**msd is required for mesoderm induction in mice**

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**SUMMARY**

Mesoderm induction is fundamental for establishing the basic body plan of the vertebrate embryo and mutations are critical for dissecting this process. Mouse embryos lacking msd (mesoderm deficiency) do not produce mesoderm but have well-defined extraembryonic and thickened embryonic ectoderm. Distribution of transcripts indicate that temporal regulation of gene expression relevant to gastrulation has begun but primitive-streak formation and mesoderm induction are blocked. Both msd-deficient embryos and embryonic stem (ES) cells fail to form highly differentiated structures of mesoderm origin, but are capable of ectodermal differentiation. Thus, the effects of the msd mutation are restricted to mesoderm formation and could result from the inability to respond to an inducing signal.

Key words: msd, mouse mutants, mesoderm induction, Brachyury (T), Evx1, goosecoid, Fgf4, Fgf5, Fgfr1, Fgfr2, Sna, H19

**INTRODUCTION**

The morphogenetic movements of gastrulation and concomitant induction of mesoderm are functionally well characterized in *Xenopus*. Numerous molecules, including members of the TGF-β (Kimelman and Kirschner, 1987; Smith, 1987; Weeks and Melton, 1987; Thomsen et al., 1992; Thomsen and Melton, 1993) and FGF families (Kimelman and Kirschner, 1987; Slack et al., 1987; Kimelman and Maas, 1992), wnt-related growth factors (Christian et al., 1991; Smith and Harland, 1991; Sokol et al., 1991; Sokol and Melton, 1992), goosecoid (Cho et al., 1991; Niehrs et al., 1993) and noggin (Smith and Harland, 1992), have the ability to induce mesoderm, influence its patterning, or potentiate the morphogenetic movements necessary for various degrees of axial structure formation (for reviews see Smith, 1989; New et al., 1991; Kimelman et al., 1992; Sive, 1993). However, it is unknown whether many of these molecules are part of an endogenous mesoderm induction pathway of the *Xenopus* embryo or if they simply simulate the normal embryonic mesoderm induction mechanism. One problem facing these functional experiments is the paucity of mutations on which background the interaction of these molecules can be studied. A strategy to overcome this obstacle has been the creation of dominant-negative mutations that inhibit protein function. Such experiments have provided evidence for endogenous activin and FGF signaling pathways required for mesoderm induction (Amaya et al., 1991; Hemmati-Brivanlou and Melton, 1992). However, these types of mutations are applicable only to certain types of molecules, such as large ligand-receptor families that may exhibit some degree of functional redundancy and are likely to be involved in macromolecular complexes.
formation of the axial mesoderm, while _exed_ is required for development of the extraembryonic ectoderm (Niswander et al., 1988, 1989).

A third region, previously called implantation-1, is the focus of this report (refer to Fig. 1). Embryos homozygous for the Ai deletions which remove implantation-1 were previously noted to die sometime between implantation and midgestation (Russell and Raymer, 1979; Russell et al., 1982). Complementation studies with the Ai or E group of albino deletions revealed that the region responsible for embryonic lethality mapped proximal to the hepatocyte-specific developmental regulator region (hsdr1) (Fig. 1).

We report here that embryos homozygous for deletions removing implantation-1 do not produce mesoderm. Furthermore, neither ectopic grafts of mutant embryos nor embryonic stem (ES) cells derived from homozygous mutant inner-cell masses formed teratocarcinomas containing differentiated tissues of mesodermal origin, suggesting that the cell autonomous defect affects the mesoderm induction pathway. For this reason, we have renamed the implantation-1 region _msd_, for mesoderm deficiency. By morphological criteria alone, it was difficult to predict accurately to what extent primitive streak formation and gastrulation had occurred in mutant embryos. For this reason, we utilized in situ hybridization techniques to define the mutant phenotype. These results provide molecular evidence that the mutant phenotype manifests itself after the onset of gastrulation, following down-regulation of _Fgf4_ in the epiblast, and prior to down-regulation of _Fgf5_, relocalization of _Fgf2_ to the embryo proper, and activation of primitive-streak markers. The morphological and in situ transcript analyses combined with the ES cell and embryo transfer data suggest that _msd_ is required for mesoderm induction.

**MATERIALS AND METHODS**

**Mice**

The _c<sup>1Dhwb</sup>_ (Ai1) and _c<sup>23DVT</sup>_ (Ai2) albino-deletion (_Df(c)_c) mice used in these experiments originated at the Oak Ridge National Labs (Russell et al., 1979, 1982) and were obtained from Dr. E. M. Rinchik. These mice have been maintained as closed colony, heterozygous stocks with chinchilla (dilute chinchilla offspring), _c<sup>ch</sup>_, and _c<sup>1DthWb</sup>_ (Ai2) albino-deletion [ _Df(ch) _] mice used in these experiments originated at the Oak Ridge National Labs (Russell et al., 1979, 1982) and were obtained from Dr. E. M. Rinchik. These mice have been maintained as closed colony, heterozygous stocks with chinchilla (_c<sup>ch</sup>_). All _Df(c) _ mice are a dilute chinchilla coat color as compared to a full chinchilla color evident in _c<sup>ch</sup>_ mice. For experimental purposes, the stocks were expanded by crossing deletion heterozygotes with C57BL/6 mice (_c<sup>ch</sup>_). All stocks with chinchilla (_c<sup>ch</sup>_). _Df(c) _ mice were a dilute chinchilla coat color as compared to a full chinchilla color evident in _c<sup>ch</sup>_mice. For experimental purposes, the stocks were expanded by crossing deletion heterozygotes with C57BL/6 mice (_c<sup>ch</sup>_). All stocks with chinchilla (_c<sup>ch</sup>_). The albino progeny were then crossed inter se to produce experimental embryos. Noon of the day of the appearance of a vaginal plug was considered 0.5 days of gestation.

**Histology**

For histological examination, embryoid bodies or embryos dissected free from decidua were washed in phosphate-buffered saline (PBS) and fixed 24 hours in 2.5% glutaraldehyde in PBS. These were then rinsed in PBS, dehydrated, and embedded in JB-4 plastic (Polyscience) according to manufacturer’s recommendations. Sections of 4.5 μm were cut and stained with Schiff/periodic acid and counterstained with 0.05% toluidine blue.

For morphological examination, four litters were dissected at 6.5 days of gestation (1 from Ai1×Ai1 and 3 from Ai2×Ai2). A total of 42 embryos were recovered, of which 30 were sectioned. It was not possible to distinguish a distinct class of mutant embryos based on morphological abnormalities (data not shown). The variation between embryos showing primitive-streak formation and mesoderm production was within the range expected for normal litters.

Between 7.5 and 10.5 days of gestation 22 litters from Ai1×Ai1 and 14 from Ai2×Ai2 were analyzed. A deletion class could be identified as early as 7.5 days of gestation and is described in detail below. A total of 224 decidua were dissected from the Ai1×Ai1 crosses. These contained 144 normal embryos, 58 embryos with the deletion phenotype, 5 resorption sites and 2 embryos with abnormalities different from the Ai1 homozygous class. A total of 159 decidua from the Ai2 cross were dissected. These contained 117 normal embryos, 29 embryos with the deletion phenotype, 5 resorption sites, and 2 embryos with abnormalities other than the Ai homozygous class.

**In situ hybridization**

Embryos from either Ai1×Ai1 or Ai2×Ai2 matings were dissected between 6.5 and 8.5 days of gestation, leaving the embryos intact in maternal decidua. Decidua were fixed overnight at 4°C, dehydrated, embedded in paraffin and sectioned to 7 μm thickness (Sassoon et al., 1988). Slide treatment, hybridization, washing and autoradiography were also performed using standard methods (Sassoon et al., 1988; Wang et al., 1992). Antisense and sense mRNA probes were prepared via in vitro transcription using T7, T3 or Sp6 polymerase and [α-<sup>35</sup>S]UTP (>1000 Ci/mmol, NEN). The probes used in this study have all been previously described, and include: partial cDNA clones encoding _Brachyury_ (T) (Herrman et al., 1990), _Evx1_ (Bastian and Gruss, 1990), _Fgf1_ (Yamaguchi et al., 1992), _Fgf2_ (Orr-URTReger et al., 1991), _goosecoid_ (Blum et al., 1992), _H19_ (Poirier et al., 1992) and _Sna_ (Nieto et al., 1992); and full-length cDNAs encoding _Fgf4_ (Hébert et al., 1990) and _Fgf5_ (Hébert et al., 1990). In situ hybridization of sense strand RNA probes did not yield specific signals.

**Production of embryonic stem-cell lines**

Embryonic stem (ES)-cell lines were established from inner-cell masses according to the in vitro culture procedures outlined by Niswander et al. (1988). Briefly, day 3.5 blastocysts were obtained by flushing uterine horns of pregnant females (Ai1×Ai1). The inner-cell masses were isolated by immunosurgery (Solter and Knowles, 1975), plated onto a feeder layer of irradiated STO fibroblast cells and cultured for 2 days in MEMα (Gibco) containing 15% fetal calf serum and 5% LIF (Leukemia-inhibitory factor)-conditioned medium. The primary colonies were then trypsinized and passaged to a new feeder layer. ES-cell lines were established by progressive passage of inner-cell-mass-derived colonies.

Because ES-cell lines were established from embryos obtained from heterozygous crosses, they could either be homozygous or heterozygous for the deletion chromosome, or homozygous for the wild-type, non-deleted chromosome. To genotype the lines, DNA was purified from ES cells that had been passaged off of STO feeders. The purified DNA was cut with EcoRI, fractionated on a 0.7% agarose gel and transferred to Nitran plus membrane (Schleicher and Schuell) using standard procedures. The transferred DNA was crosslinked to membranes using a Stratalinker (Stratagene) and baked at 80°C for 1 hour. The membranes were hybridized at 65°C in _Church Buffer_ (Church, 1984) for 16 hours with riboprobes generated from linearized plasmid DNA (see below). After hybridizing, the membranes were washed three times for 15 minutes in a 0.2x SSC, 0.1% SDS solution at 65°C. Exposure times were typically 1-2 days.

The probes used for genotyping included a T3 riboprobe prepared from BamHI linearized pv0-4AP. This 0.4 kb probe defines the genomic locus _D7Cwr11P_ which maps within the region of chromosome 7 covered by the Ai deletions (Fig. 1) (Sharan et al., 1991). Homozygous ES lines will lack hybridization signal. As a positive control for DNA loading, filters were stripped and rehybridized with a random primer labeled 4.2 kb _Asp_ 718 fragment from pA4.2c11. This probe defines the genomic locus _D7Cwr11D_ located distal to the Ai deletions (Fig. 1) (Sharan et al., 1991).
Embryoid bodies

To induce in vitro differentiation, ES cells were cultured according to the procedures of Martin (1981). Briefly, ES cells were plated onto tissue culture dishes in the absence of feeders. After 3 days of culture, colonies were dislodged from the culture dish by forceful pipetting, allowed to sediment and then grown in suspension on bacteriological Petri dishes. Media was changed daily and samples taken for histology at various times throughout the in vitro differentiation process.

Tumor production

ES-derived
ES cells (5×10^6 in 0.1 ml PBS) were injected subcutaneously into the flank regions of athymic nude mice. After 2-6 weeks, the tumors that formed (approximately 1.5 cm in diameter) were removed, fixed in formalin, embedded in paraffin, sectioned (8 µm) and stained with hematoxylin and eosin.

Embryo-derived
Embryos derived from Ai heterozygous crosses were dissected at days 7.5 and 8.5 of gestation and transplanted into the testes of Ai heterozygous or homozygous wild-type littermate males, using a pulled pipette for transfer of the embryo and for puncturing the testis.

RESULTS

Embryos lacking the msd region, located at the proximal end of the albino deletion complex (Fig. 1), were known to implant but die of unknown causes prior to midgestation (Russell et al., 1982). To determine the time and cause of death in embryos homozygous for deletions removing this region, animals heterozygous for the Ai deletions were mated and litters analyzed between 6.5 and 10.5 days of gestation. Because morphological differences between embryos homozygous for the Ai deletions analyzed were not observed, and the mutant phenotype results solely from the loss of the msd region (Russell and Raymer, 1979; Russell et al., 1982), the homozygous phenotype will be referred to collectively as the msd phenotype and embryos homozygous for the deletion as msd mutants. The numerical results of these crosses are listed in the Experimental Procedures.

\textbf{msd mutant embryos fail to produce mesoderm}

At embryonic day (E) 6.5, it was not possible to distinguish a distinct class of mutant embryos based on morphological abnormalities. The numbers of embryos without visible primitive-streak formation or mesoderm production were within the range expected for normal litters.

\textbf{E7.5 to 8.0}

Normal embryos at this stage have undergone primitive-streak formation and mesoderm production (Fig. 2A). Cells have migrated around the egg cylinder to form anterior mesoderm and into the extraembryonic cavities to line the amnion and form the allantois. The visceral endoderm overlying the extraembryonic portion of the egg cylinder appears as a polarized epithelium with apical vesicles and basal nuclei. At the distal end of the egg cylinder, the visceral endoderm flattens to form a thin layer closely associated with the embryonic ectoderm. The parietal endoderm overlies the entire embryo and consists of widely spaced squamous cells which secrete Reichert’s membrane.

In contrast, msd embryos are retarded in development and more closely resemble pregastrula embryos at E6.0-6.5 (Fig. 2B and 2C). The mutants have well-defined pseudocolumnar embryonic ectoderm and cuboidal extraembryonic ectoderm layers. In some embryos, the embryonic ectoderm has an overall thickened appearance; however, there is no structure morphologically consistent with a primitive streak. The extraembryonic ectoderm develops an asymmetric fold. It is not clear whether this asymmetry is indicative of anteroposterior axis formation. Over the embryonic portion of the egg cylinder, the visceral endoderm undergoes a transition from cuboidal to the characteristic squamous appearance (Fig. 2C). The abnormal parietal endoderm of mutant embryos consists of abundant cells that are large and round in appearance and protrude into the yolk-sac cavity.

\textbf{E8.5 to 10.5}

By E8.5 normal embryos have completed gastrulation and begun organogenesis (Fig. 2D). The primitive streak is regressing and notochord, neural tube, somites, heart and blood islands are present. In contrast, although the size of the mutant embryo has increased, the overall organization has changed little (Fig. 2E). The embryonic ectoderm appears thickened around its entire length as compared to a similar-sized wild-type at E7.5 (compare Fig. 2A and E). A squamous layer of cells in the extraembryonic space appears similar to the ectoderm layer of the amnion. The parietal endoderm layer has increased considerably in size. The number and appearance of the cells are still abnormal compared with that of wild-type embryos. In whole mounts, the mutant embryo consists of a large, expanded parietal endoderm layer surrounding a small egg cylinder.

By E9.5, development of normal embryos has progressed considerably with neurogenesis, somitogenesis and organo-
Genesis underway. In contrast, the development of mutant embryos has changed very little from earlier stages except for the continued expansion of the egg cylinder and parietal endoderm (data not shown). In some embryos, the parietal endoderm layer has expanded nearly three times that of the egg cylinder. The extraembryonic ectoderm has become more disorganized, while the embryonic ectoderm retains its pseudocolumnar form. Although many of the mutant embryos survive until E10.5 with little change, all are resorbed by E11.5.

**Primitive-streak and nascent mesoderm markers are not expressed in the msd mutant epiblast**

Although morphological studies indicated that msd embryos do not produce mesoderm, it was unclear to what extent gastrulation and primitive-streak formation had occurred. For this
reason, mRNA in situ hybridization was used to determine the extent of development relative to molecular markers of gastrulation. To ensure consistent results, in situ analysis was performed on multiple mutants identified by the criteria outlined in the preceding section describing the mdS phenotype. The results of these gene expression studies are described below and summarized in Table 1.

Transcripts normally localized to the forming primitive streak and/or nascent mesoderm were examined in mdS embryos by in situ hybridization. These included Brachyury (T), thought to affect migration of mesoderm cells in the primitive streak (Wilkinson et al., 1990; Rashbass et al., 1991; Wilson et al., 1993); Evx1, a homologue of the Drosophila pair-rule gene even skipped (Bastian and Gruss, 1990; Dush and Martin, 1992); goosecoid, a mouse homologue of a gene implicated in the organization of anterior mesodermal structures in Xenopus (Cho et al., 1991); Sna, a homologue of the Drosophila gene escargot, also expressed in the nascent mesoderm, allantois, ectoplacental cone and parietal endoderm (Nieto et al., 1992; Smith et al., 1993); and fibroblast growth factor receptor 2 (Fgfr2) (Niswander and Martin, 1992). Neither T, Evx1, goosecoid, nor Fgfr2 transcripts could be detected in mdS embryos at E7.5 and/or E8.5. Complete serial sections on sagittal and/or transverse embryo sections were utilized to ensure that gene expression in a small population of cells was not missed. An example of T expression in E6.5 normal and E7.5 mutant embryos is illustrated in Fig. 3A-D. Similarly, Sna transcripts present in the primitive streak and mesoderm of wild-type embryos were not detected in the mdS mutant embryonic ectoderm (epiblast); however, they were detected in the mdS mutant ectoplacental cone and parietal endoderm cells, similar to expression patterns seen in normal embryos (Fig. 3E-H) (Nieto et al., 1992; Smith et al., 1993).

Gene expression unrelated to primitive-streak formation and mesoderm production is normal

In wild-type embryos, Fgf4 transcripts are distributed throughout the epiblast from the time that it gives rise to primitive endoderm (approximately E4.5) until just prior to the onset of gastrulation; at this time expression appears to be down-regulated in the epiblast and ceases completely in cells on one side of the epiblast near the embryonic/extraembryonic junction. Once gastrulation begins, Fgf4 expression becomes highly restricted to the distal two thirds of the primitive streak, in both mesoderm and ectoderm layers (Niswander and Martin, 1992). Because of this dramatic change in spatial expression, we could not determine whether Fgf4 was initially expressed in the mdS mutant epiblast and down-regulated as in wild-type, or whether Fgf4 expression was initiated at all. In situ analysis of five prestreak (E6.0-6.5) litters (prior to when morphological abnormalities could be detected) indicated that Fgf4 was expressed normally in the epiblast in 39 of 44 embryos examined. 25% of these embryos should have been mdS homozygotes (11 of 44). Chi square analysis indicated that there was a greater than 90% probability that five non-expressing embryos could be found by chance. Thus, it is very likely that mdS homozygotes express Fgf4 at E6.0-6.5 and that the gene is down-regulated normally in these mutants.

Having established that mdS embryos do not express primitive-streak/early mesoderm markers, we examined the distribution of other transcripts temporally regulated during gastrulation. Distinct Sna, H19 and Fgfr2 expression patterns mark a variety of extraembryonic structures. As described above, in mdS mutants, Sna appears to be normally expressed in the existing extraembryonic tissues. The abundant non-translated H19 mRNA in normal embryos is expressed in a variety of extraembryonic cell types during gastrulation, including: ectoplacental cone, extraembryonic ectoderm, parietal and visceral endoderm, amnion ectoderm, extraembryonic mesoderm and trophoblast giant cells (Fig. 3L) (Poirier et al., 1992). H19 is similarly expressed in tissues present in mdS mutants (Fig. 3K,L).

Development of mdS mutants is arrested after the onset of gastrulation-related gene expression

In wild-type embryos, prior to primitive-streak formation, Fgfr2 transcripts are distributed throughout the epiblast and extraembryonic ectoderm (Orr-Urtreger et al., 1991). After the onset of primitive-streak formation, expression is down-regulated in the embryo proper, but transcripts continue to be expressed in the extraembryonic ectoderm, including: ectoplacental cone, extraembryonic ectoderm, parietal and visceral endoderm, amnion ectoderm, extraembryonic mesoderm and trophoblast giant cells (Fig. 3L) (Poirier et al., 1992). H19 is similarly expressed in tissues present in mdS mutants (Fig. 3K,L).

### Table 1. Gene expression in mdS mutant embryos

<table>
<thead>
<tr>
<th>Gene</th>
<th>E7.5 No. of mutant embryos examined</th>
<th>Transcript localization1 in wild-type and mutant embryos</th>
<th>Transcript localization1</th>
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<td></td>
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1 Tissues examined are abbreviated as follows: ee, embryonic ectoderm; ps, primitive streak; ve, visceral endoderm; xe, extraembryonic ectoderm; ec, ectoplacental cone; and pe, parietal endoderm.

2 Expression data are given for wild-type E7.5 embryos; see text for further details.

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epiblast, in primitive-streak mesoderm and in squamous visceral endoderm adjacent to expressing epiblast (Fig. 4G,H) (Hébert et al., 1990; Haub and Goldfarb, 1991); however, upon completion of germ-layer formation (by E7.75), expression ceases until later in development. In situ analysis of E7.5-8.5 mutant embryos revealed abundant expression of Fgf5 (Fig. 4I-L). The continued expression of Fgf5 in E8.5 mutants contrasts the absence of gene expression in wild-type E8.5 embryos.

**The effect of the msd mutation is restricted to mesoderm formation**

*msd* embryos develop normally until the beginning of gastru-

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**Fig. 3.** Gene expression in *msd* mutant embryos. In situ hybridization was performed on E6.5-8.5 mouse embryo sections using 35S-labeled antisense RNA probes corresponding to *Brachyury T* (A-D), *Sna* (E-H) and *H19* (I-L). Phase-contrast (A,C,E,G,I,K) and dark-field (B,D,F,H,J,L) photomicrographs are shown. (A-D) *Brachyury T* gene expression in wild-type E6.5 (A,B) and E7.5 *msd* mutant (C,D) embryos. Shown are transverse sections through the embryonic ectoderm located near the embryonic-extraembryonic ectoderm boundary. *T* is expressed in the forming primitive streak of the wild-type embryo, but not in the mutant. (E-H) *Sna* gene expression in mid-line sagittal sections of wild-type E7.5 (E,F) and E8.5 *msd* mutant embryos (G,H). *Sna* transcripts are localized to the primitive streak and nascent mesoderm, allantois, ectoplacental cone and parietal endoderm in the wild-type embryo, and are detected in the ectoplacental cone and parietal endoderm in the mutant. The strong hybridization signal seen in cells in the uterine cavity in E,F represents non-specific trapping of the probe and was also seen when the *Sna* sense oligonucleotide probe was hybridized to an adjacent section (not shown). (I-L) *H19* gene expression in sagittal sections of E7.5 wild-type (I,J) and E8.5 *msd* mutant embryos (K,L). *H19* is expressed in trophoblast giant cells, ectoplacental cone, extraembryonic ectoderm, visceral endoderm and amnion ectoderm in both wild-type and mutant embryos. Approximate magnification, 75×. See Fig. 2 for abbreviations.
Fig. 4. Gene expression in msd mutant embryos. In situ hybridization was performed on E7.5-8.5 mouse embryo sections using 35S-labeled antisense RNA probes corresponding to Fgfr2 (A-F) and Fgf5 (G-L). Phase-contrast (A,C,E,G,I,K) and dark-field (B,D,F,H,J,L) photomicrographs are shown. (A-D) Fgfr2 gene expression in E7.5 (A,B) and E8.5 (C,D) wild-type, and E8.5 msd mutant (E,F) embryos. (A,B) A parasagittal section of an E7.5 wild-type embryo is shown, with Fgfr2 transcripts localized to extraembryonic ectoderm (indicated by asterisk). (C,D) A sagittal section taken near the midline of a wild-type E8.5 embryo. Fgfr2 appears to be down-regulated in extraembryonic ectoderm (chorion), and transcripts are detected in somites, surface ectoderm and cranial neural ectoderm. (E,F) Sagittal section of an E8.5 msd mutant littermate of the embryo in C,D demonstrates Fgfr2 gene expression in extraembryonic ectoderm, similar to that seen in B (indicated by arrowhead). (G-L) Fgf5 gene expression in wild-type E7.5 (G,H) and E7.5 (L) and E8.5 (K,L) msd mutant embryos. (G,H) Sagittal section of an E7.5 wild-type embryo. Fgf5 transcripts are abundant throughout the embryonic ectoderm and in the most anterior portion of the primitive streak mesoderm. They are also detected in squamous visceral embryonic ectoderm. (I,J) Sagittal section of an msd mutant, a littermate to the embryo in G, H, showing similar expression in the embryonic ectoderm and visceral endoderm. (K,L) Oblique sagittal section of an E8.5 msd mutant in which Fgf5 transcripts are localized to the embryonic ectoderm. Transcripts are not expressed in the visceral endoderm in this section, which is likely due to the fact that these cells have a more cuboidal morphology characteristic of extraembryonic visceral endoderm, cells that do not normally express Fgf5 (Hebert et al., 1990; Haub and Goldfarb, 1991). Approximate magnification, 75×. See Fig. 2 for abbreviations.
lation. There appears to be a block in primitive-streak formation and mesoderm production, indicating that a gene(s) necessary for mesoderm production is removed by the deletions. Thus, we wanted to determine if cells homozygous for these deletions could form mesoderm in vitro in the absence of primitive-streak formation.

Embryonic stem (ES) cell lines were established as described in Experimental Procedures. Stem cell lines were genotyped by Southern blot analysis using DNA markers defining chromosomal loci D7Cwr11P, located within the region of DNA removed from the c^{ch} and Ai deletions (see Fig. 1). D7Cwr11D, located outside the deletions (refer to Fig. 1). The D7Cwr11P locus is deleted in c^{ch} homozygous DNA and the msd homozygous lines (Fig. 5, lane 3-6), but present in control DNAs (Fig. 5, lane 1). In contrast, the tumors obtained from all three homozygous lines consisted primarily of undifferentiated cells mixed with a poorly differentiated, pseudocolumnar epithelium resembling that of a 6.5 day epiblast (Fig. 6D). Occasionally regions of neural differentiation (Fig. 6E) and keratinized epithelium (Fig. 6F) were present, suggesting that msd cells are capable of ectodermal differentiation. Similar results were obtained when homozygous msd embryos were grafted into ectopic locations (compare wild-type embryo grafts to msd embryo grafts in Fig. 6G and H, respectively). msd embryos formed small tumors consisting of predominantly columnar ectoderm and devoid of highly differentiated mesoderm structures. In contrast, wild-type embryos formed tumors containing differentiated structures derived from all three germ layers.

**DISCUSSION**

At 7.5 days of gestation, msd embryos can be distinguished from wild-type littermates by their prestreak egg cylinder appearance and lack of mesoderm. The mutant embryos are viable for several days, during which time their size increases, but no further differentiation occurs. Investigation of gene expression relevant to gastrulation provided molecular evidence that the msd defect affects primitive-streak formation and mesoderm production. In the msd embryo, neither Brachyury (T), Sna, Evx1 nor goosecoid are expressed in the E7.5-8.5 epiblast. The primary defect in msd embryos could result from the absence of any (or a combination) of these gene products. However, because the initial formation of primitive streak and production of mesoderm is not affected in T-deficient embryos (Chelsey, 1935; Grüneberg, 1958), the msd
The phenotype is not likely to result directly from the lack of T gene product alone.

The restriction of the msd defect to mesoderm formation is substantiated further by the ability of msd embryos and homozygous msd embryonic stem cells to form teratocarcinomas lacking mesodermal derived tissues but containing cell types of ectodermal origin only, including columnar ectoderm, neural bodies or keratinized epithelium. The potential for msd cells to contribute to other lineages of the mouse embryo is currently being examined by injection of msd embryonic stem cells into the blastocyst as well as production of aggregation chimeras.
Similar experiments have been performed analysing the potential of ES cells derived from embryos homozygous for the 413.d proviral insertion to differentiate. Like msd embryos, 413.d homozygous embryos lack mesoderm (Iannaccone et al., 1992). In contrast, ES cells homozygous for the 413.d proviral insertion have the capacity to form a wide variety of differentiated tissues including those of mesoderm origin (Conlon et al., 1991) when cultured in vitro or injected subcutaneously. These results suggest that factors capable of inducing mesoderm differentiation are supplied both in vitro as well as by the subcutaneous environment and provide indirect evidence that the lack of msd ES cell mesoderm differentiation could result from the inability to respond to an inducing signal.

The correct spatial and temporal distribution of transcripts in several mutant cell types further suggests that the development of trophectoderm or other embryonic ectoderm-derived cell types is not affected. H19 and Sna transcripts are distributed normally in msd ectoplacental cone, parietal endoderm and visceral endoderm, suggesting that gene expression in these tissues is normal. In addition, Fgfr1, Fgfr2, Fgf5 and Fgf4 transcript localization suggests that transcriptional regulation is normal in the epiblast at E6.0-6.5 and E7.5.

While the temporal and spatial expression of Fgf5, Fgfr2 and Fgf4 initially appears normal in msd mutants, the distributions of these transcripts in older embryos is aberrant. For example, expression of Fgfr2 in extraembryonic ectoderm and Fgfr1 and Fgf5 in the epiblast of E7.5 msd mutants is similar to that of wild-type embryos. These spatial expression patterns continue at E8.5 in mutants. In contrast, by E8.5 the expression patterns in wild-type embryos change dramatically: Fgfr2 expression shifts to embryonic tissues undergoing a high degree of differentiation, including the somites, cranial neural ectoderm and the heart primordium; Fgfr1 expression becomes localized to somites and presomitic mesoderm; and Fgf5 expression is completely shut off. Thus, altered expression of Fgfr1, Fgf5 and Fgfr2 in msd mutant embryos reflects a developmental delay as well as a correlation between changes in gene expression and germ-layer formation.

Recent investigations demonstrated that Fgf4 gene expression is down-regulated throughout the mouse epiblast just prior to gastrulation; once gastrulation begins, Fgf4 expression becomes restricted to the distal two thirds of the primitive streak (Niswander and Martin, 1992). These results suggest that Fgf4 might be involved in maintaining the pluripotency of the epiblast cell population and may be subject to multiple modes of regulation in early postimplantation mouse development (Niswander and Martin, 1992). Absence of Fgf4 transcripts in E7.5 msd mutants demonstrates that its expression is similarly down-regulated, but subsequently fails to be localized. These results suggest that Fgf4 may be regulated by distinct mechanisms and that the msd gene product(s) is required for events leading to the expression of Fgf4 in the primitive streak.

The correct distribution of early Fgf4, Fgf5, Fgfr-1 and Fgfr2 transcripts in msd embryos provides evidence for their independence from msd at that time. In contrast, later in development, these genes are incorrectly expressed. These findings suggest that Fgf4, Fgf5, Fgfr-1 and Fgfr2 may be regulated by distinct mechanisms; the msd gene product is necessary for later expression associated with primitive streak formation and mesoderm production. In summary, the msd phenotype appears to manifest itself after down-regulation of Fgf4 and localization of Fgfr2 transcripts to the extraembryonic ectoderm, but prior to localization of Fgf4. Brachyury (T), Sna, Exv1 and goosecoid transcripts to the primitive streak, down-regulation of Fgf5 in the epiblast and a change in localization of Fgfr1 and Fgfr2.

 transcript distribution patterns in msd mutants provide molecular evidence that gene expression related to gastrulation has begun. Whether the msd gene product(s) is directly involved in these regulatory events or simply required for initiating a cascade of events necessary for germ-layer formation is unknown. The ability of msd embryos or msd embryonic stem cells to form differentiated ectodermal cell types indicates that the effects of the msd mutation are not cell lethal, and are probably cell autonomous. These data suggest that the block in the mesoderm induction pathway could result from the inability of msd cells to respond to an inducing signal. Future studies to determine whether mesoderm-inducing signals are present in msd embryos can utilize embryo recombination experiments (Ang and Rossant, 1993) and in vitro perturbation of explants (Burdasal et al., 1993) as well as assays for the ability to induce secondary axis formation (Blum et al., 1992). In addition, the effects of down-stream primitive-streak or nascent mesoderm gene expression on mesoderm induction can be studied in msd homozygous stem cells.

The albino-deletion complex represents one of the best-characterized series of mutations produced in the specific locus test (Russell, 1952; Russell et al., 1982). The completeness of complementation studies, number of deletion chromosomes available and density of molecular markers has catalyzed the search for candidate genes defined by functional regions. For example, the neonatal lethality associated with loss of the 310 kb hsdrl region has been attributed solely to loss of a single gene encoding fumarylacetacetate hydrdrolase (Fah) (Niswander et al., 1991; Kelsey et al., 1992, 1993; Klebig et al., 1992b; Grompe et al., 1993). In addition, the combined efforts of saturation mutagenesis and physical mapping studies demonstrate that the phenotype associated with deletion of the eed region is likely to result from a single gene defect within a maximum 200 kb region (Rinchik and Carpenter, 1993) and B. Holdener and T. Magnuson, unpublished data). Finally, the localization of the exed region to a 20 kb interval (S. K. Sharan and T. Magnuson, unpublished results) suggests that a single gene might be involved in this case as well. Deletion breakpoints that define the molecular limits of the msd region have been identified (Kelsey et al., 1992; Klebig et al., 1992a; Schell et al., 1992) and B. Holdener and T. Magnuson, unpublished data). Cloning these breakpoints will facilitate the identification of candidate gene(s) within the msd region required for mesoderm induction.

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