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A primary requirement for *nodal* in the formation and maintenance of the primitive streak in the mouse

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SUMMARY

The 413.d insertional mutation arrests mouse development shortly after gastrulation. *nodal*, a novel TGFβ-related gene, is closely associated with the locus. The present study provides direct evidence that the proviral insertion causes a loss of function mutation. *nodal* RNA is initially detected at day 5.5 in the primitive ectoderm. Concomitant with gastrulation, expression becomes restricted to the proximal posterior regions of the embryonic ectoderm. *nodal* RNA is also expressed in the primitive endoderm overlying the primitive streak. A few hours later, expression is strictly confined to the periphery of the mature node. Interestingly 413.d mutant embryos show no morphological evidence for the formation of a primitive streak. Nonetheless, about 25% of mutant embryos do form randomly positioned patches of cells of a posterior mesodermal character. Data presented in this report demonstrate the involvement of a TGFβ-related molecule in axis formation in mammals.

Key words: mouse, insertional mutation, TGFβ growth factor, nodal, gastrulation

INTRODUCTION

Despite intensive investigation, it has proved difficult to characterize genes acting to control gastrulation in mammalian embryos. Recent studies in other vertebrate systems, most notably *Xenopus*, have demonstrated that growth factors, particularly those belonging to the TGFβ and FGF multigene families, are required for cell fate specification at early stages of development (reviewed by Kimelman et al., 1992; Sive, 1993). Although the mammalian homologs of these molecules are known to be expressed in distinct sub-sets of cells at gastrulation (Hebert et al., 1990; Lyons et al., 1991; Niswander and Martin, 1992; Beddington and Smith, 1993; Faust and Magnuson, 1993), the biochemical details of receptor ligand interactions remain ill defined. Further insight into the signalling mechanisms responsible for growth and differentiation of specific cell types awaits the results of mutational analysis.

The generation and characterization of random or targeted mutations introduced into the mouse germ line has led to the identification of several genetic loci required for embryonic inductive and patterning events. Genomic mapping studies led to the isolation of the product of the *brachyury* (*T*) locus, which is required for correct mesodermal induction and posterior axis formation (Herrmann et al., 1990). Homozygous *brachyury* mutants lack a notochord and the posterior mesodermal tissues are reduced or absent depending on the allele (reviewed by Beddington et al., 1992). Thus, the *T* gene product seems to be required to ensure correct formation and/or specification of the mesodermal lineage. Although classical genetic studies have identified a number of spontaneous mutations that similarly interfere with early embryogenesis, the underlying defects have yet to be elucidated at the molecular level (reviewed by Lyon and Searle, 1989; Holdener-Kenny et al., 1992).

We have been studying a retrovirally induced recessive lethal mutation, termed 413.d. Homozygous mutant embryos exhibit severe tissue disturbances by 7.5 days of development, a stage that precedes the onset of the *brachyury* mutant phenotype (Conlon et al., 1991; Iannaccone et al., 1992; Robertson et al., 1992). We have demonstrated that homozygous 413.d mutant embryonic stem (ES) cells are indistinguishable from wild-type ES cells (Conlon et al., 1991) and indeed retain the ability to differentiate into a wide variety of mature mesodermal cell types in isolation. Moreover, in mosaic embryos, mutant ES cells are effectively rescued by wild-type cells and can contribute to all three primary cell lineages. These observations demonstrate that the 413.d mutation acts in a non-cell autonomous fashion, and suggest that the affected gene might encode a secreted factor essential for early post-implantation development. Consistent with this interpretation a novel member of the TGFβ super family of growth factors, termed *nodal*, was recently shown to map close to the 413.d retroviral insertion site (Zhou et al., 1993). A small group of cells occupying the node region expressed *nodal* mRNA at the late primitive streak stage. However, histologi-

cal studies had clearly indicated that the onset of morphological abnormalities occurs at a stage prior to node formation. Moreover, possible effect(s) on *nodal* gene expression and thus the connection to the mutant phenotype were not previously explored.

The present study documents a causal relationship between *nodal* gene expression and the 413.d mutant phenotype. We provide strong evidence that the developmental arrest reflects a loss of function mutation. Thus *nodal* mRNA, expressed abundantly in wild-type ES cells is not present in mutant ES cell lines. We found that *nodal* mRNA is synthesized in the embryonic ectoderm at day 5.5 of development and that concomitant with the onset of gastrulation, cells expressing *nodal* transcripts become restricted predominantly to the proximal posterior regions of the embryonic ectoderm. Additionally, low levels of *nodal* mRNA are present in the primitive endoderm overlying the embryonic ectoderm. By late streak stages, *nodal* mRNA is strictly confined to ectodermal cells at the extreme periphery of the mature node. As might be predicted by this temporally and spatially restricted *nodal* expression pattern, 413.d mutant embryos show no morphological evidence for the formation of a distinct primitive streak. These observations suggest that *nodal* functions in the patterning and organization of the primitive ectoderm. We also examined expression of several molecular markers normally localized to distinct regions, or defined sub-populations of cells in gastrulation stage embryos. These results demonstrate that the ectoderm in 413.d mutant embryos can give rise to random patches of mesodermal cells with a posterior, lateral or extra-embryonic character. Collectively these data argue that the primary role of *nodal* is not in mesoderm induction per se, but rather in the induction and/or maintenance of the primitive streak.

MATERIALS AND METHODS

Mouse strains and embryos

The 413.d mutation is maintained on the 129/Sv//Ev inbred background. Heterozygous animals, identified by Southern blot analysis (Conlon et al., 1991) or using a PCR assay were intercrossed. Embryos collected at different gestational time points were staged according to morphological criteria (Downs and Davies, 1993). The relative developmental stage of mutant embryos was assessed according to the developmental stages of heterozygous and wild-type littermates. For whole-mount analyses, embryos were dissected free of maternal tissue in phosphate-buffered saline (PBS) containing 10% fetal calf serum, and fixed overnight in 4% paraformaldehyde. Embryos were subjected to whole-mount in situ hybridization and/or immunohistochemical analyses. Following analysis and photography, embryos were washed twice in 100% methanol, and rehydrated by washing at RT for 5 minutes each in 75%, 50% and 25% methanol, followed by 2 washes in PBS. Genomic DNA was subsequently prepared from individual embryos and samples genotyped by PCR, as described in Saiki (1990) using primers as follows: the common 5′ primer (P1) corresponds to genomic sequence lying 579 bp from the 5′ LTR. This primer in combination with a 3′ primer from 5′ LTR (P2) amplifies a 590 bp band specific for the viral integration site and in combination with a 3′ primer corresponding to genomic sequence located immediately adjacent to the 3′ LTR (P3) amplifies a product of 620 bp specific for the wild-type allele. Embryos for conventional histology were collected at 6.5 days p.c., fixed in Bouin's fluid and processed according to standard protocols (Kaufman, 1990). 8 μm serial sections were stained with heamatoxylin and eosin and photographed using Kodak Ektachrome slide film. Embryos for use in conventional in situ analyses were obtained from matings between CD1 mice (Charles River). Embryos were retained within the decidua, fixed in 4% paraformaldehyde, embedded according to standard procedures (Hogan et al., 1986) and sectioned at 5 μm.

Northern analysis

Total RNA was prepared using RNAzol B (Biotecx Laboratories, Inc.), according to the manufacturers instructions. Poly(A)$^+$ RNA was selected with oligo(d)T cellulose (Sambrook et al., 1989), size fractionated on a formaldehyde agarose gel, transferred onto GeneScreenPlus (NEN Research Products) and cross-linked by UV irradiation. Filters were hybridized using a *nodal* exon 2-specific riboprobe and with a mouse β-actin probe to control for RNA loading.

In situ hybridization to tissue sections

In situ hybridization and washes were carried out essentially as described in Lyons et al. (1990), except that probes were not subjected to limited alkaline hydrolysis. The *nodal* riboprobe vector consists of nucleotides 330-660, cloned into pBluescript KS-II, and transcribed from the T3 promoter. The brachyury (T) and goosecoid probes (kindly provided by David Wilkinson and Eddy De Robertis) were previously described (Wilkinson et al., 1990; Blum et al., 1992). Slides were photographed on a Leitz DMR microscope using Kodak Ektachrome film.

Whole-mount in situ hybridization

Whole-mount in situ hybridization analysis to examine *sox-2* expression was performed according to Rosen and Beddington (1993) whereas *gooscoid* (*gsc*) and *twist* expression were analysed as described by Wilkinson (1992). The *sox-2* probe was a T3 transcript of 748 bp from a cDNA sub-clone (kindly provided by Larysa Pevny and Robin Lovell-Badge). The *twist* probe was a T3 transcript of approximately 400 bp from a cDNA sub-clone (kindly provided by Doug Spicer and Andrew Lassar). The *goosecoid* probe was used as described (Blum et al., 1992). For double-labelling experiments, embryos processed for whole-mount RNA in situ were re-fixed overnight in 4% paraformaldehyde at 4°C. Embryos were then stained with the appropriate antibodies, as described below, except that the bleaching step was omitted, and color development was carried out in the absence of nickel chloride.

Whole-mount immunocytochemistry

The polyclonal rabbit T antibody has been described by Kispert and Herrmann (1994). Polyclonal rabbit antisera specific for cdx-4 (Gamer and Wright, 1993) and Mox-1 were kindly provided by Chris Wright. Fixation and immunohistochemical staining of embryos was carried out as described by Kispert and Herrmann (1994). The double labeling protocols were described by Matthews et al. (1990). Embryos were photographed on a Wild M10 dissecting microscope using Kodak Ektachrome color slide film.

RESULTS

The 413.d proviral insertion causes a loss of nodal mRNA expression

The 413.d recessive lethal mutation, originally identified in a genetic screen of animals carrying multiple copies of a replication defective retroviral vector (Robertson et al., 1986), has been shown to co-segregate with a single provirus (Conlon et al., 1991). A unique sequence flanking probe was used to map the 413.d locus to chromosome 10 (Dr Nancy Jenkins, personal communication), within 0.6 cM of the Hβ58 locus (Lee et al., 1991). Single copy probes surrounding the provirus were used

to screen early stage embryonic cDNA libraries. Two independent cDNA clones were recovered and partially sequenced and found to correspond to *nodal*, a novel TGFβ family member recently identified by Zhou and colleagues using a similar strategy (Zhou et al., 1993).

The retrovirus has integrated into the first intron of the *nodal* gene and thus has no obvious effect on the coding potential (Zhou et al., 1993). It was therefore of considerable interest to determine whether the provirus interferes with the expression of *nodal* transcripts. As 413.d mutant embryos fail at early post-implantation stages, it has proved difficult to examine *nodal* expression in vivo. However, we found that undifferentiated ES cells strongly express *nodal* transcripts detectable by northern blot analysis. This allowed us to examine *nodal* mRNA expression in ES cell lines derived from 413.d heterozygous intercross matings (Conlon et al., 1991). As shown in Fig. 1, an exon-2 specific probe detects a single 2.0 kb mRNA in wild-type and heterozygous cell lines. This species was absent from a number of homozygous mutant ES cell lines. This result was confirmed by whole-mount RNA in situ hybridization analysis examining *nodal* expression in wild-type and mutant ES cell lines (data not shown). These data demonstrate that the 413.d provirus eliminates *nodal* mRNA synthesis, and strongly suggest that the 413.d phenotype results from a loss of function mutation.

413.d mutant embryos exhibit abnormalities at the early primitive streak stage

Histological analyses demonstrate that 413.d mutant embryos are morphologically distinct by 7.5 days p.c. (Conlon et al., 1991; Iannaccone et al., 1992; Robertson et al., 1992). To investigate the primary defect(s) underlying these tissue disturbances in mutant embryos, we developed a PCR assay using oligonucleotide primers specific for the mutant and wild-type *nodal* alleles (see Materials and Methods) that allowed us to unambiguously genotype 413.d homozygous embryos from early post-implantation stages onwards.

When mice heterozygous for the 413.d mutation were intercrossed, there was no evidence for morphologically abnormal embryos in several litters collected at the egg cylinder stage (6.0 days p.c.). The earliest sign of gastrulation is the localized thickening of the embryonic ectoderm cells to form the primitive streak. At the early gastrulation stage (approximately 6.5 days p.c.), we identified a category of embryos in which no primitive streak was evident (Fig. 2). Upon genotyping, these proved without exception to be 413.d homozygous embryos. These embryos displayed a second unusual feature; the proximal region of the conceptus, comprising the extra-embryonic ectoderm, was no longer symmetrical but often exhibited an irregular twisted surface (Fig. 2B). These abnormalities appear to be the consequence of deficiencies in the formation and/or

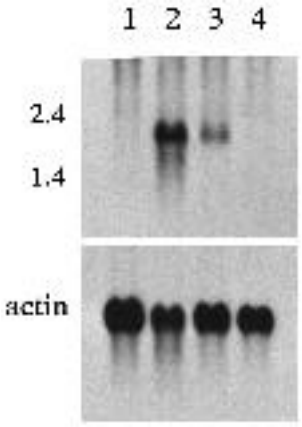

Fig. 1. Northern blot analysis of *nodal* expression in ES cells. 5 μg of poly(A)$^+$ RNA was isolated and processed as described (see Materials and Methods). The *nodal* transcript is detected with an exon 2 specific probe in undifferentiated wild-type (lane 2) and heterozygous (lane 3) ES cells but is absent in STO fibroblasts (lane 1) and homozygous mutant (lane 4) ES cells. An actin probe was used as a control for RNA loading (lower panel). The relative migration of RNA molecular mass marker fragments is indicated.

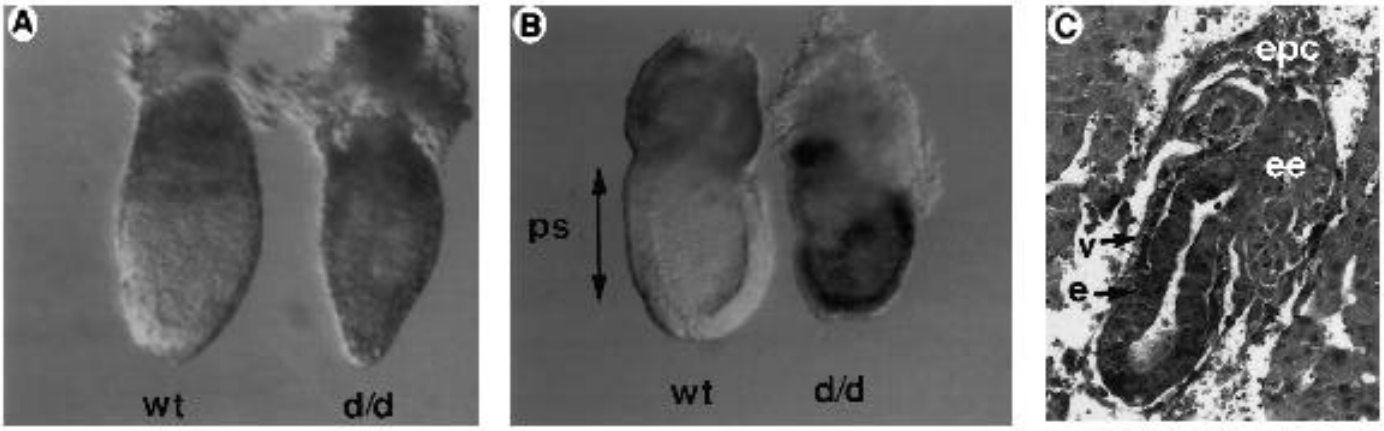

Fig. 2. Morphology of wild-type and 413.d homozygous mutant embryos at the early primitive streak stage. (A) and (B) both show the morphological appearance of a 413.d mutant embryo (d/d) compared with that of a wild-type (wt) littermate. The parietal endoderm has been reflected to reveal the extra-embryonic and embryonic regions of the embryos. Both of the normal embryos were at the mid-streak stage based on the visible extent of the primitive streak and T antibody staining. Neither of the 413.d mutant embryos had recognizable primitive streak structures, or stained positively for expression of T protein. Note the presence of the primitive streak (ps) in the normal embryo in B (indicated by vertical arrows), and the absence of a morphological streak in 413.d mutant embryos. In mutant embryos, a clear demarcation between the embryonic and extra-embryonic regions is not readily evident. (C) Representative sagittal section through a presumed 413.d mutant embryo at 6.5 days p.c. Analysis of complete serial sections failed to show any evidence of localized thickening of the embryonic ectoderm indicative of the primitive streak, or of the formation of a mesodermal layer. Note the striking disturbance of the extra-embryonic ectoderm. (epc, ectoplacental cone; ee, extra-embryonic ectoderm; e, embryonic ectoderm: v, visceral endoderm).

movement of underlying extra-embryonic mesoderm. By contrast, the embryonic ectoderm is often seen to persist as a monolayer cup of cells (Fig. 2C). The defects in the extra-embryonic ectoderm can be considered as a secondary consequence of the failure to generate or correctly pattern mesodermal cells that emerge from the streak and normally migrate proximally and laterally to generate the amniotic fold (Poelmann, 1981; Hashimoto and Nakatsuji, 1989). In mutant embryos, the extra-embryonic ectoderm is not displaced proximally towards the ectoplacental cone and the amniotic fold fails to form, subsequently causing the disorganized expansion of both the embryonic and extra-embryonic ectoderm.

Nodal mRNA expression precedes the formation of mesoderm

Next we compared the onset and pattern of *nodal* mRNA expression to that of molecules previously implicated as having important roles in primitive streak formation. *Goosecoid* (*gsc*) is expressed in the dorsal lip of the blastopore or Spemann's organizer, in *Xenopus* (Cho et al., 1991), and in Hensen's node in chick (Izpisua-Belmonte et al., 1993). Murine *gsc* transcripts are first expressed in the posterior region of the ectoderm as the primitive streak forms. During elongation of the streak, *gsc* transcripts are reported to localize to the anteriormost mesoderm, suggesting this region of the streak may represent the functional equivalent of Spemann's organizer (Blum et al., 1992). By contrast, *brachyury* (*T*) mRNA is expressed at the onset of gastrulation in the primitive streak ectoderm and nascent mesoderm. During gastrulation, *T* transcripts are present throughout the length of the streak. Following regression of the streak, *T* expression continues in the head process and notochord (Wilkinson et al., 1990).

The patterns of *nodal*, *gsc* and *T* expression were compared on adjacent sections from single embryos at 5.5 days of development. *nodal* transcripts were present at low levels throughout the embryonic ectoderm (Fig. 3A). In contrast *gsc* and *T* were not detected at this early stage (data not shown) confirming earlier reports (Blum et al., 1992; Wilkinson et al., 1990).

gsc was initially expressed in the mesoderm at the anteriormost region of the streak (Blum et al., 1992; see Fig. 3D). Additionally, we detected high levels of *gsc* mRNA in the visceral endoderm anterior to the streak. As shown in Fig. 3B, *nodal* was expressed in a gradient within the embryonic ectoderm, the highest levels of transcripts being present in the proximal portion of the

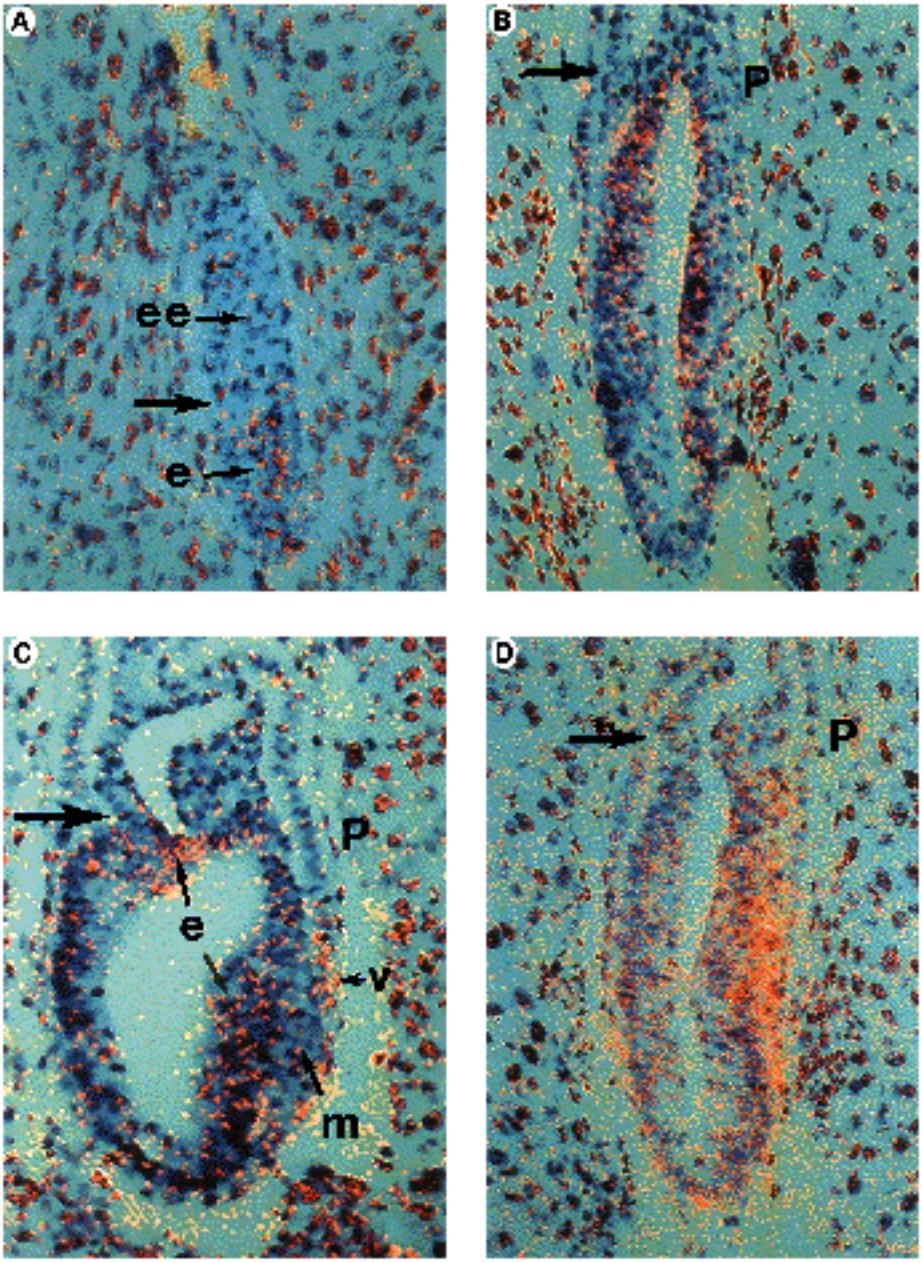

Fig. 3. Expression of *nodal* and *goosecoid* RNA in sagittal sections of normal embryos. (A) Localization of *nodal* RNA in the primitive ectoderm of the 5.5 day embryo. Sagittal section of embryo within decidum. Large arrow indicates junction between extra-embryonic and embryonic regions of the embryo. ee, extra-embryonic ectoderm; e, embryonic ectoderm. (B) Localization of *nodal* transcripts in proximal ectoderm of 6.5 day, early streak stage embryos. Large arrow indicates junction between extra-embryonic and embryonic regions. P denotes posterior side of the embryo. (C) *nodal* transcripts are predominantly localized to the proximal-posterior ectoderm (e) in the mid-streak stage embryo, and are absent from mesodermal cells exiting the streak (m). *Nodal* transcripts also accumulate in the visceral endoderm (v) overlying the posterior embryonic ectoderm. Large arrow indicates junction between extra-embryonic and embryonic regions. P denotes posterior side of the embryo. (D) Localization of *goosecoid* RNA in a section from the same embryo as that shown in B, separated by approximately 20 μm, showing high levels of expression confined to anterior mesoderm and overlying visceral endoderm. Large arrow indicates junction between extra-embryonic and embryonic regions. P denotes posterior side of the embryo.

embryo. Low levels of *nodal* mRNA were also observed in the overlying visceral endoderm. *nodal* mRNA was not expressed at detectable levels in the nascent mesodermal cell population. As primitive streak elongation proceeds, *nodal* mRNA expression continues in the proximal region of the ectoderm (approximately mid-streak stage). However, the domain of expression becomes further localized to the posterior side of the embryonic ectoderm and the adjacent endoderm (Fig. 3C). At this stage, *gsc* transcripts continue to be highly expressed in mesodermal cells present in the anterior region of the streak and in the endoderm anterior to the streak.

By the early head fold stage of development, the primitive streak reaches its maximum extent anteriorly, and the mesoderm arising from the posterior region of the streak has migrated laterally to underlie the head folds. As shown in Fig. 4, the definitive node is visible by 7.5 days p.c. at the distal end of the embryo, continuous with the anterior end of the primitive streak. At this stage *nodal* mRNA expression is restricted to the definitive node (Zhou et al., 1993; see also Fig. 4A). By comparison, *gsc* transcripts are no longer present at detectable levels (Blum et al., 1992; and data not shown), whereas *T* mRNA continues to be expressed throughout the primitive streak and in the notochordal plate (Fig. 4B). *nodal* mRNA persists as the streak regresses and the node is displaced progressively more posteriorly. As shown in Fig. 4C and D, *nodal* mRNA expression is localized to a small population of ectodermal cells lying at the lateral borders of the node of the 8.5 days p.c. embryo. By 9.5 days p.c. no detectable *nodal* hybridization signal was present.

In summary, *nodal* mRNA is predominantly expressed in gastrulation stage embryos in the proximal-posterior regions of the embryonic ectoderm coincident with formation of the primitive streak. Fate mapping studies have previously suggested that this sub-population of ectodermal cells is allocated principally to the extra-embryonic mesoderm of the VYS, amnion and allantois, i.e the earliest mesoderm to be formed (reviewed by Lawson and Pedersen, 1992). The 413.d mutant embryos exhibit defects in primitive streak formation and an abnormal pattern of mesoderm migration.

Expression of ectodermal and posterior mesodermal markers in 413.d mutant embryos

One possibility is that the embryonic ectoderm in 413.d mutant embryos lacks the ability to pattern the mesodermal cell lineage. To test this, we examined the expression of several gene products whose activities are normally confined to discrete cell populations in gastrulation stage embryos. All these genes encode putative transcription factors and hence should serve as cell-autonomous markers. Results of these experiments are summarized in Table 1.

The HMG-box-containing gene *sox-2* is expressed uniformly throughout the primitive ectoderm of the pre-streak embryo. As gastrulation commences, *sox-2* expression becomes progressively restricted to the anterior regions of the ectoderm, such that, by late streak and early head fold stages, *sox-2* transcripts are strictly confined to the neural plate (Jerome Collignon and

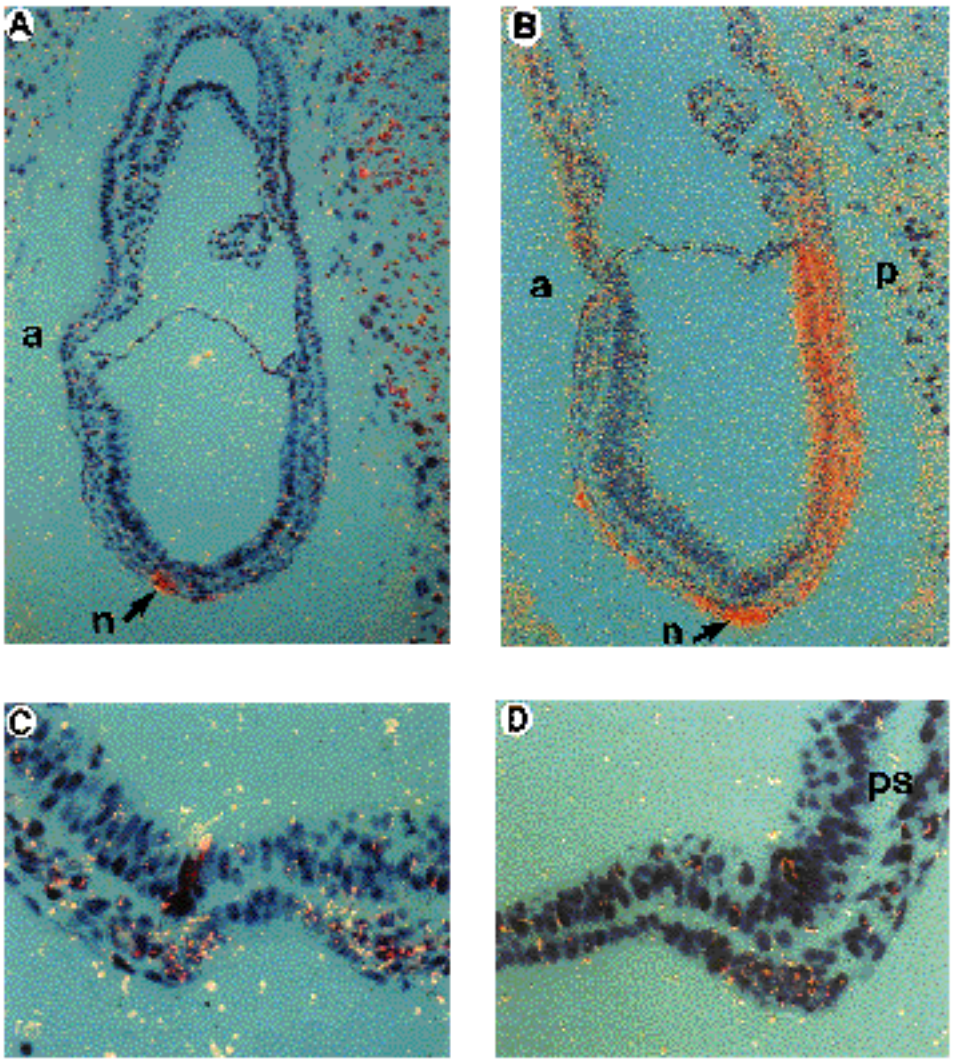

Fig. 4. *Nodal* is expressed exclusively in the node region of the late streak stage and early head fold embryo. (A) Sagittal section through 7.5 days p.c. embryo showing localization of *nodal* transcripts to the extreme anterior tip, or node (n), of the primitive streak. (B) An adjacent section to that shown in A, hybridized with a *T*-specific probe showing expression in the cells of the primitive streak, the node (n) and notochord. In both sections the anterior (a) of the embryo is to the left and the posterior (p) is to the right. (C) High power magnification of a transverse section through an early head fold stage embryo. *Nodal* transcripts are localized to a small group of ectodermal cells lying at the extreme edges of the node. *Nodal* is not expressed in the endoderm. (D) High power magnification of a sagittal section through the node of an early head fold stage embryo (8.0 days p.c.), confirming expression of *nodal* is confined to the ectodermal populations of the node. (ps, primitive streak).

Robin Lovell-Badge, personal communication; see Fig. 5A). Mutant embryos examined at 7.5 days p.c. were all found to contain patches of ectodermal cells expressing *sox-2* mRNA (Fig. 5B). Based on these findings we can conclude that cells of an ectodermal nature persist in mutant embryos.

To score for the presence of mesodermal cell types that are normally formed during early primitive streak stages, we examined T expression. T protein is transiently expressed from the onset of gastrulation in all cells ingressing into the streak and in nascent mesoderm (Kispert and Herrmann, 1994). At later stages, cells of the notochordal plate and the notochord also express T protein. Whole-mount staining showed that approximately 10% of 413.d mutant embryos express T protein at 7.5 days p.c. However, the T-expressing population appears as randomly positioned groups of cells (Figs 5D,F, 6D). T protein expression was not detected in 413.d mutant embryos at earlier stages (6.5 days p.c.).

We used double labelling techniques to examine further the spatial relationships amongst various cell sub-populations. As expected, the domains of T and *sox-2* expression were found to be non-overlapping in early to mid streak stage wild-type embryos (Fig. 5C). To determine if the boundary between distinctive cell types was maintained in mutant embryos, we similarly analyzed *sox-2* mRNA and T protein expression in individual embryos. Of 44 embryos from intercross matings analyzed at 7.5 days p.c., we recovered 10 homozygous mutants, one of which expressed both markers. As shown in Fig. 5D, cell populations expressing *sox-2* and T occupied non-overlapping regions. Thus the striking tissue disturbances observed in 413.d embryos were not associated with an intermixing of ectodermal and mesodermal cell types.

The observation of T-expression by a discrete sub-population of cells in a significant fraction of mutant embryos strongly argues that cells of a mesodermal character are formed in the absence of *nodal* expression. To confirm and extend these findings, we examined cdx-4 protein expression. Consistent with earlier reports (Gamer and Wright, 1993), the cdx-4 antibody specifically stains a small group of mesodermal cells located at the base of the allantoic bud, in mid to late-streak embryos (Fig. 6A). 12 hours later, the expression domain becomes graded, such that cdx-4-expressing cells extend laterally from the streak and proximally into the allantois (Fig. 6C). Approximately 15% of mutant embryos stained at 7.5 days p.c. contained patches of cells expressing cdx-4 protein (Fig. 6B). Although the domains of cdx-4 and T expression partially overlap in the posterior

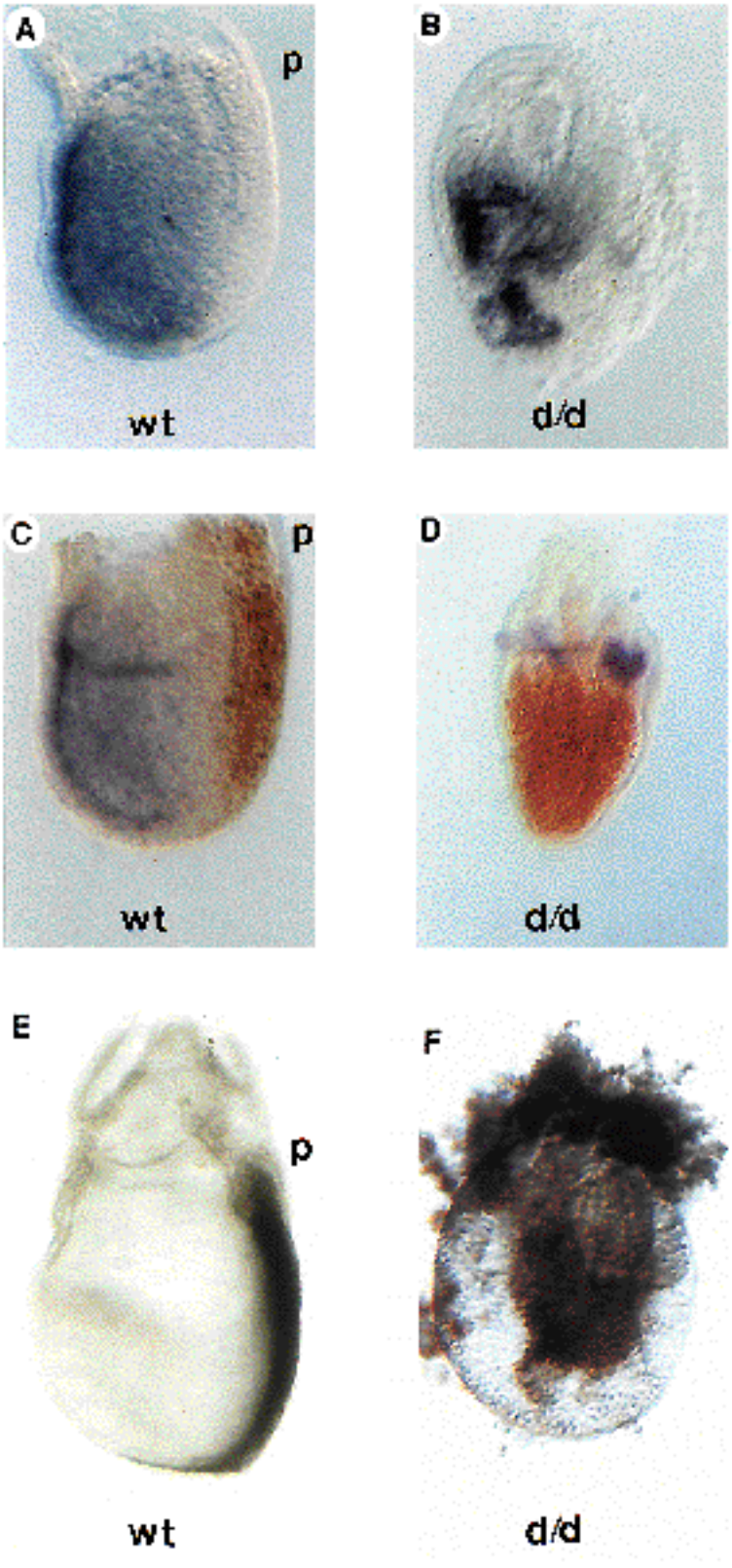

Fig. 5. Expression of *sox-2* mRNA and T protein in wild-type (wt) and 413.d mutant (d/d) embryos. (A,C,E) Wild-type embryos showing normal patterns of expression of *sox-2* mRNA (blue) and T protein (brown); a, anterior side of embryo; p, posterior side of embryo. (B,D,F) Corresponding homozygous 413.d littermates. (A) *sox-2* mRNA expression in a wild-type mid-streak stage embryo and (B) mutant littermate. (C) Double labelling of *sox-2* mRNA and T protein in wild-type mid-streak stage embryo and (D) corresponding mutant littermate. (E) Localization of T protein in wild-type early head fold stage embryo and (F) mutant littermate. Embryos have been dissected free of the parietal yolk sac for photography, except for the embryo in panel F which shows non-specific staining in the adherent tissue derived from the ectoplacental cone and decidual tissue. All wild-type embryos are viewed laterally with the posterior to the right. Examples of 413.d mutant embryos that fail to express T protein are shown in Fig. 6B and F.

proximal region of wild-type embryos (Fig. 6C) there was no evidence for co-expression of T and cdx-4 protein in the 413.d mutants. Overall, we found that 25% of 413.d mutant embryos contained cells expressing posterior mesoderm markers. Thus, we conclude that *nodal* gene expression cannot be a prerequisite for induction of the mesodermal cell lineage.

Finally, we examined several markers normally expressed by cell populations that ingress in the more anterior regions of the streak. A total of 38 embryos dissected at 6.5 days p.c. were analyzed by whole-mount in situ hybridization using a *gsc* specific probe. There was no convincing evidence for localized *gsc* mRNA expression in 10 mutant embryos examined (data not shown). However, the transient nature of *gsc* mRNA expression at this stage of development makes it difficult to rule out the possibility that mutant embryos may form cells containing *gsc* transcripts. As a marker for the formation of lateral plate, paraxial and somitic mesoderm, we examined expression of the homeobox-containing gene *Mox-1* (Candia et al., 1992). Although mRNA is present from early streak stages onwards, Mox-1 protein is first detected at late streak/early head fold in the paraxial mesoderm anterior to the node and caudal to the hind brain (Fig. 6E). At later stages mox-1 protein is most strongly expressed in the somites and pre-somitic mesoderm (data not shown). None of the 19 mutant embryos collected at 7.5-8.5 days p.c showed any expression of Mox-1 protein (Fig. 6F). We also analyzed expression of *twist* mRNA. *Twist* is first expressed in the anterior mesoderm underlying the head folds, and by late head fold stages is also expressed in the pre-somitic and somitic mesoderm (Wolf et al., 1991). We found no evidence for *twist* mRNA expression in 413.d mutant embryos.

In summary, a significant fraction of 413.d mutant embryos clearly contain discrete cell populations corresponding to nascent and posterior, lateral or extra-embryonic mesoderm. By contrast, analyzing three independent markers, we failed to

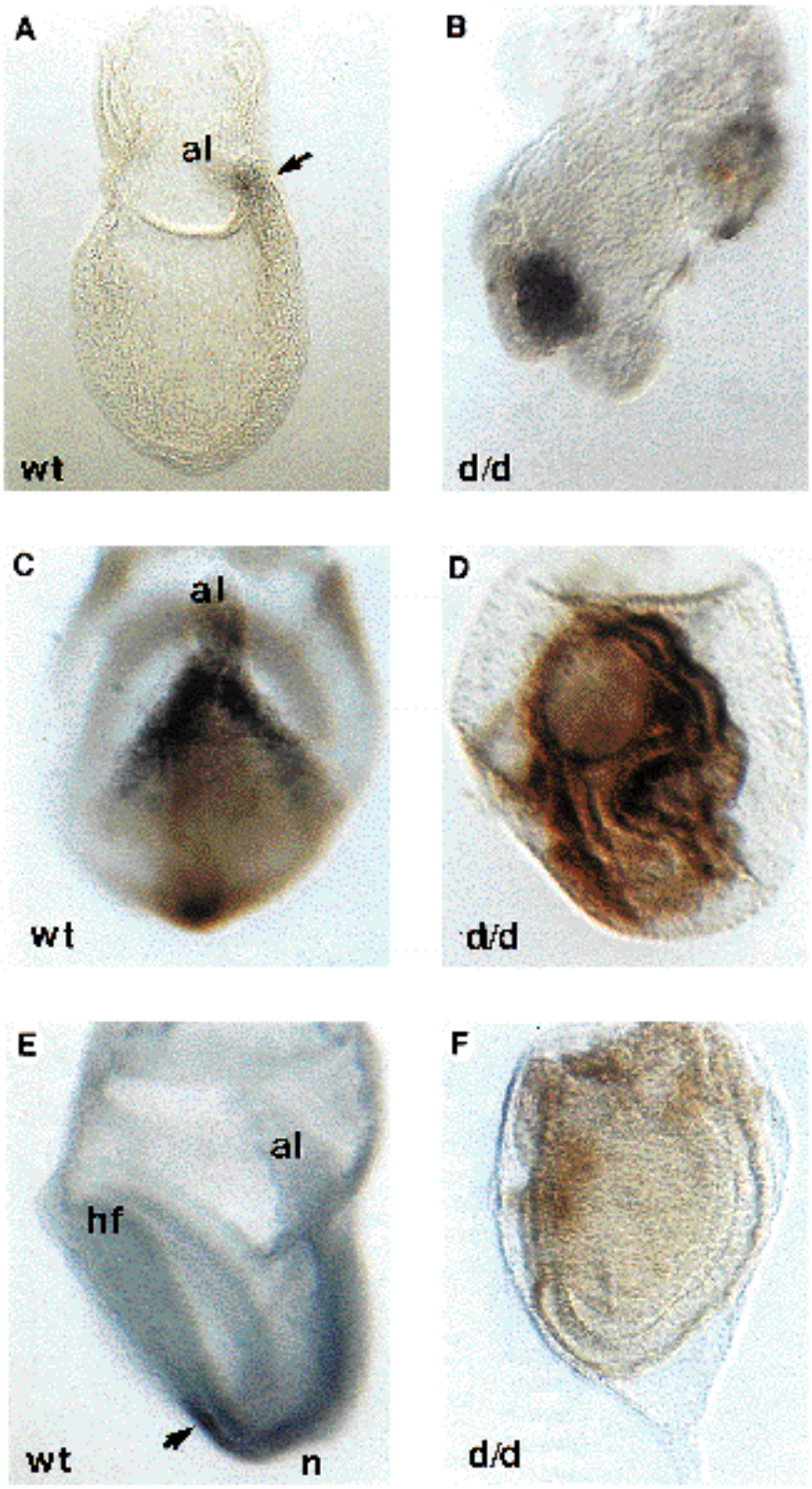

Fig. 6. Expression of T, cdx-4 and Mox-1 protein in wild-type (wt) and 413.d mutant (d/d) embryos. (A,C,E) Wild-type embryos showing normal patterns of expression; (B,D,F) corresponding 413.d mutant littermates. Whole-mount cdx-4 antibody staining in early head fold stage embryo (arrowed) (A) and corresponding mutant littermate (B). Double antibody staining with cdx-4 antibody (blue) and T antibody (brown) in a late streak stage wild-type (C) and corresponding mutant embryo (D). Note lack of cdx-4 staining in D. (E) Mox-1 antibody staining in wild-type early head fold stage embryo (arrowed) and corresponding mutant embryo (F). The mutant embryos shown in B and F were additionally analyzed for expression of T protein (faint brown staining is due to non-specific staining of superficial decidual tissue). All wild-type embryos are viewed laterally with the posterior to the right, with the exception of C, which is positioned to allow visualization of T- and cdx-4-expressing cells along the posterior axis. (al, allantois; hf, head fold; n, node).

Table 1. Expression of marker genes in 413.d mutant embryos

Marker	Technique	Frequency of positive embryos
sox-2	RNA in situ	10/10
Brachyury	antibody	4/38*
cdx-4	antibody	3/25†
Mox-1	antibody	0/19
gsc	RNA in situ	0/10
twist	RNA in situ	0/7

*Of the total of 38 embryos stained for T protein expression, 25 of these were doubly stained for cdx-4 expression, 10 were co-analyzed for *sox-2* expression and 3 were stained for T protein alone.

†Within this pool of 25 embryos, 3 were also found to express T protein exclusively. Therefore the overall number of embryos expressing posterior mesoderm markers was 6/25, or 25% of the total population.

Litters of embryos from intercrosses of animals heterozygous for the 413.d mutation were collected at approximately 7.5 days p.c. and individually scored for expression of the indicated gene products. Embryos were processed in groups of 15-20, photographed and individually genotyped by PCR.

observe any evidence for the formation of mesodermal cells derived from more anterior regions of the streak.

DISCUSSION

During early vertebrate development the process of gastrulation leads to the establishment of the body axis and the generation of the definitive germ layers of the animal. The growth, differentiation and orchestrated morphogenetic movements of cells that accompany gastrulation are thought to be directed in large part through the action of diffusible signals (reviewed by Jessel and Melton, 1992; Kimelman et al., 1992; Sive, 1993). Indeed, several growth factors capable of inducing mesodermal cell types have been identified in *Xenopus.* Substantial progress has also been made with respect to understanding how gradients of these molecules might interact to confer regional cell type specification along the body axes (Ruiz i Altaba and Melton, 1989; Green et al., 1992). Although these studies provide insight into potential role(s) of these growth factors, the exact identity of the embryonic cell sub-populations normally expressing or responding to these signalling molecules has yet to be established. Far less is known about similar pathways acting in the mammalian embryo.

We have been studying a retrovirally induced insertional mutation, termed 413.d, that identifies a gene(s) essential for post-implantation development. The key feature of mutant embryos is an apparent lack of the mesodermal cell types normally associated with the primitive streak (Conlon et al., 1991; Iannaccone et al., 1992; Robertson et al., 1992). It was recently shown that the *nodal* gene, encoding a member of the TGFβ family of secreted growth factors is closely associated with the 413.d locus (Zhou et al., 1993). The present experiments demonstrate that the 413.d phenotype results from a loss of function mutation. Thus *nodal* transcripts abundantly expressed by wild-type ES cells were not present in homozygous mutant ES cell lines. Moreover, the onset of *nodal* gene expression occurs prior to gastrulation and expression continues during early gastrulation, in keeping with the observed morphological abnormalities. The lack of *nodal* can therefore account for the specific cellular defects observed in homozygous mutant embryos.

In the mouse, cell marking experiments have shown that gastrulation is achieved by the directional and coordinated growth of the embryonic ectoderm towards the primitive streak, followed by ingression and subsequent allocation of the resulting progenitor cells (reviewed by Lawson and Pedersen, 1992). Our analysis of the 413.d mutation shows that *nodal* is required very early in this process. Mutant embryos show no evidence for the formation or continued maintenance of a discrete primitive streak. Additionally, and possibly as a secondary consequence, mutant embryos are characterized by an absence of the migration of cohorts of newly formed mesoderm, both proximally and laterally to establish the characteristic radially symmetrical body plan of the embryo. Consistent with these morphological defects, we found *nodal* transcripts in the ectoderm of pre-streak embryos, and concomitant with the initiation of primitive streak formation, *nodal* expression demarcates a discrete population of proximal-posterior ectoderm cells. These cells that are normally fated to ingress at early streak stages to generate mesodermal cells populating the posterior and extra-embryonic regions of the embryo, appear to be dramatically affected in 413.d mutant embryos. Although cells of the mesodermal lineage are formed in mutant embryos, their spatial positioning is highly aberrant. Thus the block to development in 413.d mutant embryos would appear to be associated with an abnormal pattern of mesoderm migration.

An attractive idea is that *nodal* might act as a mesoderm inducing factor. Particularly observations in *Xenopus* have shown that constitutive expression of a dominant-negative form of the activin receptor is sufficient to block mesoderm formation (Hemmati-Brivanlou and Melton, 1992). However, two lines of evidence argue that *nodal* is unlikely to initiate mesoderm induction in the mouse. Firstly, previous experiments have shown that 413.d mutant ES cells do not have an intrinsic defect in mesoderm formation per se. Homozygous mutant ES cells can differentiate with normal kinetics to form a wide spectrum of mature mesodermal cell types (Conlon et al., 1991). Furthermore, the present experiments exclude a primary role for *nodal* in early mesoderm induction pathways in vivo. Thus, we found that cells of posterior mesoderm character do form in a high proportion of 413.d mutant embryos. A significant fraction of mutant embryos examined at mid to late streak stages were found to contain patches of cells expressing T protein or cdx-4 protein. *nodal* expression is therefore not required for the formation of cell populations of a nascent or posterior mesodermal character.

A particularly noticeable feature of 413.d mutant embryos is the finding that mesodermal cell populations fail to migrate appropriately. Thus, mesoderm cells fail to move proximally and laterally from their point of origin to displace the extra-embryonic ectoderm. One possibility is that *nodal* acts in an autocrine fashion within the ectoderm to stimulate directional growth of epiblast cells towards the region of the streak. Consistent with this, *nodal* is most strongly expressed in the proximal ectodermal cells lying adjacent to the extra-embryonic regions. This population normally overlies the nascent mesoderm that migrates from the extreme posterior region of the streak (Poelmann, 1981; Hashimoto and Nakatsuji, 1989). *nodal* activity may also be required to

promote the migration of newly formed mesoderm. Mesodermal cells that have been induced in 413.d mutant embryos may fail to become displaced from their point of origin, resulting in a defect in the orderly ingression of ectoderm into the streak. A failure to maintain the primitive streak and thus the continuous production of mesoderm during gastrulation might also account for the apparent inability of 413.d mutant embryos to form mesodermal cells of a more anterior character. Thus we failed to identify cells expressing molecular markers such as *mox-1* and *twist*, normally expressed by the descendants of ectodermal cells ingressing at the more anterior regions of the streak.

Interestingly we observed that single patches of contiguous mesodermal cells were formed as opposed to several discrete populations. Thus, the 413.d mutation does not completely eliminate the underlying ectodermal patterning that presumably acts to ensure that only a single primitive streak arises. Additionally, the observation that the patches of mesoderm in mutant embryos can be quite large extends the argument against a requirement for *nodal* for the proliferation and survival of mesoderm per se. Rather *nodal* signalling seems to be required for appropriately directed mesodermal migration and subsequent interactions necessary to pattern the ectodermal and mesodermal cell lineages (Frohman et al., 1990; Ang and Rossant, 1993).

In addition to *nodal*, other molecules have been shown to influence the migratory properties of embryonic cell sub-sets. Loss of *T* gene function seems to affect the migratory properties of posterior mesoderm cells, as shown by the non-uniform distribution of *T* mutant cells in chimeric embryos (Beddington et al., 1992; Rashbass et al., 1991; Wilson et al., 1993). Similarly in *Xenopus*, over-expression of *gsc* has been shown to be associated with enhanced migratory properties of cells (Neihrs et al., 1993). Furthermore, *Xenopus* embryos treated with compounds that interfere with convergent extension movements show defects in the generation of the body axes (Gerhart et al., 1989). Cell movements during gastrulation are thought to be dictated in part by interactions of cells with the extra-cellular matrix (Burdsal et al., 1993). There is considerable evidence that members of the mammalian TGFβ growth factor family regulate expression of extra-cellular matrix molecules (reviewed by Massague, 1990). It is tempting to speculate that *nodal* similarly acts to induce synthesis of extra-cellular matrix proteins in specific target cell populations that can affect their movement.

In conclusion, *nodal*, a member of the family of TGFß related growth factors has been shown to play a role in cell interactions that regulate early development of the mammalian embryo. Whether or not our current model proves to be correct, the availability of mutant mice lacking *nodal* expression provides a suitable background for studying the role of TGFβ-related molecules in differentiation and patterning of cells at early stages of mouse development.

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