The eed mutation disrupts anterior mesoderm production in mice

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SUMMARY

Mouse embryos homozygous for the mutation embryonic ectoderm development (eed) exhibit a growth defect and fail to gastrulate normally. While extraembryonic mesoderm is produced extensively, very little embryonic mesoderm is detected in eed mutant embryos, and there is no subsequent organization of mesoderm into node, notochord, or somites. The phenotype is consistent with a defect in the distal primitive streak. Here we report additional phenotypic analyses that include mRNA in situ hybridization of genes whose expression reflects the function of different regions of the primitive streak and their derivatives. These studies have confirmed that mesoderm derived from the proximal primitive streak is specified appropriately. Despite the absence of a morphologically distinct node, sparse axial mesoderm cells in eed mutant embryos are specified, as reflected by expression of Brachyury (T), Sonic hedgehog, and Tcf3b/HNF-3β, and definitive endoderm is produced. Specification of these cell types is also independent of correct expression of nodal, Fgf4, and gsc. Finally, T and Evx1 display ectopic expression in cells not normally fated to ingress through the primitive streak. The data presented are discussed in terms of mechanisms for establishment of the eed phenotype, and are consistent with the eed gene product playing an early role in primitive streak formation and/or organization.

Key words: mammalian embryogenesis, mouse mutants, gastrulation, mesoderm induction, primitive streak, germ cell lineage, node, axial mesoderm, eed, Brachyury (T), Evx1

INTRODUCTION

Gastrulation is a process through which the definitive embryonic germ layers – the ectoderm, mesoderm, and endoderm – are formed, resulting in establishment of the basic body plan. In mouse, gastrulation occurs from approximately embryonic day (E) 6.5-7.5, as cells of the primitive ectoderm (epiblast) delaminate on the posterior side of the embryo and ingress through the primitive streak. The fate of epiblast cells of pre- and early-streak mouse embryos has been determined via clonal analysis of epiblast cells in situ (Lawson et al., 1991). This and previous work (Snow, 1977) demonstrate that increased epiblast cell proliferation likely directs growth towards the primitive streak, as well as epiblast cell ingestion. As the primitive streak extends to the distal tip of the embryo, mesodermal cells migrate anteriorly around the surface of the epiblast, contributing to head and trunk mesoderm; mesoderm migrating into the extraembryonic region of the embryo drives the formation of the amnion, chorion, and allantois.

While mouse gastrulation is morphologically well defined, its molecular control is poorly understood. Numerous homologues of genes implicated in Xenopus laevis mesoderm induction, organization, or specification demonstrate specific expression patterns in the mouse gastrula (reviewed by Beddington and Smith, 1993; Faust and Magnuson, 1993). These include, for example, members of the fibroblast growth factor (Fgf) gene family and their receptors (Hébert et al., 1991; Orr-Urtreger et al., 1991; Niswander and Martin, 1992; Yamaguchi et al., 1992); nodal, a member of the transforming growth factor (TGF)-β superfamily (Zhou et al., 1993; Conlon et al., 1994); and putative transcription factors, including Brachyury (T) (Wilkinson et al., 1990), even-skipped homeobox gene-1 (Evx1) (Bastian and Gruss, 1990; Dush and Martin, 1992), goosecoid (Gsc) (Blum et al., 1992), and transcription factor β (Tcf3b), also called hepatocyte nuclear factor-3β (Hnf3b)1 (Ang et al., 1993; Monaghan et al., 1993; Ruiz i Altaba et al., 1993; Sasaki and Hogan, 1993).

Mutational analysis is critical for establishing essential roles for these genes. For example, targeted gene disruption via homologous recombination in embryonic stem cells has verified the importance of Tcf3b during gastrulation and node formation (Ang and Rossant, 1994; Weinstein et al., 1994). However, analysis of gene function by this approach can be complicated if the gene has an additional, essential role earlier in development, as has been shown for Evx1 (Spyropoulos and Capecechi, 1994). A strategy that has successfully yielded the identification of novel genes involved in gastrulation has been analysis of spontaneous or induced mutations producing dramatic phenotypes during gastrulation and early organogenesis. This approach has lead to the characterization of nodal and T, which are required for normal primitive streak formation and function (Iannaccone et al., 1992; Conlon et al., 1994; Herrmann, 1994).

1In keeping with the nomenclature established by the Committee on Standardized Genetic Nomenclature for Mice [(1993) Mouse Genome, vol. 91, pp. 149-150], Hnf3b will herein be referred to as Tcf3b.
We have taken the latter approach and characterized a deletion mutant, eed (embryonic ectoderm development), which exhibits a dramatic phenotype during gastrulation (Niswander et al., 1988). Embryos homozygous for deletions removing eed are able to initiate mesoderm production, but the primitive streak appears abnormal.

The eed phenotype is most obvious at E8.5 (see Fig. 1): although wild-type embryos at this stage have undergone extensive neural and axial development (Fig. 1A), eed mutants possess a smaller, presumably undifferentiated embryonic ectoderm, and exhibit sparse embryonic mesoderm (Fig. 1B). Furthermore, there is no organization of mesoderm into somites, node, or notochord, and there is no morphological evidence of neural induction. In contrast, production of mesoderm in the proximal part of the embryo is undiminished, with extensive formation of the extraembryonic structures – amnion, chorion, and allantois. By E9.5 no mutant embryos are observed in litters derived from eed/+ inter se matings. Fate mapping studies of the wild-type mouse gastrula suggest that the deficiency of embryonic mesoderm and absence of axial structures (node, notochord, somites) reflect a defect in the distal primitive streak (Lawson et al., 1991). Consistent with this hypothesis is the unimpeded production of extraembryonic mesoderm, which is derived from epiblast cells traversing the proximal primitive streak (Lawson et al., 1991).

The eed mutation maps to the albino deletion complex on mouse chromosome 7. Because of the complexity of the phenotype and large size of the eed region (≥170 kilobases; B. Holdener and T. Magnuson, unpublished data), the possibility exists that more than one deleted gene is responsible for the eed phenotype. Indeed, two loci (l(7)5Rn and l(7)6Rn) mapping to the eed interval have been identified via an N-ethyl-N-nitrosourea (ENU) saturation mutagenesis screen (Rinchik, 1991; Rinchik and Carpenter, 1993). Three l(7)5Rn alleles have been isolated, and embryonic homozygous for one of these alleles (l(7)5Rn5354SB) demonstrate a similar phenotype as eed deletion homozygotes (B. Holdener and T. Magnuson, unpublished data). Since ENU-induced mutations are generally intragenic (Popp et al., 1983; Russell and Rinchik, 1987; Peters et al., 1990; Russell et al., 1990; Provost and Short, 1994), these data suggest that the eed phenotype probably results from a single-gene defect.

To explore the nature of the gastrulation defect in eed mutants, we have undertaken additional phenotypic analyses that include mRNA in situ hybridization of gene expression relevant to this process. These studies have confirmed that tissues derived from the proximal part of the primitive streak are correctly specified. Moreover, sparse mesodermal cells derived from the more distal regions of the primitive streak also appear to be appropriately specified in eed mutant embryos. Head process (notochord precursor) mesoderm, as well as definitive endoderm, are present, and their production is independent of correct expression of nodal, Fgf4, and Gsc.

Furthermore, the primitive streak markers T and Evx1 have expanded their domains of expression in eed mutant embryos to include cells not normally fated to traverse the primitive streak. The findings presented here are consistent with the eed gene product acting early in gastrulation to regulate genes required for proper primitive streak formation and/or organization.

MATERIALS AND METHODS

Mouse strains and embryos

The c<sup>ML</sup> albino-deletion and l(7)354SB mice used in this study originated at the Oak Ridge National Labs (Russell et al., 1982; Rinchik and Carpenter, 1993) and were obtained from Drs L. B. Russell and E. M. Rinchik. These mice have been maintained as closed colony, heterozygous stocks with chinchilla (c<sup>ch</sup>), c<sup>ML</sup>c<sup>ch</sup> and c<sup>ch</sup>/c<sup>ch</sup> (where + = 354SB mutation) stocks are a dilute chinchilla coat color as compared to a full chinchilla color evident in c<sup>ML</sup>c<sup>ch</sup> mice. For experimental purposes, the stocks were expanded by crossing heterozygous mutant stocks with chinchilla mice to produce c<sup>ML</sup>c<sup>ch</sup> or c<sup>ch</sup>/c<sup>ch</sup> (dilute chinchilla) and c<sup>ML</sup>c<sup>ch</sup> (chinchilla) offspring. Dilute chinchilla offspring were crossed inter se to produce experimental embryos. No significant differences in gene expression were detected between embryos homozygous for the ENU-induced allele and deletion homozygotes.

Noon of the day of the appearance of the vaginal plug was designated E0.5.

Histology and in situ hybridization to sectioned embryos

Pregnant females were sacrificed at 6.5-9.0 days of gestation and embryos left intact in their decidua for processing. For in situ hybridization, embryos were fixed in 4% paraformaldehyde overnight, dehydrated, embedded in Paraplast (Oxford), and sectioned according to standard procedures (Zeller, 1989). An entire litter was embedded in a single block, from which 6 μm sections were cut. At this point E8.5 and some E7.5 eed mutant embryos were identified by their unique morphology, as described in the Introduction and Results. To identify E6.5-7.5 eed mutants not displaying an obvious phenotype, hybridization was performed using a cDNA clone (φmid2) which maps to the region removed by the c<sup>ML</sup> deletion (A. Schumacher and T. Magnuson, unpublished data). In situ analysis revealed that this cDNA (φmid2) was ubiquitously expressed in wild-type embryos at E6.5-8.5 but no transcripts were detected in E8.5 eed mutant embryos (C. Faust, A. Schumacher, and T. Magnuson, unpublished data). To genotype E6.5-7.5 eed mutants, embryos were sectioned either sagit tally or transversely and probed for φmid2 expression and the gene of interest on adjacent 6 μm sections.

For identification of primordial germ cells, embryos were fixed in 90% ethanol overnight and subsequently processed as described above. Embryo sections were stained for non-specific alkaline phosphatase activity using the protocol of McGadey (McGadey, 1970).

mRNA in situ hybridization to sectioned embryos

Probe preparation, in situ hybridization and autoradiography were performed as per Sassoon and Rosenthal (Sassoon and Rosenthal, 1993). Antisense strand probes were prepared via in vitro transcription using T3, T7, or Sp6 polymerase and α-<sup>35</sup>S-UTP (1000 Ci/mmol, NEN). To ensure that hybridization was specific as per the washing conditions employed, sense strand probes were also prepared and hybridized to adjacent sections in the initial stages of this study. Except for φmid2, the probes used in this study have been described previously. Probes for the following genes were obtained, and we gratefully acknowledge those investigators who gave them: Flk1, from J. Rossant (Yamaguchi et al., 1993); Igf2, from A. Elstratiadi (De Chiara et al., 1991); Evx1, from P. Gruss (Bastian and Gruss, 1990); Fgf4, from G. Martin (Niswander and Martin, 1992); T, from B. Herrmann (Herrmann et al., 1990); Tcf3b and -3a, from B. Hogan (Sasaki and Hogan, 1993); Shh, from A. McMahon (Echelard et al., 1993); nodal, from M. Kuehn (Zhou et al., 1993), and Gsc, from E. De Robertis (Blum et al., 1992). Probes were exposed for 1-2 weeks (Flk1, I. Igf2, Gsc, and Shh), or 3-4 weeks (nodal, Evx1, Tcf3b and -3a, and Fgf4).

Sectioned embryos were scored for expression using a Nikon Optiphot microscope equipped with dark-field optics. For publication, sections were imaged on a Biorad MRC-600 confocal microscope.
using the transmitted light mode, with the reflection block in PMT1. Bright-field and dark-field images were scanned in the PMT2 channel, and merged in Adobe Photoshop to obtain the final image.

**Whole-mount in situ hybridization and immunohistochemistry**

Preparation of digoxigenin probes and whole-mount in situ hybridization were performed essentially as per Conlon and Herrmann (Conlon and Herrmann, 1993), with the following exceptions. The peroxide, glycine and borohydride steps were eliminated, and embryos were treated with 10 μg/ml proteinase K for 10 minutes (E7.5 wild type/E8.5 mutants) or 15 minutes (E8.5 wild type).

A polyclonal rabbit T antiserum, kindly provided by B. Herrmann, was used for whole-mount immunohistochemistry as per Kispert and Herrmann (Kispert and Herrmann, 1994). Whole-mount embryos were photographed on a layer of 1% agarose, using a Nikon SMZ-2T microscope and Kodak Ektachrome 160T film.

**RESULTS**

Previous work had indicated that a mutant class of eed homozygotes could not be identified until E8.5, when wild-type littermates were initiating organogenesis (Niswander et al., 1988; Fig. 1A, B); however, some mutant embryos exhibit a striking phenotype at E7.5 (compare Fig. 1C and D). These mutants appear lopsided, with large amounts of mesoderm located proximally in the embryo and very little residing distally. The mesoderm layer of the proximal primitive streak is abnormally thickened, and the streak also appears kinked. Analysis of multiple E7.5 litters demonstrated variation in the extent of development of the eed mutant phenotype. While some E7.5 embryos have a large excess of proximal mesoderm, the majority of mutants generally resemble an E6.0-6.5 egg cylinder-stage embryo. Embryos exhibit a well defined embryonic ectoderm, and a small amount of mesoderm is present proximally on one side of the embryo. In some embryos there appears to be a delay in the transition of cuboidal to squamous visceral endoderm. The size of the mutant embryos varies, from about one-fourth to two-thirds the size of their wild-type littersmates.

At E8.5 eed homozygotes resemble E7.5 embryos with respect to overall size, the apparent lack of differentiation of embryonic ectoderm and mesoderm, and the extent of development of the extraembryonic structures (Fig. 1B,C). Like E7.5, there is some variation among mutant embryos with respect to size and the amount of embryonic mesoderm produced. Embryos often appear skewed, wherein the proximodistal axis is visibly longer anteriorly. While extraembryonic development is quite extensive, it is not completely normal – the allantois is exaggerated in size and often not well defined, and the amnion is abnormal, often contiguous with the extraembryonic mesoderm; nevertheless, epiblast cells traversing the proximal part of the primitive streak seem to be able to adopt their normal fates. To investigate this at a molecular level, we adopted two approaches: staining for the
presence of primordial germ cells, precursors of the germ cell lineage; and mRNA in situ analysis of genes whose expression reflects the function of the proximal primitive streak.

**Primordial germ cells are specified in eed mutant embryos**

Clonal analysis has indicated that primordial germ cells (PGCs), like extraembryonic mesoderm, descend from epiblast precursors that traverse the proximal primitive streak (Lawson and Hage, 1994). Thus, we were interested in determining if this cell type was specified in eed mutant embryos. In E7.0-7.5 wild-type embryos, PGCs, identified by their alkaline phosphatase (ALP) activity (Chiquoine, 1954; Ginsburg et al., 1990), are clustered at the base of the allantois; by E8.5, PGCs have migrated from the allantois into the embryonic hindgut (Ginsburg et al., 1990, and references therein; Fig. 2A). ALP-positive cells were identified in the extraembryonic mesoderm of E8.5 eed mutant embryos (Fig. 2B). The presence of these cells in eed mutant embryos indicates that epiblast cells traversing the proximal primitive streak are able to adopt a primordial germ cell fate.

**Flik1 expression indicates that mesoderm derived from the proximal primitive streak is appropriately specified in eed mutant embryos**

*Flik1*, an early marker of endothelial cell precursors (Yamaguchi et al., 1993), is initially expressed at E7.5 in mesoderm derived from cells traversing the most proximal part of the primitive streak (Lawson et al., 1991). These include the nascent mesoderm of the proximal primitive streak, extraembryonic yolk sac mesoderm, allantois, and proximoanterior embryonic mesoderm (see Fig. 2C). As development proceeds, *Flik1* expression is seen additionally in a variety of embryonic tissues undergoing vasculogenesis (Yamaguchi et al., 1993).
Flk1 expression in E8.5 eed mutants was similar to E7.5 wild-type, in that transcripts were detected in the proximal primitive streak, extraembryonic mesoderm, and proximoanterior embryonic mesoderm (Fig. 2D). These data indicated that specification of mesoderm derived from the proximal primitive streak is occurring normally. This conclusion was supported by expression analysis of insulin-like growth factor II (Igf2), which demonstrates a similar expression pattern to Flk1 in wild-type embryos (Lee et al., 1990; data not shown).

The distal primitive streak markers goosecoid and Fgf4 are not expressed in the primitive streak of eed mutant embryos

Having confirmed our previous morphological observations, i.e., that the proximal primitive streak appears to function normally, we turned to the analysis of markers of the distal primitive streak and its derivatives. The first marker examined was goosecoid (Gsc), which is expressed in a very defined window during gastrulation (Blum et al., 1992). Prior to any obvious delamination (E6.25-6.4), Gsc transcripts are localized to squamous visceral endoderm and the proximoposterior epiblast where the primitive streak will form (Blum et al., 1992; Conlon et al., 1994; Fig. 3A). As the streak forms and extends to the distal tip of the embryo (E6.5-7.5), Gsc transcripts are found in the distal streak, the node (lying at the distal-most tip of the streak), and axial mesoderm (arrowhead). (A) In E6.4 embryos, Gsc transcripts are detected in the posterior epiblast (ee) and squamous visceral endoderm (ve). xe, extraembryonic ectoderm. (B) At E7.0 Gsc expression in visceral endoderm continues, and expression is also seen in the distal primitive streak, node (n), and axial mesoderm (arrowhead). (C) In E7.5 eed embryos, only expression in the squamous visceral endoderm can be detected (arrowheads). (D-E) φmid2 expression in E7.0 wild-type (D) and E7.5 eed (E) embryos. (D) In E7.0 wild-type embryos φmid2 is ubiquitously expressed, but transcripts are absent from E7.5 eed embryos (E), permitting genotyping. The sections shown in D and E are 6 µm adjacent to B and C, respectively. (F-H) Fgf4 expression in E7.5 wild-type (F), E7.5 eed (G), and E8.5 eed (H) embryos. (F) Oblique sagittal section demonstrating Fgf4 expression in the distal primitive streak. (G, H) Midline sagittal sections of eed mutant embryos reveal no Fgf4 transcripts in the primitive streak. Bar, 50 µm.
mesoderm extending anteriorly from the node (Fig. 3B). Gsc transcription ceases abruptly by approximately E7.75 (head fold stage), only to resume much later in development (Gaunt et al., 1993).

As expected, no Gsc transcripts were detected in E8.5 eed mutant embryos (data not shown). Interestingly, E6.5-7.5 mutant embryos \((n = 7)\) demonstrated very little, if any, Gsc expression above background levels in the posterior epiblast, as determined by both sagittal (Fig. 3C) and transverse sections through the egg cylinder. Expression in squamous visceral endoderm, however, was maintained. To distinguish mutant embryos at this early stage, embryos were genotyped using a cDNA (φmid2) mapping to the eed region (A. Schumacher and T. Magnuson, unpublished data; see Materials and Methods). φmid2 transcripts were ubiquitously expressed in wild-type embryos (Fig. 3D), but were absent in mutant littersmates (Fig. 3E).

In wild-type mouse embryos, Fgf4 expression is initiated quite early, beginning in the late blastocyst stage (Niswander and Martin, 1992). After implantation Fgf4 transcripts are detected throughout the epiblast, but at the onset of primitive streak formation, expression becomes restricted to the distal two-thirds of the streak (see Fig. 3F); expression continues in the streak at E8.5 (Niswander and Martin, 1992). E6.5-7.5 eed mutant embryos \((n = 7)\), identified by a lack of φmid2 expression, exhibited no Fgf4 gene expression (Fig. 3G). In addition, no Fgf4 transcripts could be detected in E8.5 eed mutants \((n = 10;\) Fig. 3H). Serial 6 µm sections were taken to confirm that expression was not missed. In pre-streak mutant embryos \((E6.0-6.25)\), Fgf4 expression was detected throughout the epiblast, similar to wild-type littersmates (data not shown). Thus, Fgf4 expression prior to gastrulation appears normal in eed mutant embryos, but is not subsequently initiated in the primitive streak.

Sparse axial mesoderm cells are correctly specified in eed mutant embryos

While distal primitive streak gene expression is disrupted in eed mutant embryos, it is not completely ablated. This was indicated via whole-mount immunohistochemical analysis of Brachyury (T) protein. In E7.5 wild-type embryos, T protein is expressed throughout the primitive streak, node and notochord precursor cells (the headprocess/notochord precursor, herein referred to as the notochord precursor), which extends anteriorly from the node at the distal midline (Kispert and Herrmann, 1994; Fig. 4A, right). In E8.5 eed mutant embryos similar expression was detected in the primitive streak and sparse mesoderm cells extending anteriorly from the streak (Fig. 4A, left). These data suggest that a notochord precursor is formed in eed mutant embryos, despite the absence of a morphologically distinct node, the major source of these cell types (Selleck and Stern, 1991; Beddington, 1994).

Finding that T was expressed in axial mesoderm cells of eed mutant embryos was intriguing, and we were interested in learning to what extent this mesoderm is specified. Thus, two additional markers with specific expression in these tissues, Tcf3b and Shh, were examined. mRNA in situ hybridization using both whole mount and sectioned embryos was performed on E8.5 mutant and E7.5 wild-type embryos (Fig. 4B-D,F). At E7.5 Tcf3b is expressed in the node, notochord precursor, and prechordal mesoderm, which lies rostral to the notochord (Fig. 4B, left) (Ang et al., 1993; Monaghan et al., 1993; Ruiz i Altaba et al., 1993; Sasaki and Hogan, 1993). In mutant embryos Tcf3b transcripts were detected in the primitive streak and midline mesoderm extending anteriorly (Fig. 4B, right and 4F). Expression appeared to be in a band 3-5 cells wide at the midline.

In wild-type embryos Shh transcripts are first detected at E7.5 in the notochord precursor (Fig. 4C, right), and thus are present in a subset of the Tcf3b expressing domain. Later, during somite formation, Shh expression spreads caudally into the node, overlapping with Tcf3b (Echelard et al., 1993). In some eed mutant embryos, Shh transcripts were detected in a subset of the Tcf3b-expressing domain lying more anteriorly in the embryo, similar to wild-type expression at E7.5 (Fig. 4C, left; Echelard et al., 1993). In others, Shh expression overlapped more completely with Tcf3b, extending farther posteriorly. Variation in Shh expression likely reflects differences in the extent of development among E8.5 eed mutant embryos with respect to wild-type. Comparison of Shh, T, and Tcf3b gene expression in frontal sections of E8.5 eed embryos revealed that while all three genes are expressed in distal midline mesoderm (Fig. 4D-F), Shh and Tcf3b expression extends more anteriorly than T (data not shown). Such expression could reflect the presence of prechordal mesoderm, but this cannot be confirmed until a good marker restricted to this cell population is available.

eed mutant embryos are able to initiate production of definitive endoderm

Tcf3b and a related molecule, Tcf3a (also called HNF-3α; see Footnote in Introduction) are also markers of the definitive endoderm lineage (Ang et al., 1993; Monaghan et al., 1993; Ruiz i Altaba et al., 1993; Sasaki and Hogan, 1993). Definitive endoderm is derived in part from cells traversing the most distal region of the primitive streak (Lawson et al., 1991). A significant amount of definitive endoderm also arises from the node (Selleck and Stern, 1991; Beddington, 1994), where it emerges concomitantly with the notochord precursor, displacing embryonic visceral endoderm proximoanteriorly (Poelmann, 1981b; Lawson and Pedersen, 1987; Tam and Beddington, 1992). Analysis of sectioned mutant embryos revealed abundant expression of both Tcf3b (Fig. 4F) and Tcf3a (not shown) in the endoderm layer extending anteriorly from the distal tip of the embryo. Both Tcf3b and Tcf3a are expressed at low levels in primitive visceral endoderm (Ang et al., 1993), but expression is upregulated in definitive endoderm. Therefore, it is likely that strong expression of these genes indicates the presence of definitive endoderm in eed mutant embryos.

nodal is expressed throughout the epiblast of eed mutant embryos

While eed mutant embryos lack a morphologically distinct node, they are still able to produce and specify some axial mesoderm, suggesting that some node activity might be present. One gene that has been suggested to be important for the establishment and/or maintenance of this structure is nodal, a member of the TGFβ superfamily (Zhou et al., 1993). In pre- to early-streak wild-type embryos, prior to node formation, nodal is expressed throughout the proximal epiblast and visceral endoderm (Conlon et al., 1994; Fig. 5A).
As gastrulation proceeds, *nodal* expression becomes limited to epiblast cells lying more posteriorly, so that by mid- to late-streak expression is detected only on the posterior side of the epiblast, adjacent to primitive streak mesoderm. Subsequently, epiblast expression is downregulated and *nodal* transcripts become restricted to cells surrounding the node (Fig. 5B). Examination of E7.5 *eed* mutants revealed that *nodal* was expressed throughout the proximal epiblast and visceral endoderm (data not shown). In E8.5 *eed* mutant embryos, *nodal* transcripts were still detected throughout the proximal part of the epiblast (Fig. 5C). Thus, *nodal* expression appears to be correctly initiated in *eed* mutant embryos, but subsequently expression is not properly downregulated in the epiblast.
the most proximoanterior epiblast (compare Fig. 6A and B), genes overlap. These data are intriguing, since misexpression was observed in E8.5 mutants, no Brachyury or Evx1 transcripts were detected (data not shown). This is likely to reflect a developmental delay as compared to wild-type littermates, which normally express these genes at E6.5.

**DISCUSSION**

Our earlier morphological work described the eed phenotype as a retardation of embryonic ectoderm growth and absence of axial structures (Niswander et al., 1988). Since the deficient tissues are normally derived from epiblast cells fated to traverse the distal primitive streak, we hypothesized that the defect might lie in the failure of their progenitors to ingress through the streak. The results of the mRNA in situ hybridization analyses presented here are consistent with a defect in distal primitive streak function, but they also suggest alternative mechanisms for the establishment of the eed phenotype. A comparison of gene expression between eed and wild-type embryos is summarized in Fig. 7.

Two lines of evidence strongly suggest that epiblast cells traversing the proximal primitive streak of eed mutant embryos are able to adopt their normal fates. First, Flk1 and Igf2, two genes expressed in derivatives of the proximal primitive streak, are expressed normally with respect to the extraembryonic mesoderm cell types present in eed mutant embryos (see Fig. 7A). Second, PGCs, descendants of the presumptive extraembryonic mesoderm cells of the epiblast, are also present. Although PGCs are clearly specified, differences with wild-type embryos were observed: PGCs of eed mutant embryos were not always present in a cluster, and were often distributed more anteriorly. Such differences could reflect the overabundance and disorganization of extraembryonic mesoderm in eed mutant embryos as compared to wild-type.

Absence of detectable Gsc and Fgf4 mRNA in the primitive streak of eed mutant embryos (see Fig. 7A,B) likely represents a defect in distal primitive streak function. Our analyses, however, do not distinguish whether lack of expression of these genes directly affects the establishment of the phenotype.
or merely reflects an absence of a population(s) of distal primitive streak cells. In wild-type embryos both Fgf4 and Gsc are expressed in the primitive streak very early in gastrulation (Blum et al., 1992; Niswander and Martin, 1992), but their potential functions in this process are unclear. The spatial and temporal expression of Gsc during gastrulation is highly conserved among vertebrates (Cho et al., 1991; Izpisua-Belmonte et al., 1993; Stachel et al., 1993; Schulte-Merker et al., 1994); nevertheless, Gsc expression does not appear to be required for mouse gastrulation, since embryos harboring a null mutation for Gsc survive well beyond this point without any apparent defects (R. Behringer, personal communication).

Anterior mesoderm production, while inhibited in eed mutants, is not completely abolished, perhaps due to the continued activity of other molecules, like Tcf3b, in specifying anterior fates early in gastrulation. Sparse midline mesoderm cells lying anterior to the primitive streak of E8.5 eed mutant embryos appear to be specified as notochord precursor, as exemplified by expression of Tcf3b, T, and Shh (see Fig. 7A). Furthermore, Tcf3b and Tcf3a expression in endoderm extending anteriorly from the streak suggest that mutant embryos are able to initiate definitive endoderm production.

The node represents a major source of notochord and definitive endoderm in the mouse embryo (Beddington, 1994). Furthermore, the node demonstrates organizing activity, such that when grafted to an ectopic site, it induces a secondary neural axis containing notochord, definitive endoderm, and somites (Beddington, 1994). Thus, the ability to form a notochord precursor and definitive endoderm in eed mutants indicates that some node activity is present, even though a morphologically distinct node structure is not apparent. This activity could be attributed to the appropriate expression of Tcf3b, which in
wild-type embryos is required for node function. Targeted inactivation of Tcf3b results in both loss of morphological structure and functional activity of the node; Tcf3b$^{−/−}$ mutants do not initiate the formation of a notochord precursor (Ang and Rossant, 1994; Weinstein et al., 1994). The paucity of axial mesoderm and definitive endoderm, as well as lack of further organization of these tissues in eed mutant are consistent with diminished node function. Because of its restricted expression pattern and putative nature as a signaling molecule, nodal might be important for the establishment and/or maintenance of a structurally definitive node (Zhou et al., 1993; see Sasaki and Hogan, 1993). Thus, it is tempting to speculate that inappropriate expression of nodal in E8.5 eed mutant embryos (Fig. 7A,B) affects node function. Consistent with its essential role early in gastrulation (Iannaccone et al., 1992; Conlon et al., 1994), initial expression of nodal in the eed mutant epiblast appears normal (Fig. 7B); however, at later stages it fails to be down-regulated and localized around the node. Further analysis is required to determine if eed is directly involved in the regulation of nodal in the epiblast, and whether or not absence of localized nodal expression results in failure to form a node.

The gene expression data presented thus far are consistent with a model in which the eed gene product plays a direct role in the production of mesoderm from the distal primitive streak. Perhaps without the eed gene product to induce complete anterior mesoderm formation, the embryo can still initiate gastrulation, but produces mesoderm of mostly posterior, or caudal, character. Support for this model comes from studies of Xenopus laevis embryos, where absence of early signals specifying dorsal fates leads to inhibition of proper organizer function during gastrulation, resulting in the default production of ventral/posterior mesoderm (see Gerhart et al., 1989; Kimelman et al., 1992; Slack and Tannahill, 1992). The problem with this model, however, is that it does not readily explain the morphologically apparent overproduction of extraembryonic mesoderm, as well as ectopic expression of T and Evx1 observed in the proximal epiblast of eed mutant embryos (Fig. 7B). In fact, data regarding the functions of T and Evx1 in both mouse and Xenopus embryos are consistent with a model in which their misexpression plays a dominant role in the establishment of the eed phenotype.

Besides being required cell autonomously for the differentiation and maintenance of notochord (Rashbass et al., 1991), a number of studies suggest that the T gene is required for proper epiblast cell ingression and migration of mesoderm cells from the proximal primitive streak (Herrmann and Kispert, 1994, and references therein). Additional support for the role of T in posterior mesoderm formation is provided by studies of the Xenopus T homologue Xbra. Injection of Xbra mRNA into the animal pole of one-cell stage embryos results in misexpression of Xbra throughout the embryo and a disruption of gastrulation; although blastopore formation occurs, no involution of mesoderm at the marginal zone is detected, and a layer of ectopic mesoderm contiguous with the marginal zone is formed over the animal hemisphere (Cunliffe and Smith, 1992). Older embryos often lack heads and develop an additional tail. The ectopic mesoderm is of posterior character, exhibiting small amounts of muscle and expressing Xhox3, the Xenopus homologue of Evx1. These data are consistent with a model in which Xbra/T is sufficient to act as a genetic switch from an ectodermal cell fate to a posterior/ventral mesodermal cell fate (Herrmann and Kispert, 1994).

The phenotype observed in Xenopus embryos misexpress-
ing Xbra is not unlike that of mouse eed mutants. Gastrulation is disrupted in both embryos, and posterior mesoderm appears to be produced at the expense of anterior. At the onset of gastrulation in eed mutant embryos, T is ectopically expressed in the proximoanterior epiblast, a region sharing a similar fate with animal hemisphere cells in Xenopus — that of surface ectoderm (Dale and Slack, 1987; Tam, 1989; Lawson et al., 1991). These cells do not normally ingress through the primitive streak, and one possibility is that in eed mutants ectopic T expression in these cells initiates a cellular program that influences them to ingress. Because of their proximal location in the mutant embryo, they would likely give rise to extraembryonic mesoderm after traversing the streak (Lawson et al., 1991), resulting in the large deposition of mesoderm observed proximally. Alternatively, they might undergo direct delamination to form mesoderm, bypassing the primitive streak. One way to determine how proximal epiblast cells behave in eed mutant embryos would be to perform lineage analysis like that used to construct a fate map of the pregastrula mouse embryo (Lawson et al., 1991). Furthermore, creation of T−/− eed double homozygotes will be helpful in elucidating the effect of ectopic T expression on the establishment of the eed phenotype.

Ectopic Evx1 expression might also play a role in establishing the eed phenotype. Like the mouse, Xenopus Xho3 is expressed in a graded fashion along the anteroposterior axis (Ruiz i Altalba and Melton, 1989a). Inhibition of Xho3 function prevents the normal development of posterior structures (Ruiz i Altalba et al., 1991), while abolishing its normal expression gradient by ectopic expression anteriorly results in embryos in which anterior mesoderm patterning is inhibited (Ruiz i Altalba and Melton, 1989b). Inhibition of anterior development is similar to that seen upon misexpression of Xbra; however, ectopic mesoderm is not induced, and embryos undergo normal gastrulation movements, producing an apparently normal amount of embryonic mesoderm (Ruiz i Altalba and Melton, 1989b). Thus, evidence from Xenopus supports a role for Xho3 in specifying cell fates along the anteroposterior axis, rather than potentiating cell movement during gastrulation. If such a role is conserved in mouse, ectopic expression of Evx1 in eed mutant embryos might reflect specification of a wider range of epiblast cells to a posterior fate. Assuming that a gradient of Evx1 expression is normally involved in specifying positional values of nascent mesoderm, it is possible that disrupting this gradient by an increase in Evx1 expression early in streak formation could lead to the inhibition of gene expression required for the specification of anterior mesoderm. In this manner the distal primitive streak defect hypothesized in eed mutant embryos might be a downstream effect of the dominant misexpression of T and Evx1. Furthermore, if ectopic expression of T and Evx1 does indeed reflect a dominant posteriorization of the embryo, the normal role of the eed gene product during gastrulation would likely be in the negative regulation of one or both of these genes.

Finally, it is possible that, in addition to its effects on primitive streak function, the eed gene product has an independent role in controlling epiblast growth. Alternatively, growth inhibition could directly influence primitive streak function in eed mutants, since in the wild-type embryo epiblast cell ingestion is thought to be driven by growth of the epiblast toward the primitive streak (Lawson et al., 1991). eed might directly regulate cell growth or affect expression of other gene(s) that direct epiblast cell growth and ingestion during gastrulation. One gene whose expression is affected in eed mutants and might be involved in cell growth is Fgf4. Although the function of Fgf4 in gastrulation is unknown, targeted disruption of the gene encoding the FGF type 1 receptor, which can bind the FGF4 protein (Mansukhani et al., 1990), has revealed that FGF signaling is required for proper embryonic growth, mesoderm production, and patterning during gastrulation (T. Yamaguchi and J. Rossant, personal communication). To implicate a growth defect as primary for the establishment of the phenotype, assays will be conducted to examine cell growth and proliferation in the eed mutant embryo (see Snow, 1977; Poelmann, 1981a,b; Lawson et al., 1991; Mac Auley et al., 1993).

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