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A targeted mouse *Otx2* mutation leads to severe defects in gastrulation and formation of axial mesoderm and to deletion of rostral brain

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SUMMARY

Mouse *Otx2* is a *bicoid*-class homeobox gene, related to the *Drosophila orthodenticle (otd)* gene. Expression of this gene is initially widespread in the epiblast at embryonic day 5.5 but becomes progressively restricted to the anterior end of the embryo at the headfold stage. In flies, loss of function mutations in *otd* result in deletion of pre-antennal and antennal segments; which leads to the absence of head structures derived from these segments. To study the function of *Otx2* in mice, we have generated a homeobox deletion mutation in this gene. Mice homozygous for this mutation show severe defects in gastrulation and in formation of axial mesoderm and loss of anterior neural tissues. These results demonstrate that *Otx2* is required for proper development of the epiblast and patterning of the anterior brain in mice, and supports the idea of evolutionary conservation of the function of *Otd/Otx* genes in head development in flies and mice.

Key words: *Otx2*, neural tube, patterning, gastrulation, notochord, prechordal mesoderm, node, gene targeting

INTRODUCTION

The patterning and development of the vertebrate neural tube is a complex process involving both cell extrinsic and cell intrinsic events. Among the cell extrinsic events, the mesoderm in the organizer region, namely the dorsal blastopore lip in amphibians, Hensen's node in birds and the node in the mouse, is able to induce neural differentiation in the surrounding ectoderm tissue (Spemann, 1938; Waddington, 1933; Beddington et al., 1994). Furthermore, in vitro experiments have demonstrated that the prechordal mesoderm and notochord, descendants of the organizer, can also induce and pattern the neural tube along the anteroposterior axis in *Xenopus* embryos (reviewed in Slack and Tannahill, 1992; Doniach, 1993; Ruiz i Altaba, 1994). Support for a role of the prechordal mesoderm in the induction of forebrain and midbrain has also come from the analysis of the phenotype of homozygous *Lim1* mutant embryos, where lack of prechordal mesoderm cells in these mutant mice is suggested to be responsible for the subsequent loss of anterior brain tissues (Shawlot and Behringer, 1995). However, the role of notochord in anteroposterior (A-P) patterning of the neural tube is still in question, since embryos mutant for the winged-helix domain gene, *HNF-3β*, lack an organized node and notochord but show relatively normal A-P patterning of the CNS (Ang and Rossant, 1994; Weinstein et al., 1994).

A major contribution to the identification of cell intrinsic molecules responsible for neural tube regionalization has come from cloning of genes homologous to homeobox-containing genes within the *HOM-C* complex in *Drosophila* (reviewed in Lawrence and Morata, 1994), namely the *Hox* genes in mice. The function of these genes in the regional specification of hindbrain and vertebrae has been demonstrated using both loss-of-function and gain-of-function genetic experiments (reviewed by Krumlauf, 1994). *Hox* genes are not expressed in the forebrain and midbrain, suggesting that other classes of homeobox genes are involved in the development of these rostral regions. Recently, two new classes of homeobox genes, related to the *Drosophila orthodenticle (otd)* (Finkelstein et al., 1990) and *empty spiracles (ems)* gene (Dalton et al., 1989), have been cloned. These genes, *otx1*, *otx2*, *emx1* and *emx2*, are expressed in nested domains in the forebrain and midbrain regions (Simeone et al., 1992a). Since *otd* and *ems* have been shown to participate in a regulatory network required for head formation in flies, it has been suggested that the conserved murine genes serve similar roles in the patterning of rostral brain in mice (Finkelstein and Boncinelli, 1994).

The mouse *otd*-related genes, *Otx1* and *Otx2*, belong to the bicoid-class of homeobox genes and the amino acid sequence of their homeobox differ by two and three amino acids from that of the *otd* gene respectively (Simeone et al., 1993). These genes are expressed in overlapping domains in the anterior

CNS with the domain of *Otx1* expression encompassed within the *Otx2* domain. *Otx2* is already expressed by embryonic day 5.5 (E5.5), while the expression of *Otx1* mRNA is not detected until early E8.0. *Otx2* expression at E5.5 is widespread in the epiblast, which gives rise to the embryo proper. From the early primitive streak to headfold stages, *Otx2* expression in the ectoderm becomes restricted to the anterior end of the embryo (Simeone et al., 1993, Ang et al., 1994). We and others have previously shown that this *Otx2* expression in the anterior ectoderm depends on interactions with the underlying mesoderm at the anterior end of the embryo in mice and *Xenopus* (Ang et al., 1994; Pannese et al., 1995; Blitz and Cho, 1995). *Otx2* expression was also found in anterior mesendoderm tissues, including axial mesoderm tissues, such as notochord and prechordal mesoderm (Ang et al., 1994; Simeone et al., 1995). The earlier expression of *Otx2*, compared to *Otx1*, and its expression in axial mesoderm tissues that possess neural patterning capabilities suggests that this gene could be involved in the patterning of anterior neural tissues.

To begin to dissect the roles of *Otx2* in vivo, we have generated a homeobox deletion in the gene using homologous recombination in ES cells. This mutation results in early gastrulation defects. Homozygous *Otx2^{hd}* mutant embryos also lack the prechordal mesoderm and notochord precursors by the headfold stage. By E8.25, rostral deletion of the neural tube anterior to rhombomere 3 was clearly apparent. These defects are consistent with multiple roles for *Otx2* in gastrulation and the patterning of rostral brain in mice.

MATERIALS AND METHODS

Targeting vectors

A 1 kb mouse *Otx2* partial cDNA probe was used to isolate two overlapping genomic clones, containing the entire coding region of the *Otx2* gene from a 129SV/J genomic library. To construct the targeting vector, pPNTO2, a 1.7 kb *Bgl*II-*Xba*1 piece of 5′ homology was first subcloned into the *Bam*HI-*Xba*I site of pPNT (Tybulewicz et al., 1991). The 3′ 4.4 kb homology *Stu*I fragment was subsequently cloned into the *Xho*I site of the above vector, with the PGKneo and PGKtk cassettes in the opposite transcriptional orientation compared to the endogenous *Otx2* gene.

Generation of the mutation

The culture, selection and electroporation with *Not*I-linearized pPNTO2 of R1 ES cells was carried out as described (Wurst and Joyner, 1993). ES cell colonies that were resistant to 150 μg/ml of G418 and 2 μm gancyclovir were analyzed by Southern blotting for homologous recombination events. Genomic DNA from these cell lines was digested with *Spe*I and *Eco*RI and probed with a 2.0 kb *Hin*dIII-*Xba*I 3′ flanking probe and a 1.7 kb *Bgl*II-*Xba*I 5′ internal probe. Hybridization was carried out in 50% formamide, 200 mM phosphate buffer (pH 7.3), 1 mM EDTA, 10 mg/ml of BSA, and 7% SDS at 63°C.

Chimeras were generated by blastocyst injection and ES-morulla aggregation with targeted ES lines. Chimeric males were bred to CD1 females to establish F_1 heterozygotes. Embryos from F_1 heterozygous intercrosses were typed either by Southern analysis or by PCR of yolk sac DNA. To detect a 1.3 kb band present in the wild-type allele but deleted in the *Otx2^{hd}* allele, the following primers were used: sense strand (5′-ATGATGTCTTATCTAAAGCAACCGCCTTACG-3′) and antisense strand (5′-TCATTGGGTCATCAGTATAAACCA-3′). The *Otx2^{hd}* mutant allele was detected as a 650 bp neo fragment using a set of primers corresponding to sense strand oligonucleotide in the neo gene (5′-ATCTCCTGTCATCTCACCTTGC-3′) and antisense PGK poly(A) sequence (5′-ACCCCACCCCCACCCCCGTAGC-3′). Samples were amplified for 35 cycles (94°C 40 seconds; 55°C 1 minute; 72°C 1.5 minutes) for the wild-type allele and for 40 cycles (94°C for 1 minute; 65°C for 1 minute; 72°C for 2 minutes) for the mutant allele. Amplified bands were visualized by agarose gel electrophoresis and ethidium bromide staining.

In situ hybridization, immunohistochemistry and histology

Whole-mount in situ hybridization was performed as described previously (Conlon and Herrmann, 1993). For histology and section in situ hybridization, embryos were fixed overnight in 4% paraformaldehyde in PBS. They were then processed and embedded in wax and sectioned at 7 μm. Slides were then dewaxed, rehydrated and stained with hematoxylin and eosin. In situ hybridization of sections was performed as described by Guillemot and Joyner (1993). The RNA probes used were:- *Flk-1* (Yamaguchi et al., 1993), a 1.0 kb *Pem-1* cDNA containing the entire protein coding sequence, *gsc* (Blum et al., 1992), *Lim1* (Barnes et al., 1994), *Brachyury* (Hermann, 1991), *Mox-1* (Candia et al., 1992), *HNF-3β* (Ang et al., 1993), *Emx-2* (Simeone et al., 1992b), a 2.1 kb *BF-1* cDNA containing the entire protein coding sequence, *Netrin-1* (Serafini et al., 1994); *Krox-20* (Wilkinson et al., 1989b) and *Hoxb-1* (Wilkinson et al., 1989a).

Whole-mount immunocytochemistry was performed according to the method of Davis et al. (1991), except that embryos were fixed in 4% paraformaldehyde in PBS for 2 hours. The En-2 antiserum was used at a dilution of 1:50. The secondary antibody was peroxidase-coupled goat anti-rabbit (Jackson laboratory) which was used at a dilution of 1:200.

RESULTS

Targeted disruption of *Otx2* in ES cells by homologous recombination

A positive/negative targeting vector containing 1.7 kb of 5′ and 4.4 kb of 3′ genomic sequences (Fig. 1A), was designed to delete the homeodomain region of *Otx2*, and replace it by the PGKneo cassette from the pPNT vector (Tybulewicz et al. 1991). Therefore the PGKneo insertion should truncate the Otx2 coding sequence immediately after the first exon which contains 32 amino acids. This allele is referred to as the *Otx2^{hd}* allele.

The linearized targeting vector was electroporated into ES cells (Nagy et al., 1993) and clones were selected for resistance to G418 and gancyclovir. 350 double resistant clones, obtained after electroporation of the pPNT02 targeting vector, were analyzed by Southern blot using a 5′ internal and a 3′ external probe. Five cell lines, including 5-23, and TE123, yielded the 3.5-kb *Spe*I band and 13.2-kb *Eco*RI band expected from a homologous recombination event (Fig. 1B). Using a genomic probe spanning exon 2 (Fig. 1A), the deletion of the homeobox region was confirmed in the genomic DNA of homozygous *Otx2^{hd}* mutants by Southern blot analysis (data not shown).

The 5-23, TE123 and C-12 targeted ES cell lines were used to generate chimeras by either ES cell-morulla aggregation (Nagy et al., 1993) or by blastocyst injection. These chimeras transmitted the mutation to their progeny. Mice generated from all three lines showed identical phenotypes. The analysis

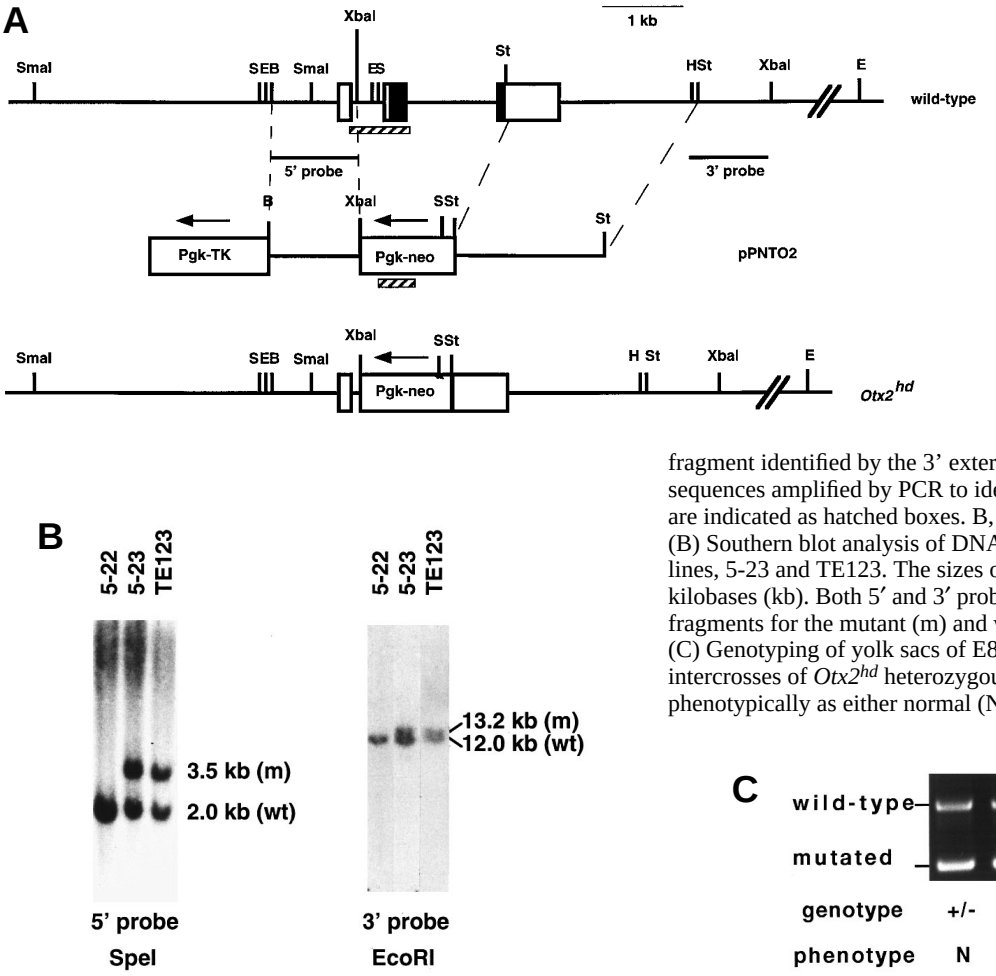

Fig. 1. Targeted disruption of the *Otx2* gene. (A, B) The targeting vector, pPNTO2, contains 7.1 kb of genomic sequences of the *Otx2* genomic locus. The open boxes represent the coding region while the solid boxes indicate the homeodomain. The 5′, 3′ and exon 2 probes used for Southern blot analysis are indicated. Homologous recombination between pPNTO2 and the wild-type allele replaces the homeodomain by the PGKneo gene, resulting in a bandshift from 2 to 3.5 kb upon *Spe*I digestion with the 5′ probe. Digestion of genomic DNA with *Eco*RI shifts the restriction fragment identified by the 3' external probe from 12 to 13.2 kb. The sequences amplified by PCR to identify the wild-type and $Otx2^{hd}$ alleles are indicated as hatched boxes. B, *Bgl*II; E, *Eco*RI; S, *Spe*I; St, *Stu*I. (B) Southern blot analysis of DNA from control line 5-22 and targeted ES lines, 5-23 and TE123. The sizes of the DNA bands are indicated in kilobases (kb). Both 5′ and 3′ probes detected predicted restriction fragments for the mutant (m) and wild-type (wt) allele. (C) Genotyping of yolk sacs of E8.5 embryos from heterozygous intercrosses of $Otx2^{hd}$ heterozygous mice. Embryos were scored phenotypically as either normal (N) or mutant (M).

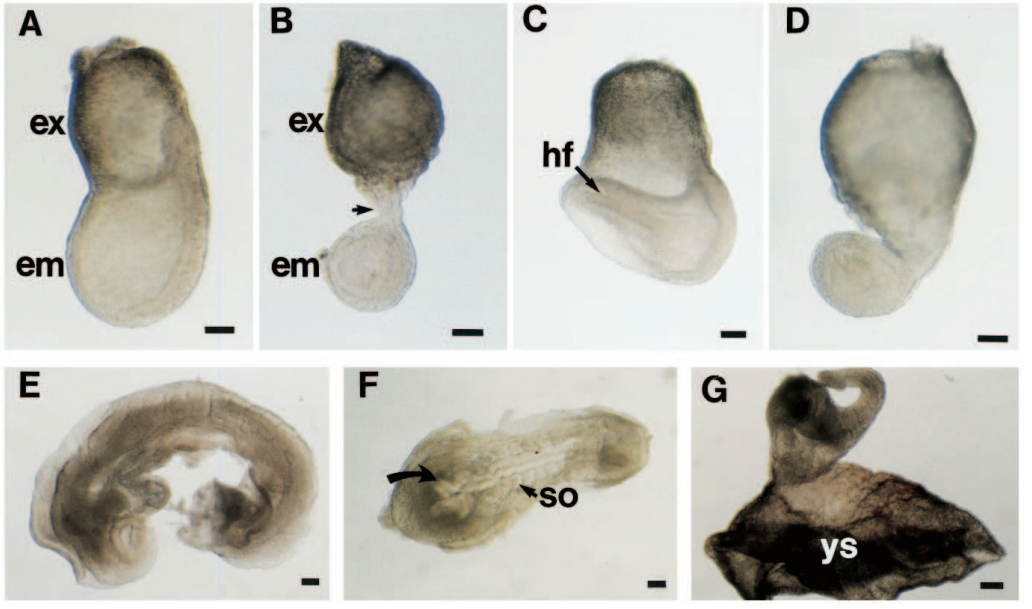

Fig. 2. Phenotype of *Otx2* homozygous embryos. A,C,E show wild-type or heterozygous embryos and B,D,F,G show homozygous embryos at E7.5-8.5. (A,B) E7.5 embryos. In mutant embryos, a constriction is seen between embryonic and extraembryonic regions (arrow in B), and the embryonic portion (B) is smaller than in wild-type embryos (A). (C,D) E7.7.5 embryos. Headfolds seen at the anterior end of normal embryos (arrow in C) at this stage are not visible in mutant embryos (D). (E,F,G) E8.5 embryos. Examples of less severely (F) and severly (G) affected homozygous mutants. The curved arrow points to the abnormal anterior neural tube (F). The severely affected mutant embryos are completely excluded from the yolk sac (G). em, embryonic region; ex, extraembryonic region; hf, headfold; so, somites, ys, yolk sac. Anterior is to the left. Scale bar, 100 μm.

reported here is primarily from the 5-23 line. All analyses were carried out on a mixed CD1/129 background.

The *Otx2^{hd}* mutation leads to embryonic lethality

When heterozygous animals were crossed with wild-type CD1 females and their progeny was genotyped at 3 weeks of age, heterozygous mice were obtained with at a frequency of 141/311, less than the expected 50% ratio. These heterozygous mice appeared normal and were fertile. However, when offsprings from heterozygous intercrosses were harvested at E9.5 ane E10.5, a small fraction (8/149) of normal size embryos showed an open neural tube defect at the forebrain and midbrain levels. Caudal to the midbrain region, these embryos looked identical to wild-type embryos (data not shown). All these embryos have been genotyped to be heterozygous animals. The *Otx2^{hd}* mutation thus result in a heterozygous phenotype that is weakly penetrant on the CD1/129 background, and this heterozygous phenotype may explain the slightly lower number of heterozygous animals obtained at 3 weeks of age. This phenotype will be analyzed in more detail elsewhere.

When intercrosses of *Otx2^{hd}* heterozygous animals were analyzed at birth, no homozygous newborn animals were found, indicating that Otx2 is required for embryonic development (Table 1). To characterize the embryonic lethality, we analyzed litters from heterozygous intercrosses from E7.25 to E10.5. Homozygous mutant embryos were present between E7.25 and E9.5 at roughly the expected frequency of 25%, however, at E10.5, the proportion of mutants obtained declined to 17% and these mutant embryos were either severely growth retarded or being resorbed (Table 1). Thus, the *Otx2^{hd}* mutation leads to embryonic lethality around E10.5. The results from a typical genotyping analysis of yolk sacs from E8.5 embryos generated from *Otx2^{hd}* heterozygous intercrosses is illustrated in Fig. 1C.

Table 1. Genotype of mice resulting from *Otx2^{hd}* heterozygous intercrosses

Stage	+/+	*Otx2^{hd}*/+	*Otx2^{hd}*/*Otx2^{hd}* (%)
7.5	12	27	13 (25)
8.5	24	43	22 (25)
9.5	16	32	14 (23)
10.5	15	23	8 (17)*
Postpartum	18	36	0

*Embryos were either severely growth retarded or being resorbed.

Morphological and histological analysis of the *Otx2^{hd}* homozygous phenotype

By E7.25-E7.5 (mid- to late-streak stage), homozygous embryos were morphologically distinguishable from normal embryos. The abnormal looking embryos were smaller in the embryonic portion than their littermate (Fig. 2A,B), and these embryos were confirmed to be homozygous mutant by PCR analysis (Table 1). Sections through mutant embryos at this stage demonstrated clearly that these embryos had initiated gastrulation and mesoderm had formed all around the embryo. Embryonic ectoderm, mesoderm and endoderm cells are present in mutant embryos; however some of the ectoderm cells appeared disorganized and lost their characteristic epithelial structure (Fig. 3A-C).

In the extraembryonic region, the yolk sac looked abnormal and displayed a characteristic ruffled appearance in the endoderm layer (Fig. 3B,C), which reflected a reduced, discontinuous underlying extraembryonic mesoderm layer. The yolk sac was also constricted in the position normally occupied by the amnion, thus isolating the extraembryonic from the embryonic region (Fig. 3A-C). The allantois was poorly developed and formed a round ball of cells, in contrast to its fairly long and extended appearance in normal embryos at this stage. It was also often disconnected from the embryonic portion of the conceptus (Fig. 3C and data not shown).

At E7.75 (headfold stage), the headfolds forming at the rostral end of wild-type embryos were not apparent in homozygous mutant embryos (Fig. 2C, D). At E8.5 (somite stage), variations in the phenotype of the mutants were found. We have divided the mutants at this stage into a less severe class, which includes embryos enclosed in the yolk sac, and a more severe class whereby the embryos are completely excluded from the yolk sac (Fig. 2G). In the less severely affected class of mutants, there was also fairly good development of somites posteriorly and a rudimentary heart (Fig. 2F and data not shown). At the anterior end, the neural tube looked extremely abnormal with either numerous folds or a single fused structure instead of open neural folds (Figs 2F, 5E). Compared to wild-type or heterozygous embryos, no forebrain or midbrain tissue could be identified suggesting that deletion of neural tissue had occurred anteriorly. The somites were not always normal; in some embryos they were fused at the midline or were irregularly shaped (Fig. 5E). The more severely affected homozygous mutant embryos were smaller than the first class of mutants, wrapped up anteriorly in definitive endoderm and showed improper segmentation of mesoderm into somites (Fig. 2G). Some of the mutants in this class were very thin and spiral-shaped and showed no sign of organogenesis and segmentation of mesoderm into somites (data not shown). The two different classes of mutants occurred at approximately the same frequency.

Defects in gastrulation and extra-embryonic structures in *Otx2^{hd}* homozygous embryos

The small size and abnormal morphology of E7.25 embryos demonstrated that defects had already occurred in homozygous mutants at this early stage. To determine if defects occurred in the primitive streak, we analyzed the expression of the genes *goosecoid (gsc)* and *Brachyury*. *goosecoid* is a homeobox gene expressed in the anterior primitive streak region in mid- to late-streak stage embryos (Fig. 4A and Blum et al., 1992). *goosecoid*-expressing cells were found ectopically located in the proximal region of homozygous mutants in one case and was absent in three other embryos analysed (Fig. 4B and data not shown). Previous studies have demonstrated that *gsc* is expressed transiently in the anterior primitive streak in wild-type embryos (Blum et al., 1992). Since it is difficult to accurately stage the homozygous mutant embryos due to their abnormal morphology, we cannot distinguish whether the mutant embryos that fail to

express *gsc* do so because they are older than the late-streak stage or because *Otx2* is required for the maintenance of *gsc* expression at this early stage. The *Brachyury* gene is expressed along the entire proximal-distal extent of the primitive streak at the mid- to late-streak stage (Hermann, 1991). In some homozygous mutants, *Brachyury* expression was only found in the proximal region in a similar position as *gsc*-expressing cells in *Otx2^{hd}* homozygous mutants (data not shown), while others showed almost complete extension but a thickening of the region of the primitive streak labeled (Fig. 4F). Together, these results demonstrate that *Otx2^{hd}* homozygous mutants show severe early gastrulation defects, characterized by a lack of proper primitive streak organization.

To assess the presence in mutants of the different extraembryonic tissues, we analysed the expression of the extraembryonic markers *Flk1* and *Pem*. *Flk1* is expressed in wild-type E7.5 embryos in nascent mesoderm of the proximal part of the primitive streak, extraembryonic yolk sac mesoderm, and proximoanterior embryonic mesoderm (Fig. 3E and Yamaguchi et al., 1993). *Flk-1* expression in homozygous mutant embryos at this stage was detected in all these tissues indicating that specification of mesoderm derived from the proximal primitive streak is occurring normally. However, in some cases there was abnormal accumulation of mesoderm cells in the proximal region between the embryonic and extraembryonic regions (Fig. 3H). *Pem* is a homeobox gene expressed specifically in extraembryonic tissues (Fig. 3F and Lin et al., 1994). All the *Pem*-expressing tissues (yolk sac, chorion, primitive endoderm and ectoplacental cone) could be recognized in the mutant embryos, but the yolk sac looked abnormal in the mutants between the embryonic and extraembryonic regions (Fig. 3I).

Prechordal mesoderm and notochord are severely affected in *Otx2^{hd}* mutant embryos

To determine if the different populations of embryonic mesoderm tissues were present in mutants at E8.5, the markers *Brachyury*, *Mox-1* (Candia et al., 1992) and *Lim1* (Barnes et al., 1994) were used to identify axial, paraxial and lateral mesoderm cells, respectively. Both *Mox-1* and *Lim-1* expression could be detected in mesoderm cells of mutant E8.5 embryos, indicating that the homozygous mutants contain paraxial and lateral mesoderm cells (Fig. 4J,L). *Mox-1* expression in the somites in posterior regions was similar in homozygous mutants and their littermates (Fig. 4I,J). Anteriorly however, *Mox-1* expression spread across the midline, as expected from the observed fusion of somites. In four out of five embryos analyzed, *Brachyury* expression in homozygous mutants was absent in the anterior midline, at the normal position of the notochord (Fig. 4M,O). In one case, *Brachyury*-expressing cells were present anteriorly but appeared to bud off and diverge from the axial notochord (Fig. 4N). Thus, axial mesoderm cells are severly affected in *Otx2^{hd}* homozygous embryos, while paraxial and lateral mesoderm cells do develop more normally. The appearance of fused somites is consistent with axial mesoderm defects since it has also been seen in other notochordless mouse mutants and notochordless chick embryos (Dietrich et al., 1993; Ang and Rossant, 1994; Teillet and le Douarin, 1983; Rong et al., 1992).

We next tested whether axial mesoderm defects occurred at earlier stages than those examined above. *Lim1*, *Brachyury*, and *HNF-3β* (Ang et al., 1993; Monaghan et al., 1993; Sasaki and Hogan, 1993) are all expressed in the node and head-process in wild-type embryos at E7.5 (Fig. 4C,E,G). *HNF-3β* and *Lim1* are also expressed in midline cells anterior to the notochord known as prechordal mesoderm cells (Fig. 4C,G – arrows). In homozygous mutants, all three genes were expressed in the node and in a few cells extending anteriorly at a short distance from the node (Fig. 4D,F,H). This was in sharp contrast to wild-type embryos in which labelled head-process cells had migrated much further anteriorly (Fig. 4C,E,G). In particular, the anterior-most midline expression of *Lim-1* and *HNF-3β* in the prechordal mesoderm cells were missing in the mutants (arrows in 4D and H). These results suggest that the defects in axial mesoderm cells observed at E8.5 are due to a failure in the generation of these cells at earlier stages. Prechordal mesoderm fail to develop and notochord development is incomplete in E7.5 *Otx2^{hd}* homozygous embryos.

Deletion of anterior brain regions rostral to rhombomere 3

To determine precisely how much rostral brain tissue was deleted in mutant embryos, several A-P region-specific neural markers were used to characterise *Otx2^{hd}* homozygous embryos. For these studies, we used only E8.25-E8.5 mutant embryos from the less severe class, where the head is free of underlying yolk sac and is not surrounded by endoderm tissue. At E8.5, the *forkhead*-related gene, *BF1* (Tao and Lai, 1992), and the homeobox gene, *Emx-2*, which are normally specifically expressed in the telencephalon, and part of the diencephalon for *Emx-2* (Simeone et al., 1992b), were not expressed in the neural tube of homozygous mutant embryos, thus demonstrating the absence of these tissues in mutant embryos (data not shown). Lack of *HNF-3β* and *netrin1* (Serafini et al., 1994), in the rostral portion of the neural tube of mutants embryos demonstrated the absence of ventral midbrain tissue (Fig. 5B,E and data not shown). Furthermore, we analyzed mutant embryos for En protein expression by whole-mount antibody staining. No expression of En was observed in three E8.5 mutant embryos, indicating that the midbrain and anterior hindbrain regions were missing in homozygous mutants (Fig. 5A,D). These results demonstrate that forebrain, midbrain and anterior hindbrain are missing in *Otx2^{hd}* homozygous mutants.

To further characterize at what level in the hindbrain deletion had occurred, homozygous E8.5 mutants of the less severely affected group were analyzed for expression of two hindbrain markers, *Hoxb-1* (Wilkinson et al., 1992a) and *Krox-20* (Wilkinson et al., 1992b). *Hoxb-1* was expressed in rhombomere 4 and the posterior spinal cord in both mutant and wild-type animals (Fig. 5C,F). *Krox-20* was expressed in two bands across the neural tube in mutants as in wild-type or heterozygous embryos. However in mutants the first band of expression occurred close to or at the anterior-most end of the embryo (Fig. 5B,E and data not shown). These results demonstrate that in homozygous mutants, neural tissues anterior to rhombomere 3 have been deleted.

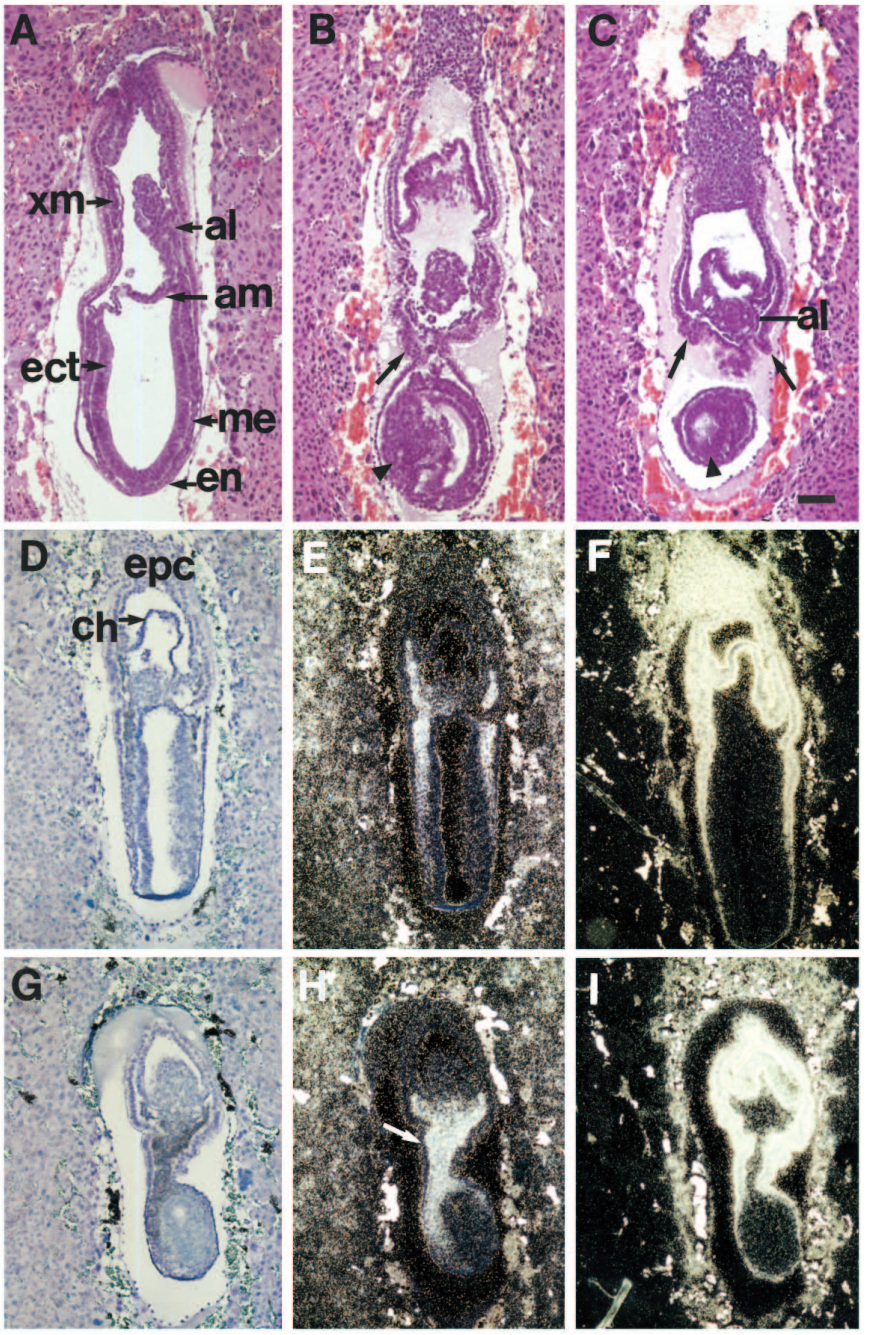

Fig. 3. Sections of wild-type and mutant embryos in decidua analyzed by histology or in situ hybridization. Near sagittal sections of late streak stage wild-type (A) or homozygous mutant embryos (B,C). Embryonic ectoderm (ect), mesoderm (me) and endoderm (en) cells can be seen in sections of homozygous mutants, however some of the ectoderm cells looked disorganised and lost their characteristic epithelial structure (arrowhead). The yolk sac endoderm cells appeared ruffled in homozygous mutants (B,C) compared to wild-type embryos (A) and separated the extraembryonic from the embryonic regions (arrows in B,C). The allantois (al) was often disconnected from the embryo in mutants unlike normal embryos (C). (D-I) Oblique sections of wild-type and mutant embryos analysed with the *Flk1* and *Pem1* probes. (E,H) Adjacent sections of wild-type and mutant embryos showing accumulation of *Flk-1*-expressing mesoderm cells in the proximal region of the mutant embryo (arrow in H). *Pem1* expression was detected in extraembryonic tissues both in wild-type (F) and mutant embryos (I). Scale bar, 100 μm. ect, ectoderm; me, mesoderm; en, endoderm; xm, extraembryonic mesoderm; am, amnion; al, allantois; epc, ectoplacental cone; ch, chorion.

Fig. 4. Whole-mount analysis of mesoderm markers in wild-type or heterozygous embryos (A,C,E,G,I,K,M) and homozygous mutant embryos (B,D,F,H,J,L,N,O) at E7.25-E8.5 (anterior is to the left in A-H, and M-O or to the top in I-L). (A,B) *gsc* expression in E7.25 embryos. (A) *gsc* expression in anterior primitive streak cells in normal embryos. (B) *gsc*-expressing cells were present but only in the proximal region of the mutant embryo. (C,D) *Lim1* expression in E7.25 embryos. (C) In normal embryos, *Lim1* is expressed in the mesodermal wings, node, head process and prechordal mesoderm cells (arrow in C). In mutants, *Lim1* is expressed in the same areas except there was no expression in the prechordal mesoderm region (arrow in D). (E,F) *Brachyury* expression in E7.75 embryos at the headfold stage. (E) *Brachyury* is expressed in the primitive streak, node and head process in normal embryos. (F) In mutant embryos, *Brachyury* expression is expanded in the primitive streak (open arrowhead), in the node (arrow) and in a few headprocess cells extending anteriorly from the node (arrowhead). (G,H) *HNF-3β* expression in E7.75 embryos. (G) In E7.75 wild-type embryos, *HNF-3β* is expressed in the node, head process and prechordal mesoderm (arrow). (H). In mutant embryos, *HNF-3β* is expressed in the node, in a shortened headprocess but not in the prechordal mesoderm area (arrow). (I,J) *Mox-1* expression in E8.5 embryos. (I) *Mox-1* is expressed in the somitic mesoderm of wild-type embryos. (J) In mutants, *Mox-1* expression anteriorly is spread across the midline (arrow). (K, L) *Lim1* expression in E8.5 embryos. *Lim1* is expressed in the lateral mesoderm of normal embryos (K) and mutant embryos (L). (M,N,O) *Brachyury* expression in E8.5 embryos. In normal embryos, *Brachyury* is expressed in the notochord and in the posterior primitive streak region. In mutant embryos, *Brachyury*-expressing cells are displaced from the midline anteriorly (arrowheads in N), or missing from the anterior and trunk regions (O). However, *Brachyury* was expressed in the primitive streak of mutants (N,O). Scale bar, 100 μm.

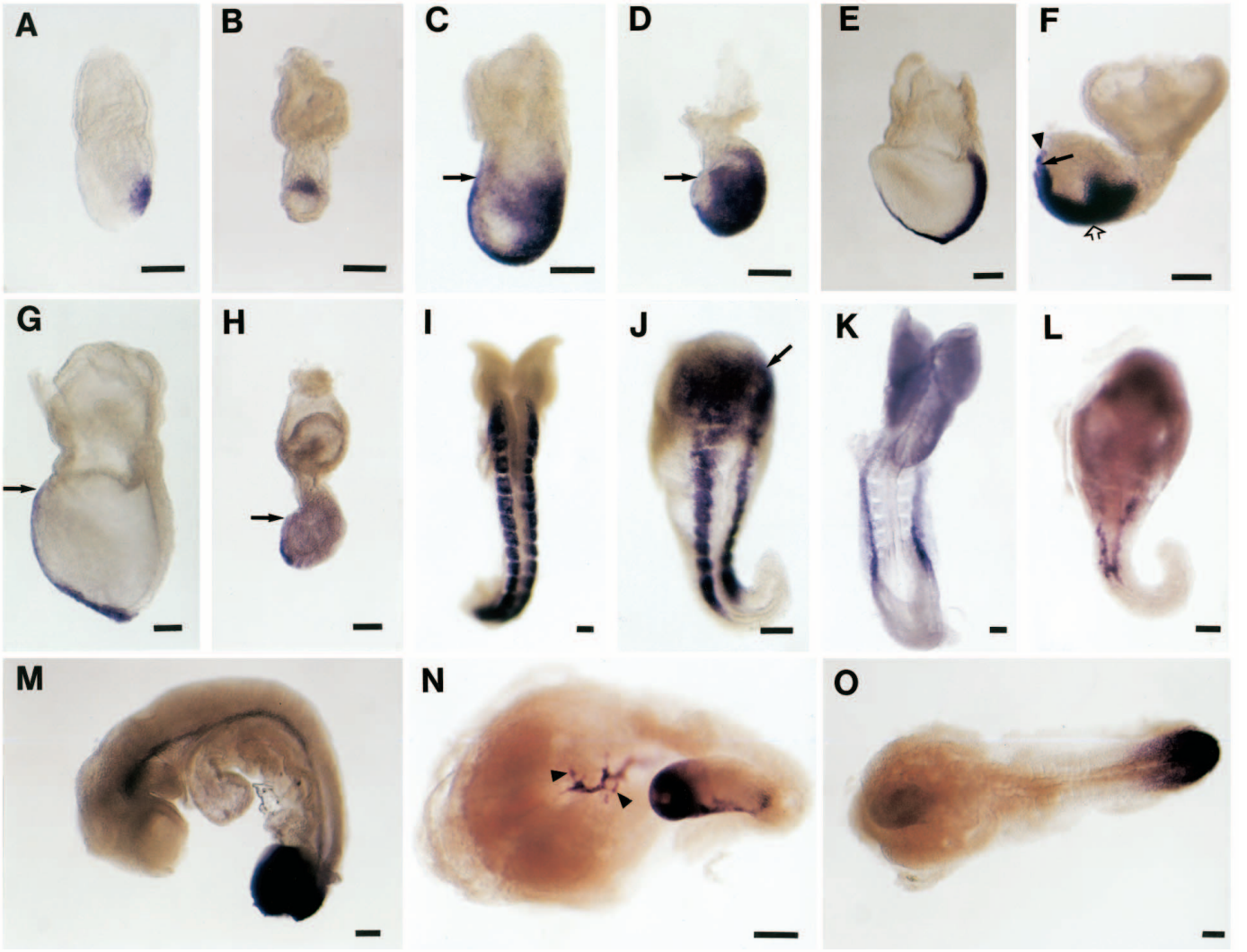

Fig. 4.

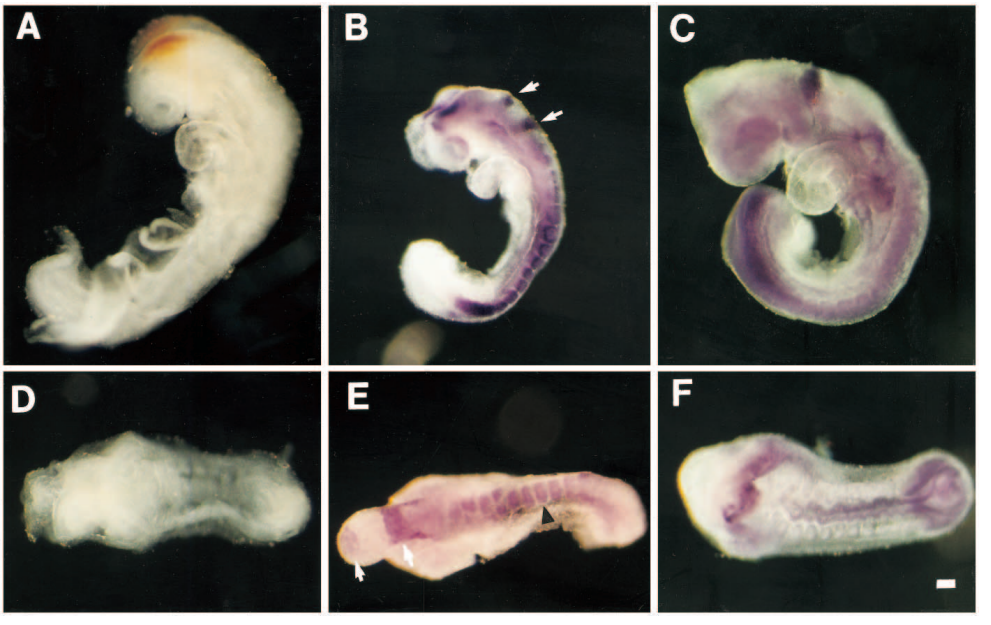

Fig. 5. Whole-mount RNA in situ hybridization and antibody staining of anteroposterior CNS markers in E8.5 wild-type or heterozygous embryos (A,B,C) and E8.5 homozygous mutant embryos (D,E,F). (A,D) En is expressed in normal embryos (A), but is missing from mutant embryos (D). (B,E). *Krox-20* and *Netrin-1* expression in embryos. Expression of *Krox-20* is detected in both rhombomeres 3 and 5 in wild-type (white arrows in B) and mutant embryos (white arrows in E). *Netrin-1* expression was not seen in the CNS but was expressed in the fused somitic mesoderm of mutant embryos (black arrowhead). (C,F) *Hoxb-1* expression in embryos. *Hoxb-1* is expressed in rhombomere 4 and posterior spinal cord in both normal (C) and mutant embryos (F). Scale bar, 100 μm.

DISCUSSION

Deletion of the homeobox region of the *Otx2* gene produced an embryonic lethal phenotype in mice. The phenotype was characterized by severe gastrulation defects, absence of prechordal mesoderm, reduced notochord production and severe anterior truncations. All mutant embryos were severely growth retarded or resorbed by E10.5, presumably because the separation of embryonic and extraembryonic regions resulted in defective yolk sac circulation. Given that *Otx2* is broadly expressed at the pre-streak and early streak stage embryos, and is later restricted to anterior structures, this phenotype implicates *Otx2* in several different aspects of early postimplantation patterning.

Early gastrulation defects in *Otx2^{hd}* homozygous mutants

In the mid- to late-streak stage mutant embryos, the incomplete elongation of the primitive streak, the accumulation of mesoderm cells between embryonic and extraembryonic region of the embryo and the ectopic location of *gsc*-expressing cells in the proximal region, indicate that *Otx2* is required in some manner for the normal organization of the streak. *Otx2^{hd}* homozygous mutants also showed defects in extraembryonic structures. The ruffling in the yolk sac is most likely caused by defects in the migration and accumulation of extraembryonic mesoderm.

The smaller size of the embryonic portion of mutant embryos at the mid-streak stage suggests that proliferation of the epiblast tissue could also be affected or delayed in absence of *Otx2*. To examine whether cell proliferation in the epiblast is affected at this early stage, BrdU incorporation experiments will be performed. In addition, the severe constriction observed between embryonic and extraembryonic regions in *Otx2^{hd}* mutants suggest that other processes besides proliferation are also affected in the mutants. A very similar extraembryonic-embryonic constriction was also observed in *HNF-3β* mutants (Ang and Rossant, 1994; Weinstein et al., 1994) and to a lesser extent in *Lim-1* mutants (Shawlot and Behringer, 1995), both of which also affect node and notochord development, suggesting that alteration of these processes is somehow involved in the generation of the constriction.

Otx2 is required for proper prechordal mesoderm and notochord development

We have demonstrated that the prechordal mesoderm is missing in *Otx2^{hd}* homozygous mutant embryos at the headfold stage using *Lim-1* and *HNF-3β* genes as markers for this tissue. These results demonstrate that *Otx2* is an essential regulator of prechordal mesoderm development. Recent phenotypic studies on *Lim1* homozygous mutants have also demonstrated an essential role for the *Lim1* gene in prechordal mesoderm development. Since *Otx2* and *Lim1* are both expressed in prechordal mesoderm in headfold stage embryos and are required for its development, it will be interesting to study whether these two genes might function in the same genetic pathway.

Defects in notochord development were also observed in *Otx2^{hd}* homozygous embryos. In the late streak and headfold stage embryos, there was limited midline extension of the head process. In later embryos, the notochord formed only at the most posterior end in some cases, while in other cases, notochord cells were present but misplaced lateral to the midline. Anterior notochord normally express *Otx2* at the midbrain level (Ang et al., 1994), perhaps explaining the anterior notochord defects observed in mutant embryos. However abnormalities in the development of notochord cells, which normally do not express *Otx2* at the hindbrain and trunk levels, cannot be readily explained. It seems likely that the failure of later notochord development reflects earlier defects in headprocess development. Interestingly, studies of *Otx2* in *Xenopus* and chick have demonstrated *Otx2* expression in the organizer tissue of these species, namely dorsal blastopore lip and the Hensen's node respectively (Pannese et al., 1995; Blitz et al., 1995; Bailly-Cuif et al., 1995). Thus, by analogy to the situation seen in these species, *Otx2* might be expressed in the presumptive node at the anterior end of the primitive streak in early streak stage mouse embryos (Lawson et al., 1991), this expression being obscured by the simultaneous widespread expression of *Otx2* in the epiblast at this stage. Lost of *Otx2* in this structure may be responsible for the notochord defects at later stages.

Otx2 is required for rostral brain development during mouse embryogenesis

Otx2^{hd} homozygous mutants that failed to become enclosed by visceral yolk sac by E8.5 due to the severe constriction between embryonic and extraembryonic regions, were extremely abnormal and in some cases seemed to lack any axial organization. However, the less severely affected embryos that were still enclosed in the yolk sac, allowed delineation of specific anterior defects in the mutants.

By E8.25, deletions in the anterior neural tube rostral to rhombomere 3 were clearly apparent in these homozygous mutants. Loss of forebrain, midbrain and anterior hindbrain was demonstrated using early molecular markers for these tissues such as *BF1, Emx-2* and En. This phenotype could have been predicted, given the anterior expression of *Otx2* and its relationship to the *Otd* gene in *Drosophila*. Loss of function mutations in *otd* in flies lead to deletion of anterior head structures (Finkelstein et al., 1990). These results support evolutionary conservation of the function of these genes in head development in flies and mice.

This interpretation of the phenotype assumes that *Otx2* acts autonomously in the anterior CNS and is required for the specification of these regions. However, this hypothesis cannot readily explain why structures posterior to the expression domain of *Otx2* are also deleted. *Otx2* expression caudally marks the mid-hindbrain boundary. However, deletions of the hindbrain region, containing the En-expressing domain of the metencephalon and close to the anterior border of rhombomere 3, occur in mutant embryos. This could either be due to an early expression of *Otx2* in cells fated to become hindbrain at the late-streak stage or to a dependence of anterior hindbrain development on more rostral neural tube. Comparison of the fate-map studies of late streak stage embryos with the domain of *Otx2* expression at this stage suggest that this domain does not include hindbrain territories (Tam, 1989). However a more extensive study is necessary to exclude this possibility.

Alternatively, loss of anterior neural tissue could be an indirect consequence of the loss of notochord and prechordal mesoderm in the *Otx2^{hd}* homozygous mutants. Classical

embryological studies have demonstrated a role for both these tissues in the induction and patterning of the neural tube (reviewed by Slack and Tannahill, 1992; Doniach, 1993; Ruiz i Altaba, 1994). A role for the prechordal mesoderm was supported by the phenotype of *Lim1* null homozygous mutants (Shawlot and Behringer, 1995). In contrast, a role for the notochord tissue in A-P neural tube patterning has not been confirmed by mutant studies in mice. Mouse embryos lacking the *HNF-3β* gene do not develop a notochord, yet they showed expression of rostral brain markers (Ang and Rossant, 1994; Weinstein et al., 1994). Together, these results suggest an essential role for the prechordal mesoderm, but not the notochord, in patterning of the anterior neural tube in mice. Furthermore, the *Lim1* mutant phenotype is remarkably similar to that of the *Otx2^{hd}* homozygous mutants, in that deletions of the anterior neural tube occur at about the same anteroposterior level (Shawlot and Behringer, 1995). Thus, loss of rostral brain tissues observed in *Otx2^{hd}* homozygous mutants could be a consequence of the lack of prechordal mesoderm development.

To distinguish between these hypotheses, we are planning to recombine mesoderm tissue from homozygous mutant *Otx2^{hd}* embryos with ectoderm tissue from wild-type embryos to determine if the mutant mesoderm tissue can induce expression of anterior neural markers such as *En* genes. The reverse experiment of recombining ectoderm tissue from *Otx2^{hd}* homozygous embryos and mesoderm from wild-type embryos will be performed to determine if the *Otx-2* negative ectoderm tissue can respond to inducing signals from the mesoderm. The use of the in vitro tissue recombination assay (Ang and Rossant, 1993, 1994) to analyze *Otx2^{hd}* mutants should allow us to dissect the roles of *Otx2* in the ectoderm and mesoderm tissues at the late-streak stage.

In conclusion, *Otx2^{hd}* homozygous mutants show complex defects in gastrulation, axial mesoderm and rostral brain development that implicate *Otx2* in the pathways of primitive streak organization, axial mesoderm development as well as anterior head development. Further experiments will be required to dissect out these different roles.

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