast of wild-type embryos at 6.5 dpc, but is then downregulated (Hebert *et al.,* 1991). We detected *Fgf5* transcripts throughout the epiblast of *HNF3β* mutants as well (Table 2; data not shown).

Three *Wnt* genes are also expressed throughout the primitive streak. Expression of *Wnt3a* is at fairly high levels throughout the streak and is required for normal development of streak derivatives (Takada *et al.,* 1994). Expression in mutant embryos was essentially identical to that shown for *Fgf8;* i.e., a domain of hybridization signal adjacent to the embryonic/extraembryonic junction (Table 2). We also detected similar expression of *Wnt5a* and *5b* in such a proximal domain, consistent with their expression throughout the streak of wild-type embryos (Takada *et al.,* 1994) (Table 2). In short, all the *Wnts* and all the *FGFs* known to be expressed in the primitive streak of wild-type embryos are also expressed in the truncated, proximally restricted streak of *HNF3β* mutants.

We examined the nature of RA signaling in the absence of the node. We introduced into *HNF3β* heterozygotes a transgenic reporter for RA signaling, which carries a retinoic acid response element (RARE) fused to the *lacZ* gene (Rossant *et al.,* 1991). This reporter begins to be expressed shortly after node formation, showing a very sharp A-P boundary (Fig. 4A). In 7.5-dpc *HNF3β* homozygotes the same pattern of expression is seen (Fig. 4B). At 8.5 dpc, wild-type embryos continue to show a sharp A-P boundary of *lacZ* expression, occurring at the level of the hindbrain (Fig. 4C). In addition, a bilateral anterior domain of expression marks the early optic vesicles (Rossant *et al.,* 1991). Homozygous littermates show a sharp expression boundary at the same relative A-P level, despite the much smaller size (Fig. 4D). Two small clusters of bilaterally symmetric expression occur anteriorly, most likely representing the location of the deficient optic vesicles (arrows). These results indicate that lack of the node has no discernable effect on the generation of a posterior domain of RA signaling activity, as measured by *RARE-lacZ* expression.

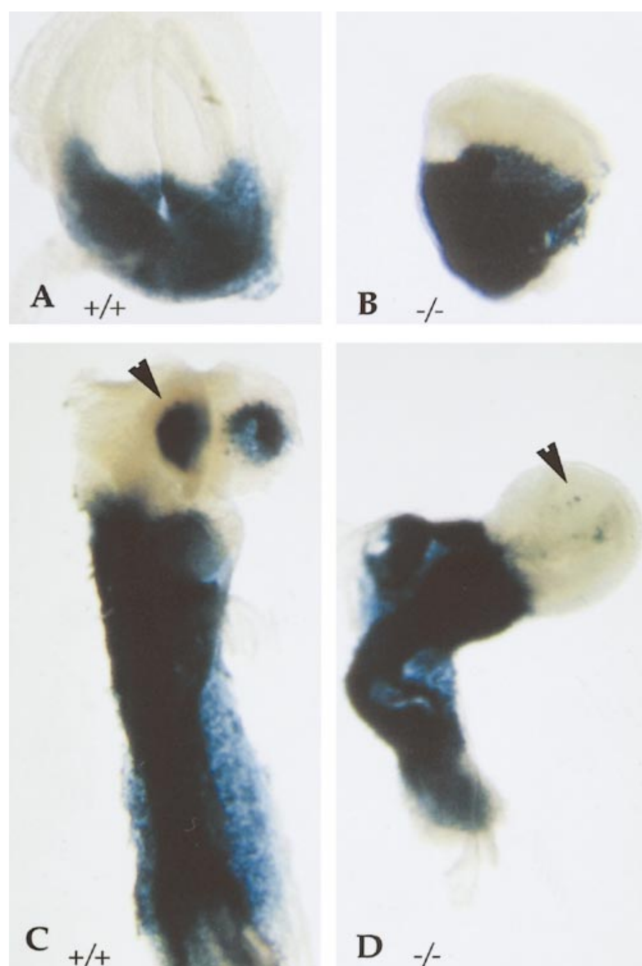

FIG. 4. Expression of a retinoic acid response marker shows normal spatiotemporal pattern in the absence of the node. Wild-type and *HNF3β* mutant littermates heterozygous for a *RARE-lacZ* transgene were stained for *lacZ* activity. A and B are anterior views, proximal at top. C and D are ventral–lateral views, with anterior at top. (A) Wild-type embryo at E7.5. Expression of the transgene shows a very sharp A-P boundary anterior to the node. Expression is uniformly strong posterior to the boundary and completely lacking to the anterior. (B) Homozygote at E7.5. Expression of the transgene shows the same sharp A-P boundary and posterior intensity of expression as in wildtype. (C) Wild-type embryo at E8.5. The trunk of the embryo shows strong expression from just caudal to the preotic sulcus to the open posterior neuropore. In the head, expression is limited to the optic eminence on either side of the embryo (arrowhead). (D) Homozygous embryo at E8.5. Expression shows very similar restrictions at both the anterior and the posterior limits of trunk expression and occurs at similar intensity to wildtype. A few cells in two bilateral domains in the anterior of the embryo also express *lacZ* (arrowhead). These may correspond to the expression in the optic eminence of wild-type embryos.

Anterior Visceral Endoderm Persists in HNF3β Homozygous Embryos

Chimera analysis has revealed that extraembryonic functions of *HNF3β* are responsible for some of the roles this gene plays in gastrulation and may enhance anterior development (Dufort *et al.,* 1998). Of particular interest is the anterior visceral endoderm, which has been implicated as a source of signals promoting anterior fate in the underlying ectoderm (Thomas and Beddington, 1997). A mouse homolog (*Cer1*) of a secreted frog protein which can induce ectopic heads, Cerberus (Bowmeester *et al.,* 1996), is expressed in the AVE of gastrulating mouse embryos (reviewed by Beddington and Robertson, 1998). The wild-type expression pattern of *Cer1* is highly dynamic during gastrulation, with an early domain of expression in the AVE (Fig.

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3I), followed by expression in the forming definitive endoderm (Fig. 3J), then fading out completely until somitogenesis. Three of five 7.5-dpc mutant embryos hybridized with a *Cer1* probe, showed expression—in each case along the anterior midline in the AVE, in small patches (Fig. 3K). This expression indicates that the AVE is present in *HNF3β* mutants, although the definitive anterior endoderm does not develop (Ang and Rossant, 1994). Moreover, a putative secreted patterning factor produced by the AVE is expressed in its proper location in at least some *HNF3β* homozygotes.

The Early Node and Node Precursors Can Induce Engrailed Expression in Naïve Ectoderm

We used the induction of an *Engrailed2-lacZ (En-lacZ)* transgene (Logan *et al.,* 1993) in cultured early-streak anterior ectoderm explants (Ang and Rossant, 1993) as an assay for neural induction and patterning by the mammalian organizer and other portions of the primitive streak at various stages of gastrulation (Figs. 5 and 6; Table 3). As reported previously (Ang and Rossant, 1993), early-streak ectoderm alone failed to express *En* (Fig. 5), but, by late streak, expression was observed in isolated anterior ectoderm, suggesting that induction and specification of *En* expression had occurred by that time (Table 3). In addition, we cultured early-streak and midstreak ectoderm along with associated AVE (a putative anterior-inducing tissue) (Beddington and Robertson, 1998) and observed no induction of *En* expression, indicating that the AVE alone is not sufficient for *En* induction (Fig. 5; Table 3). As a positive control for induction, pre- to early-streak (PES) anterior ectoderm was recombined with headfold stage total anterior mesendoderm, previously shown to induce *En* in this system (Ang and Rossant, 1993). All such recombinants expressed *lacZ* after 3 days (Table 3).

The ability of node and node precursors to induce *En* expression was then tested, using recombinants with early-streak anterior ectoderm alone or anterior ectoderm with attached AVE. No differences in results were seen between ectoderm alone or ectoderm plus AVE, showing that the AVE does not influence the ability of the node to induce En. Results from the two types of experiments were thus pooled. We find that the early node is a potent source of *En*-inducing signals (Table 3; Fig. 6J). Tissue used as the node explant was isolated from the distal tip of late-streak to early-bud stage embryos (Downs and Davies, 1993), as shown in Fig. 6B. Collectively, 92% of recombinants expressed *En.* Many of the genes which are expressed in the early node are also expressed in the progenitor of the node, the anterior end of the elongating primitive streak at midstreak stages (reviewed by Tam and Behringer, 1997). We tested the ability of explants from the anterior streak at midstreak stages to induce *En* expression (Fig. 6A). Three of four recombinants expressed *En* after 3 days of culture (Table 3; Fig. 6I). In contrast, early/midheadfold nodes, which are morphologically defined (Fig. 6C), did not induce *En* expression (Table 3; Fig. 6K). We also tested the inductive ability of posterior primitive streak, which occurs at the opposite end of the mesodermal-forming region from the node. Late-streak posterior primitive streak explants, which included approximately the fifth of the streak just distal to the embryonic/extraembryonic junction (Fig. 6B), exhibited little *En*-inducing activity (1/14). However, both morphologically defined nodes and posterior primitive streak explants expressed node or streak markers, respectively, after 48 h culture (Figs. 6G and 6H), indicating that they retain at least some of their molecular characteristics.

In summary, we find that the anterior streak and early node can induce *En* expression in the presence or absence of the AVE, whereas posterior streak cannot. However, the definitive node, visible at headfold stages, had little if any ability to induce *En,* although it might induce more posterior markers not tested here.

En-Inducing Activity Appears to Be Diffusely Localized in HNF3β Mutants

Because the definitive node, its derivatives, and its constellation of secreted factors are absent in *HNF3β* mutant embryos, we sought to determine what alternative sources of neural-inducing and patterning signals might exist in these mutant embryos. One possibility seemed to be that some as yet unidentified neural inducer might be expressed in the distal tip of gastrulating *HNF3β* embryos, despite the node's absence. A second scenario consistent with our data is that neural-inducing signals emanate from the truncated primitive streak domain, which remains proximal in *HNF3β* mutants. We tested the ability of distal and proximal mutant explants to induce *En* expression in PES anterior ectoderm explants (Fig. 6D, Table 3). None of the recombinants exhibited robust expression of *En,* but some activity was detected in recombinants containing both distal and proximal portions of early day 7 *HNF3β* mutant embryos (Fig. 6L). Neither distal nor proximal explants from older mutant embryos induced *En* (Table 3). Diffuse, low-level *En*-inducing activity thus persists in *HNF3β* mutants as opposed to the highly focused, strong activity of the early wild-type node.

DISCUSSION

The importance of the Spemann organizer as a source of signals for induction and patterning of the major body axes in vertebrate embryos has been demonstrated by ectopic grafting experiments in many different species (Lemaire and Kodjabachian, 1996). In all such experiments, the organizer equivalent can induce new axial structures, including neural tissue, from the host embryo. However, the results of the converse experiment of organizer removal have not been so clear-cut. Organizer ablation via microdissection in chick (Psychoyos and Stern, 1996, and references therein), fish (Shih and Fraser, 1996), and mice (Davidson *et al.,* 1999) have all shown that formation of the neural tube and the

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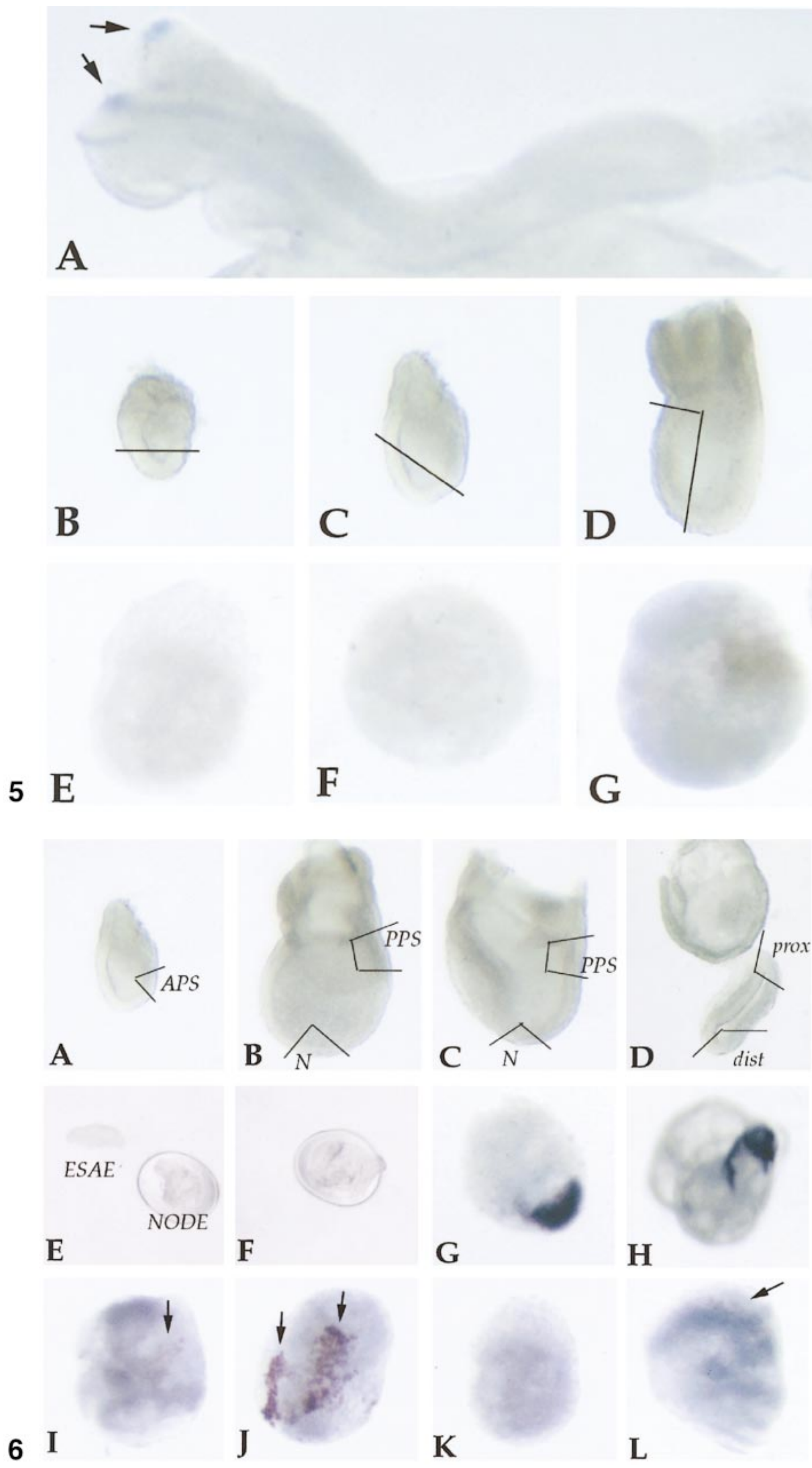

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anterior–posterior body axis can still occur after organizer removal. However, in the chick, at least, the node (the organizer equivalent) can regenerate even after very large pieces of tissue are removed (Psychoyos and Stern, 1996) and, in all experiments, it is difficult to be sure that all organizer material has been removed from the embryo. The genetic ablation of the node, which occurs in *HNF3β* embryos (Ang and Rossant, 1994; Weinstein *et al.,* 1994), provides a unique opportunity to examine the effect of organizer ablation in any vertebrate embryo because organizer formation appears to be blocked well before the appearance of a morphological node. We found no evidence for a node-like constellation of gene expression after the early- to midstreak stage. Since *HNF3β* is absolutely required for definitive node development, there is no possibility of residual or regenerating node material in these embryos.

Here we show that the loss of the node and its derivatives does not lead to loss of the ability to form and pattern the neural tube along the A-P axis. Using a much more extensive series of markers than examined before, we have shown that regionally restricted markers of the nervous system are expressed in the same order and with very similar boundaries of expression in *HNF3β* mutant and wild-type embryos. The most anterior markers examined, such as *Six3* and *Fgf8,* showed more variability in extent of expression, and anterior truncation of expression domains was apparent in a number of embryos. However, in all cases, some expression was observed, indicating that some degree of anterior patterning had occurred. Because *HNF3β* mutant embryos die around E9.5, it is not possible to say how well the regionalization of the neural tube would be translated into the morphological structures of the brain. However, it is unlikely that development would be normal, because the embryos show severe defects in D-V patterning of the neural tube in the absence of the notochord (Ang and Rossant, 1994; Weinstein *et al.,* 1994). This leads to loss of ventral structures in the brain and spinal cord and would impact on the further morphogenesis of the nervous system throughout. Thus, the loss of the node and its derivatives impacts on later development of the nervous system, but does not seem to adversely affect its initial induction and A-P patterning.

We have asked how these results can be reconciled with current models for the role of organizer signals in neural patterning. There is some consensus that the signals for head induction and trunk/tail induction are different and, in the mouse at least, appear to be physically located in different structures (reviewed by Beddington and Robertson, 1998). Experiments in *Xenopus* have suggested that anterior head induction requires the activity of factors that can block both Wnt and BMP-type activity (Glinka *et al.,* 1997; Piccolo *et al.,* 1999), while trunk organizer activity involves BMP antagonists only (Harland and Gerhardt, 1997). The anterior activity has been associated with the expression of Cerberus, a potent BMP and Wnt antagonist, and Dickkopf, a Wnt

FIG. 5. Specification of *Engrailed2* expression occurs at the mid- to late-streak stage and is not modified by anterior visceral endoderm. A–D show lateral views of embryos with anterior to the left. (A) *En2-lacZ* expression at its earliest appearance, at around the four-somite stage. Expression is found in only two dorsal, bilateral regions at the midbrain/hindbrain junction (arrows). No expression is detected anywhere earlier. (B–D) Embryos were dissected from prestreak to late-streak stages, and putative anterior neural ectoderm was explanted and cultured for 2–3 days. The black lines indicate the position of the cuts made to excise the ectoderm and overlying visceral endoderm. (B) Pre/early-streak embryo, from which the distal tip of the epiblast was explanted. (C) Midstreak embryo, with explant isolated by cutting diagonally from anterior to the streak to more proximal ectoderm. (D) Late-streak embryo; explant taken as the tissue anterior to the distal tip but posterior to the extraembryonic tissue. Mesendoderm was removed from the ectoderm prior to culture. (E) Early-streak anterior ectoderm with adherent visceral endoderm cultured for 3 days and stained for *lacZ* activity. No expression is detected. (F) Midstreak anterior ectoderm with adherent AVE cultured for 2 days and stained for *lacZ* activity. No expression is detected. (G) Late-streak anterior ectoderm and adherent AVE cultured for 2 days and stained. A large sector of cells expresses *lacZ* (red).

FIG. 6. The early node and its precursor are potent inducers of *Engrailed* expression in naïve ectoderm, but other axial regions of wild-type and mutant embryos are not. (A–D) The regions of embryos within the black lines were excised and recombined with early-streak anterior ectoderm from *En-lacZ/+* embryos to test their inductive ability. Anterior is to the left and proximal at top of each. (A) Anterior primitive streak (APS) from midstreak stage embryos was excised as indicated. (B) The early node (N) and the posterior primitive streak (PPS) were excised from late-streak to early allantoic-bud stage embryos. (C) The mature (morphological) node and posterior streak were also excised from early- to midheadfold stage embryos as shown. (D) The distal tip (dist) and posterior proximal (prox) portions of *HNF3β* embryos dissected at E7.5, at a time when wild-type sibling embryos varied in stage from early allantoic bud to early headfold stage. (E) An early streak anterior ectoderm explant (ESAE) is shown ready for recombination with a node explant. (F) The explants are recombined in a well pushed into a plastic petri dish and become adherent to each other, soon forming an integrated recombinant. (G) Node explant from a *C101/+* early-headfold stage embryo cultured for 2 days and stained for *lacZ* activity. Expression of this node marker was maintained in culture. (H) A posterior primitive streak explant from a wild-type embryo cultured for 2 days and hybridized with an antisense probe for *brachyury.* The explant continued to express this marker after culture. (I) Explant recombinant derived from ESAE and anterior primitive streak from a midstreak stage embryo. *En* expression occurred in a few patches, each composed of several cells (arrow). (J) Explant recombinant of ESAE and early allantoic-bud stage node. Three large patches of intense staining were detected (arrows). (K) Explant recombinant of ESAE and midheadfold node. No *En* expression was detected. (L) Explant recombinant of ESAE and *HNF3β* mutant distal tip. A small scattering of cells expressed *En* in this recombinant. Half of such recombinants showed similar very weak expression, the others none. Similar results occurred with posterior proximal recombinants.

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TABLE 3
Specification and Induction of *Engrailed2-lacZ* Expression in Anterior Ectoderm Explants and Explant Recombinants

Explants	*En-lacZ* expression
ES ant. ecto.	0/5
ES ant. ecto. + ant. visceral endo.	1/26
MS ant. ecto. + ant. visceral endo.	0/5
LS ant. ecto.	4/4
ES ant. ecto. + headfold ant. mesendo.	4/4
ES ant. ecto. + midstreak ant. streak	3/4
ES ant. ecto. + late-streak node	11/12
ES ant. ecto. + headfold node	0/7
ES ant. ecto. + late-streak post. streak	1/14
ES ant. ecto. + *3β* late-streak distal tip	2/5
ES ant. ecto. + *3β* late-streak proximal streak	2/5
ES ant. ecto. + *3β* headfold distal tip	0/4
ES ant. ecto. + *3β* headfold proximal streak	0/3

Note. The left column indicates anterior ectoderm (ant. ecto.) explants from *En-lacZ*/+ embryos cultured alone or recombined with other tissues explanted from embryos not bearing *lacZ.* The right column reports the number of cultured specimens positive for *lacZ* expression after 3 days, of the total number assayed. In the case of recombinants, only those cases in which cocultured explants had physically aggregated after 24 h were cultured further. Unless derived from *HNF3β* homozygous embryos (*3β*), nonectoderm explants were isolated from wild-type embryos of the stages indicated. ES, early-primitive-streak stage; MS, mid-primitive-streak stage; LS, late-primitive-streak stage.

antagonist, while Noggin and Chordin, BMP antagonists, are thought to make up the trunk organizer activity. In the mouse, there is recent evidence that the AVE may be important for head development (Beddington and Robertson, 1998), and it expresses *Cerl,* a Cerberus-related gene, as well as *mDkk1,* a Dickkopf family member (Glinka *et al.,* 1998; unpublished observations). Noggin and Chordin, on the other hand, are expressed in the node and derivatives, consistent with the capacity of the node to induce trunk structures, but not anterior markers in ectopic grafts (Beddington, 1994; Tam *et al.,* 1997). We addressed the head organizer and trunk organizer activity of *HNF3β* mutant embryos in the absence of a definitive node by examining the expression of the putative signals associated with both activities. *Cerl* was expressed in its correct anterior domain in mutant embryos, but expression was weak and poorly maintained, consistent with the variable extent of anterior development in the mutants. *HNF3β* is expressed throughout the visceral endoderm and is required for its correct function. When *HNF3β* mutant embryos were provided with wild-type primitive endoderm in tetraploid aggregates, normal streak morphogenesis was restored and more normal anterior development also occurred (Dufort *et al.,* 1998). Thus, any AVE activity remaining in *HNF3β* mutant embryos seems unlikely to be sufficient to explain the development of a complete A-P axis. Indeed, we showed by explant experiments that the AVE alone is not sufficient to induce and pattern *En* expression in anterior ectoderm in wild-type embryos, suggesting that the AVE cannot act alone to pattern anything more than perhaps the most anterior regions of the CNS.

We examined carefully the expression of the node-associated BMP antagonists, *Nog* and *Chd,* and found that the only areas of expression of these markers that persisted in mutant embryos were extraembryonic. Using a *Nog-lacZ* knock-in allele as a sensitive assay for *Nog* expression (McMahon *et al.,* 1998), we were unable to detect any expression in the embryo itself from E7.0 on. *Chd* is normally expressed earlier than *Nog* in the putative organizer derivatives at the anterior of the streak and, although we could not detect *Chd* expression at 7 days of development, we did observe transient expression associated with the very early streak. Thus, if the activity of these molecules is needed for neural induction and A-P patterning, the requirement can only be transient. Prolonged *noggin/chordin* expression is not needed for axis development. However, later expression of these genes may be involved in full maturation of neural patterning, as suggested by the anterior patterning defects seen in *noggin;chordin* double mutants (Bachiller *et al.,* submitted for publication).

Thus both AVE and node-associated signals are only transiently expressed and are poorly localized in *HNF3β* mutant embryos, suggesting that one of the roles of the node is indeed as an organizer—organizing and localizing the different sources of signals in the developing embryo. Our explant–recombination experiments with mutant and wild-type streak tissue support this. The mature node or its precursor, the anterior of the elongating primitive streak, can induce *En* expression when combined with naïve ectoderm, whereas more posterior primitive streak tissue does not. In contrast, both anterior and posterior streak tissue from *HNF3β* mutant embryos has *En*-inducing capacity, albeit weak. Although the organized node fails to form in *HNF3β* mutants, it appears that there is early disperse-inducing activity. This activity, in combination, perhaps, with weak AVE signals, is sufficient to initiate neural axis development in *HNF3β* mutant embryos. Once axis development begins, then other sources of signals for A-P patterning become established and can presumably explain the continued development of more posterior markers in *HNF3β* mutant embryos. Members of the FGF and Wnt family as well as RA have been proposed to be important for posterior development (Doniach, 1995), and examination of these factors in *HNF3β* mutants revealed that all were still expressed. The activity of these factors is associated with primitive streak and paraxial mesoderm, rather than the node itself, and so is not ablated in the mutants. However, the domains of expression of these factors are often reduced, suggesting again that the node helps to organize the signal-

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ing sources of the embryo, possibly by promoting the convergence–extension needed to elongate the vertebrate body axis (Keller *et al.,* 1992) and align the different tissues of the developing axis.

In sum, our results show that neural induction and A-P patterning can be initiated in mammals without the presence of the mature node, as a morphological entity, and without prolonged expression of the constellation of signaling molecules associated with classic organizer function. This suggests that the events that initiate neural induction occur much earlier in development and may be associated more with the Niewkoop-type activity that initiates the axial asymmetry of the embryo than with the Spemann organizer activity per se. However, currently in mammals there are very few data on how axis development is first initiated or even which tissues are involved. Further examination of very early stages of development in *HNF3β* mutants may help reveal candidate pathways that are still intact in these embryos. It is clear that the early events of axis induction occur in *HNF3β* mutants and are sufficient to induce transient expression of anterior signals like *Cerl* and node signals like Chordin. In the presence of other posterior streak-associated signals like FGFs, Wnts, and RA, the embryonic axis can extend and show reasonable A-P patterning. The role of the node and its derivatives, therefore, is probably to help maintain and refine the pattern initiated earlier in development.

The axial mesoderm arising from the node is absolutely critical for some aspects of axial patterning as shown by the severe dorsal–ventral patterning problems in the neural tube and somites (Ang and Rossant, 1994; Weinstein *et al.,* 1994) and the defective left–right patterning (Dufort *et al.,* 1998) in *HNF3β* embryos. These defects are exactly as predicted from experimental manipulations in other species (Dodd *et al.,* 1999; Ramsdell and Yost, 1998). It is only the weak effects on neural induction and A-P patterning that are unexpected in these mutants. It is not yet clear whether the reduced role for the organizer in neural induction and patterning proposed here is true across vertebrates or reflects differences between amniotes and lower vertebrates. Interestingly, it has recently been proposed in chick that BMP antagonism is not sufficient for neural induction but may play more of a role in defining the limits of the neural plate induced by other means (Streit *et al.,* 1998). It is possible that Noggin and Chordin expression in the node has been conserved across evolution but has lost its original role in neural induction and gained a role in stabilization or refinement of neural pattern. Ectopic grafts of organizers across evolution would still induce axial structures in all cases by their ability to block BMP activity, but the endogenous roles of the organizer may have been modified. Further analysis of organizer function across evolution is needed to determine the origin of neural patterning signals in different species.

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