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 anterior-posterior (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-
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 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 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 Nogβ, 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).
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 digoxigenin-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 (Bachillier et al., submitted for publication), En2 (Davis et al., 1998), Fgf3 (Wilkinson et al., 1988), Fgf4 (Niswander and Martin, 1992), Fgf5 (Hebert et al., 1991), Fgf8 (Crosley 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 (McA hon 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).

**Table 1**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Conserved expression domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Six3</td>
<td>FB/extreme ant.</td>
</tr>
<tr>
<td>Bf1</td>
<td>FB/extreme ant.</td>
</tr>
<tr>
<td>Pax6 (ant.)</td>
<td>FB/extreme ant.</td>
</tr>
<tr>
<td>Otx2</td>
<td>FB, M B</td>
</tr>
<tr>
<td>Fgf8</td>
<td>MB</td>
</tr>
<tr>
<td>En2</td>
<td>M/B/HB junction</td>
</tr>
<tr>
<td>Wnt1</td>
<td>M/B/HB junction</td>
</tr>
<tr>
<td>Krox20</td>
<td>HB; rhomb. 3 and 5</td>
</tr>
<tr>
<td>Hoxb1 (ant.)</td>
<td>HB; rhomb. 4</td>
</tr>
<tr>
<td>Fgf3</td>
<td>HB; rhomb.</td>
</tr>
<tr>
<td>Pax6 (post.)</td>
<td>HB (post.)/SC (ant.)</td>
</tr>
<tr>
<td>Hoxb5</td>
<td>SC: middle</td>
</tr>
<tr>
<td>Hoxb9</td>
<td>SC: post. 2/3</td>
</tr>
<tr>
<td>Hoxb1 (post.)</td>
<td>SC: post. end</td>
</tr>
</tbody>
</table>

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
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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**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.
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 mid-streak 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 (Hemmati-Brivanlou et al., 1994), is also expressed in Xenopus embryos. In addition to the domain of Chd expression, Follistatin (Foll) is expressed throughout the primitive streak (Fig. 3E), in the same pattern as shown above for Nog. Shh, Cobi (Gasca et al., 1995), and other markers of the node per se. Expression appears by midstreak 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, Cobi (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 littersmates 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 mid-streak littersmates (Fig. 2L). This is similar to the pattern of expression of Gsc in HNF3β mutants, as previously reported (Ang and Rossant, 1994).

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 (McMahan 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 (McMahan 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.

**TABLE 2**

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Axial expression</th>
<th>Exp'd in 3p?</th>
<th>Other exp sites</th>
<th>Other exp in 3p?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shh</td>
<td>Late node, HP</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nog</td>
<td>Node, HP</td>
<td>–</td>
<td>Amnion</td>
<td>+</td>
</tr>
<tr>
<td>Chd</td>
<td>Ant. streak, node</td>
<td>–</td>
<td>6.5: ant. streak</td>
<td>+</td>
</tr>
<tr>
<td>Foll</td>
<td>Entire streak</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cer1</td>
<td>AVE</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nodal</td>
<td>Perinodal</td>
<td>–</td>
<td>6.5: prox. streak</td>
<td>+</td>
</tr>
<tr>
<td>BMP7</td>
<td>Ant. streak, node</td>
<td>–</td>
<td>Extraemb.</td>
<td>+</td>
</tr>
<tr>
<td>BMP4</td>
<td>Post. streak</td>
<td>+</td>
<td>Extraemb.</td>
<td>+</td>
</tr>
<tr>
<td>Fgf5</td>
<td>Epiblast</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fgf3</td>
<td>Entire streak</td>
<td>+</td>
<td>Extraemb.</td>
<td>+</td>
</tr>
<tr>
<td>Fgf4</td>
<td>Ant. 3/4 streak</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fgf8</td>
<td>Entire streak</td>
<td>+</td>
<td>Headfolds</td>
<td></td>
</tr>
<tr>
<td>Wnt3a</td>
<td>Entire streak</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wnt5a</td>
<td>Post. streak</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wnt5b</td>
<td>Post. streak</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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; extraembryonic tissue; post. streak, posterior streak.
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
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. Fg3 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
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.

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 (re- viewed 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. 4). In the posterior, expression is highly dynamic during 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 (viewed 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.
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 Cε1 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 reorganized 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 Naive 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 mid-streak 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. 6). 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 mid-streak stages (reviewed by Tam and Behringer, 1997). We tested the ability of explants from the anterior streak at mid-streak 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/mid-headfold nodes, which are morphologically defined (Fig. 6C), did not induce En expression (Table 3; Fig. 6K). We also tested the induc-
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 Fgfb, 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 (Glinskia 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

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**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 (ESA) 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/+–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 ESA 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 ESA and early allantoic-bud stage node. Three large patches of intense staining were detected (arrows). (K) Explant recombinant of ESA and midheadfold node. No En expression was detected. (L) Explant recombinant of ESA 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.
TABLE 3
Specification and Induction of Engrailed2-lacZ Expression in Anterior Ectoderm Explants and Explant Recombinants

<table>
<thead>
<tr>
<th>Explants</th>
<th>En-lacZ expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES ant. ecto.</td>
<td>0/5</td>
</tr>
<tr>
<td>ES ant. ecto. + ant. visceral endo.</td>
<td>1/26</td>
</tr>
<tr>
<td>MS ant. ecto. + ant. visceral endo.</td>
<td>0/5</td>
</tr>
<tr>
<td>LS ant. ecto.</td>
<td>4/4</td>
</tr>
<tr>
<td>ES ant. ecto. + headfold ant. mesendo.</td>
<td>4/4</td>
</tr>
<tr>
<td>ES ant. ecto. + mid streak ant. streak</td>
<td>3/4</td>
</tr>
<tr>
<td>ES ant. ecto. + late-streak node</td>
<td>11/12</td>
</tr>
<tr>
<td>ES ant. ecto. + headfold node</td>
<td>0/7</td>
</tr>
<tr>
<td>ES ant. ecto. + late-streak post. streak</td>
<td>1/14</td>
</tr>
<tr>
<td>ES ant. ecto. + 3β late-streak distal tip</td>
<td>2/5</td>
</tr>
<tr>
<td>ES ant. ecto. + 3β late-streak proximal streak</td>
<td>2/5</td>
</tr>
<tr>
<td>ES ant. ecto. + 3β headfold distal tip</td>
<td>0/4</td>
</tr>
<tr>
<td>ES ant. ecto. + 3β headfold proximal streak</td>
<td>0/3</td>
</tr>
</tbody>
</table>

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 may 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 naive 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 dispersal-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-
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 Nieuwkoop-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 Wnts, Wnt, 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 (Duftort 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 due to the presence of amniotes or lower vertebrates. It reflects differences between amniotes and lower vertebrates. It 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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