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# Maintenance of the Specification of the Anterior Definitive Endoderm and Forebrain Depends on the Axial Mesendoderm: A Study Using *HNF3β/Foxa2* Conditional Mutants

**Marc Hallonet,*[,1] Klaus H. Kaestner,†[,1] Luis Martin-Parras,* Hiroshi Sasaki,‡ Ulrich A. K. Betz,§[,2] and Siew-Lan Ang*[,3]**

**Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/Université Louis Pasteur, C.U. de Strasbourg, 67404 Illkirch Cedex, France; †Department of Genetics and Penn Diabetes Center, University of Pennsylvania School of Medicine, 415 Curie Boulevard, Philadelphia, Pennsylvania 19104; ‡Laboratory of Developmental Biology, Institute for Molecular and Cellular Biology, Osaka University, Osaka Japan; and §Institute of Genetics, University of Cologne, Weyertal 121, D-50931 Cologne, Germany*

**In mouse embryo, the early induction of the head region depends on signals from the anterior visceral endoderm (AVE) and the anterior primitive streak. Subsequently, node derivatives, including anterior definitive endoderm and axial mesendoderm, are thought to play a role in the maintenance and elaboration of anterior neural character. *Foxa2* encodes a winged-helix transcription factor expressed in signaling centers required for head development, including the AVE, anterior primitive streak, anterior definitive endoderm, and axial mesendoderm. To address *Foxa2* function during formation of the head, we used conditional mutants in which *Foxa2* function is preserved in extraembryonic tissues during early embryonic stages and inactivated in embryonic tissues after the onset of gastrulation. In *Foxa2* conditional mutants, the anterior neural plate and anterior definitive endoderm were initially specified. In contrast, the axial mesendoderm failed to differentiate. At later stages, specification of the anterior neural plate and anterior definitive endoderm was shown to be labile. As a result, head truncations were observed in *Foxa2* conditional mutants. Our results therefore indicate that anterior definitive endoderm alone is not sufficient to maintain anterior head specification and that an interaction between the axial mesendoderm and the anterior definitive endoderm is required for proper specification of the endoderm. *Foxa2* therefore plays an integral role in the formation of axial mesendoderm, which is required to maintain the specification of the forebrain and the anterior definitive endoderm.** © 2002 Elsevier Science (USA)

***Key Words:*** **Cerberus; SII6; foregut; head formation; forebrain induction.**

## INTRODUCTION

The formation of the head and of the anterior central nervous system (CNS) involves complex tissue interactions. Recent findings suggest that the induction and specification of the anterior region of the mouse embryo occurs during gastrulation and depends on the anterior visceral endoderm (AVE) and the anterior primitive streak (reviewed in Beddington and Robertson, 1999; Tam and Steiner, 1999; de Souza and Niehrs, 2000). Removal of the AVE at the onset or during the early stages of gastrulation severely compromises development of the anterior CNS (Thomas and Beddington, 1996). Absence of the anterior primitive streak in *Wnt3* mutant embryos results in loss of anterior and posterior neural tissue, despite apparently normal AVE development (Liu *et al.*, 1999). At later stages, embryonic derivatives, including the anterior axial mesendoderm which originates in the posterior epiblast at the level of the anterior primitive streak (Beddington, 1994; Lawson *et al.*,

[1] M.H. and K.H.K. contributed equally to this work.

[2] Present address: Bayer AG, Pharma Research Antiinfectives, Wuppertal Germany.

[3] To whom correspondence should be addressed. Fax: 33 388653201. E-mail: siew-lan@igbmc.u-strasbg.fr.



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1991; Sulik *et al.*, 1994), are also thought to be required for the maintenance of the initial anterior specification (Ang and Rossant, 1993a; Ang *et al.*, 1994; Camus *et al.*, 2000; Shawlot *et al.*, 1999). Experimental evidence further suggests that anterior midline tissues, including the anterior mesendoderm, play a role in the refinement of the patterning of the anterior region of the vertebrate embryo (Camus *et al.*, 2000; Rowan *et al.*, 1999).

The molecular mechanisms underlying the tissue interactions that occur during head formation are beginning to be understood. For example, null mutation of the transcription factors *Otx2* or *Lim1*, both of which are expressed in the AVE and anterior mesendoderm, results in the specific loss of anterior structures (Acampora *et al.*, 1995; Ang *et al.*, 1996; Matsuo *et al.*, 1995; Perea-Gomez *et al.*, 2001; Shawlot and Behringer, 1995). Another good candidate as a regulator of inductive signaling during head formation is Foxa2, a nuclear protein of the winged-helix family of transcription factors expressed in the anterior midline signaling structures (Kaufmann *et al.*, 1994; reviewed in Kaufmann and Knochel, 1996; Lai *et al.*, 1991). *Foxa2* is first expressed in visceral endoderm, and after the onset of gastrulation, it is expressed at the anterior level of the primitive streak, in definitive endoderm, in the node, and in axial derivatives including axial mesendoderm, notochord, and floor plate (Ang *et al.*, 1993b; Lai *et al.*, 1991; Monaghan *et al.*, 1993; Sasaki and Hogan, 1993). Ectopic expression of *Foxa2* under the control of the *Engrailed-2* promoter resulted in the differentiation of an ectopic floor plate, demonstrating that *Foxa2* is sufficient to specify the ventral differentiation of the neural tube in the midbrain (Sasaki and Hogan, 1994). Loss-of-function experiments and analysis of tetraploid embryonic stem (ES) cell chimeric mouse embryos demonstrated that *Foxa2* is required in the extraembryonic visceral endoderm for primitive streak elongation and that *Foxa2* is required cell autonomously in the embryonic epiblast at the node level for the specification of axial mesendoderm precursors (Ang and Rossant, 1994b; Weinstein *et al.*, 1994; Dufort *et al.*, 1998). Consistent with the conclusion that *Foxa2* is involved in the differentiation of axial structures, functional Foxa2 binding sites have been identified on the *shh* promoter (Epstein *et al.*, 1999; Muller *et al.*, 1999).

Here, we have studied the function of *Foxa2* at the head level outside the visceral endoderm (VE), in the anterior axial mesendoderm in particular, by using an alternative loss-of-function strategy based on the *Cre-loxP* system (Nagy, 2000). The purpose of this approach was to preserve *Foxa2* function during the early stages of embryogenesis in extraembryonic tissues, inactivating the gene in embryonic tissues only at later stages. We therefore generated and analyzed conditional mutants in which the *Foxa2* allele flanked by *LoxP* sites (Sund *et al.*, 2000), and noted *Foxa2$^{flox}$* hereafter, was selectively inactivated by the *Cre* recombinase expressed in most embryonic tissues under the regulation of the *Nestin* rat promoter (Betz *et al.*, 1996). In these conditional mutants, *Cre* recombinase activity was restricted to embryonic derivatives beginning at 6.5 dpc. The conditional inactivation of *Foxa2* at this stage did not perturb anterior neural plate specification or the formation of the anterior definitive endoderm, but did result in a failure of axial mesendoderm formation. As a consequence, the specification of the anterior neural plate was labile. It has recently been proposed that the anterior definitive endoderm, derived from the node, is required for the maintenance of anterior character (Martinez-Barbera and Beddington, 2001). Our results therefore indicate that the anterior definitive endoderm is not sufficient alone to maintain anterior head specification and suggest that reciprocal interactions between anterior midline tissues, during gastrulation, are required to stabilize the initial induction/specification of the anterior neural plate.

## MATERIALS AND METHODS

### *Production of Conditional Mutant Embryos*

To inactivate *Foxa2* in epiblast derivatives, we used mice carrying a *Foxa2* allele, which is flanked by *loxP* sites designated *Foxa2$^{flox}$* (Sund *et al.*, 2000), maintained on a C57BL/6-J × 129/Sv background. The *Foxa2$^{flox}$* allele has wild-type activity and can be converted to a null allele by *Cre*-mediated recombination (Meyers *et al.*, 1998). We generated compound animals heterozygous for *Foxa2$^{flox}$* and a null Foxa2 mutation, designated *Foxa2$^{null}$* (Ang and Rossant, 1994b). These animals also carried a transgene driving *Cre* recombinase expression under the regulation of the rat *Nestin* promoter from the *Nestin::Cre* transgenic mouse line, balancer 1 (Betz *et al.*, 1996). *Nestin::Cre* males, heterozygous for the *Foxa2$^{null}$* allele (Ang and Rossant, 1994b), were crossed to females homozygous for the *Foxa2$^{flox}$* allele (Sund *et al.*, 2000). *Foxa2$^{null}$/Foxa2$^{flox}$*; *Nestin::Cre* embryos have the potential to convert the *Foxa2$^{flox}$* allele to a null allele in cells and their progeny, resulting in a complete loss of *Foxa2* activity. *Foxa2$^{null}$/Foxa2$^{flox}$*, *Nestin::Cre* embryos were therefore considered as *Foxa2* conditional mutants. Embryos carrying other genotypes were considered as controls. PCR genotyping was performed as previously described for the *Cre* allele (Betz *et al.*, 1996) and for the null *Foxa2* allele (Ang and Rossant, 1994b).

The time and localization of the *Cre* activity was analyzed by crossbreeding *Nestin::Cre* transgenic mice (Betz *et al.*, 1996) *with loxP* reporter mice R26R (Soriano, 1999). In these mice, *lacZ* is expressed only after *Cre*-mediated recombination and is used as a tracer for *Cre*-recombinase activity. These two mouse lines were maintained in a mixed CD1 outbred background. Staging of early postimplantation mouse embryos was according to Downs and Davies (1993).

### *In Situ Hybridization and Immunostaining*

Whole-mount *in situ* hybridization and *Foxa2* immunostaining were performed as previously described (Filosa *et al.*, 1997). Five to seven-micrometer-thick microtome or cryostat serial sections were collected after embedding in JB4 or OCT, respectively.

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## RESULTS

### The Cre Recombinase Activity Is Specific for Embryonic Derivatives

The efficiency and specificity of *Cre* expression was analyzed by crossbreeding *Nestin::Cre* transgenic mice (Betz *et al.*, 1996) with R26R reporter mice (Soriano, 1999). At the onset of gastrulation at the early-streak stage, *lacZ* activity was observed bilaterally in the most posterior epiblast, a region devoid of Foxa2 expression (data not shown). At the late bud stage, *lacZ* activity was mosaic and restricted to the embryonic ectoderm and mesoderm (Figs. 1A–1C). Several hours later at the headfold stage, lacZ activity was uniformly detected in the ectoderm, in the neural plate (Figs. 1D and 1E), in the anterior axial mesendoderm (Figs. 1E and 1F), and in the node (Fig. 1D, and data not shown). Strikingly, lacZ activity was absent from the anterior definitive endoderm (Figs. 1B and 1C) and the visceral endoderm (Figs. 1A and 1D).

In order to identify cells that could account for the developmental defects observed in conditional mutants, we also examined expression of Foxa2 in these β-galactosidase (β-gal)-stained compound embryos. At the headfold stage, all Foxa2-positive cells were sharing *lacZ* activity, except in the lateral and ventral foregut (Figs. 1E and 1F).

### The AVE Is Functional in Conditional Foxa2 Mutants

Foxa2 protein persists in the AVE and anterior definitive endoderm of late bud/early headfold-stage mutant embryos (Figs. 2B and 2D). The constriction observed in homozygous null mutants between extraembryonic and embryonic tissues during gastrulation that results from abnormal visceral endoderm function (Dufort *et al.,* 1998) was absent in conditional mutants (Figs. 2B and 2D). Moreover, primitive streak elongation appeared unaffected in conditional *Foxa2* mutant embryos (Figs. 2B and 2D). Altogether, these observations strongly suggested that *Foxa2* function was maintained during the first steps of gastrulation and that the visceral endoderm, including the AVE, remained unaffected in conditional mutant embryos.

### The Anterior Head Is Sequentially Truncated in Conditional Mutants

No obvious morphological difference between conditional mutant and control embryos could be detected prior to the headfold stage (data not shown). Some conditional mutant embryos survived until 11.5 dpc, 2 days later than the disappearance of null mutants (data not shown). All embryos showed variable ventral midline defects and anterior head truncations (Figs. 3B, 3D, and 4D–4F).

Morphological observations suggested that anterior structures were induced in all conditional *Foxa2* mutants (Figs. 3B and 3D), in contrast to previous observations of null mutants (Ang and Rossant, 1994b; Weinstein *et al.,* 1994). Conditional mutant embryos displayed a reduced lateral extension of the neural folds at the headfold stage (Figs. 3B and 3D) and ventromedial fusion of the telencephalic bulging at 8.5 dpc (arrowhead in Figs. 4D–4F). Formation of the ventral prosencephalon was accordingly affected in conditional *Foxa2* mutant embryos.

In order to elucidate the molecular mechanisms involved in malformation of the head of conditional mutant embryos, we studied the expression of forebrain markers, including *Six3* (Fig. 3A; Oliver *et al.,* 1995), *Hesx1* (Figs. 3C and 4A; Thomas *et al.,* 1998), *BF1* (Figs. 4B and 5A; Tao and Lai, 1992), and *Fgf8* (Fig. 4C; Crossley and Martin, 1995; Shimamura and Rubenstein, 1997). The expression of *Six3* and *Hesx1* was induced in the neural plate of all conditional mutants examined at the headfold stage (Figs. 3B and 3D). This suggested that the induction and initial specification of the most anterior neural plate occurred in conditional mutants. Nevertheless, the territory of expression of all markers studied was smaller than in control embryos. The Foxa2 protein was systematically absent from axial neural structures from 7.75 to 8.5 dpc (Figs. 2F and 4D-4F). This observation was consistent with the observation that lacZ activity was detected in the medial structures of *R26R*; *Nestin::Cre* embryos and suggested that *Foxa2* was inactivated by *Cre* recombinase in the midline structures of conditional mutants.

At 8.5 dpc, expression of *Hesx1* and *BF1* began to decline and was absent in some mutant embryos (Figs. 4D and 4E, respectively). Fgf8 expression was severely reduced or missing in the anterior neural ridge, but appeared normal in the midbrain, branchial arches, and tailbud of mutant embryos (Fig. 4F). In three cases at 9.5 dpc, the expression of the telencephalic marker *BF1* disappeared and no cerebral hemispheres formed (Fig. 5B). In conclusion, truncation of the head was associated with reduced expression of the most anterior neural plate markers.

### The Axial Mesendoderm Is Severely Affected in Conditional Mutants

The axial mesendoderm expresses Foxa2 (Ang *et al.,* 1993b; Monaghan *et al.,* 1993; Hogan and Sasaki, 1993) and originates in the node (Lawson *et al.,* 1986; Lawson and Pedersen, 1992). Both tissue recombination experiments and studies of chimeric and mutant mouse embryos have shown that the axial mesendoderm is required for normal patterning of the anterior neural plate (Ang *et al.,* 1994; Shawlot *et al.,* 1999; Camus *et al.,* 2000; Jin *et al.,* 2001). We therefore tested whether the axial mesendoderm was affected in conditional mutants.

Consistent with the lacZ expression observed in the anterior primitive streak and in axial mesendodermal from 6.75 dpc onward in *lacZ*$^{flox}$; Nestin:*Cre* transgenic embryos, Foxa2 protein expression was missing from the mutant anterior primitive streak and from axial mesendodermal structures from 6.75 dpc onward (Figs. 2B and 2D). Histological examinations confirmed the absence of any identi-

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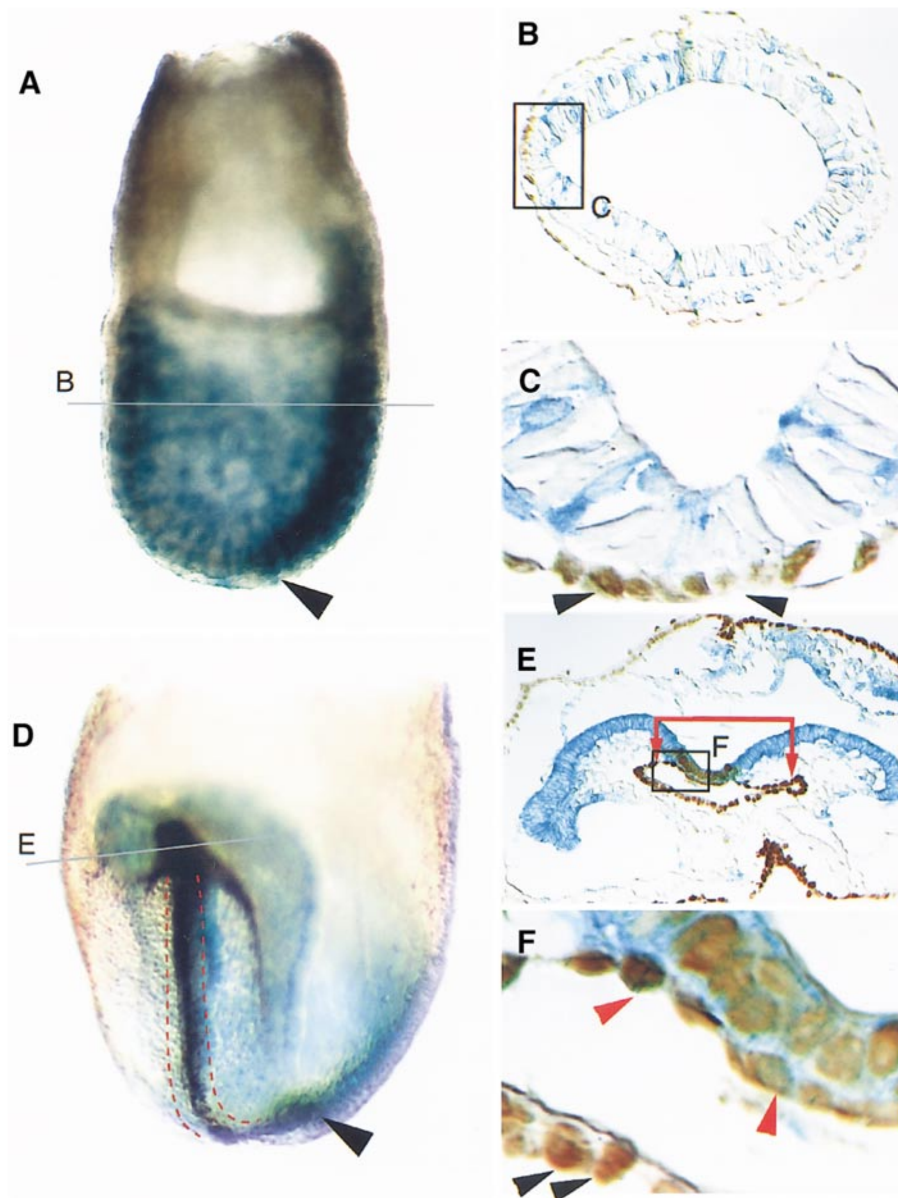


**FIG. 1.** In embryonic derivatives, all Foxa2-positive cells are coexpressing lacZ, except anterior definitive endoderm and its descendants, the ventral foregut. Whole-mount double staining of lacZ activity with Xgal (blue) and of Foxa2 by immunocytochemistry (brown) on *R26R*; *Nestin::Cre* compound embryos. *LacZ* serves as a tracer for *Cre* recombinase activity. (A–C) Early headfold stage *R26R*; *Nestin::Cre* compound embryos. (B) Transverse section of the embryo shown in (A) at the head level. $\beta$-Galactosidase ($\beta$gal) activity was mosaic in the ectoderm. (C) Higher magnification of the view shown in (B). $\beta$gal activity was absent from the anterior definitive endoderm (arrowheads). (D–F) Late headfold stage *R26R; Nestin::Cre* compound embryos. (E) Transverse section of the embryo shown in (D) at the head level. $\beta$gal activity was uniformly detected in the ectoderm and in the neural plate. (F) Higher magnification of the view shown in (E). $\beta$gal activity was absent from the derivatives of the anterior definitive endoderm, located in the lateral and ventral foregut pocket (arrowhead). The red dashed lines in (D) and the red arrows in (E) border the *lacZ*-positive dorsal foregut (red arrowheads in F) that are derived from axial mesendoderm.

fiable axial structure expressing Foxa2 protein at the level expected for anterior axial mesendoderm (Fig. 2F).

We also determined whether axial mesendoderm was formed in the absence of *Foxa2* by using molecular markers. *Gsc*, which is specifically expressed in the anterior-most axial mesendoderm in wild-type embryos (Fig. 6A; Blum *et*

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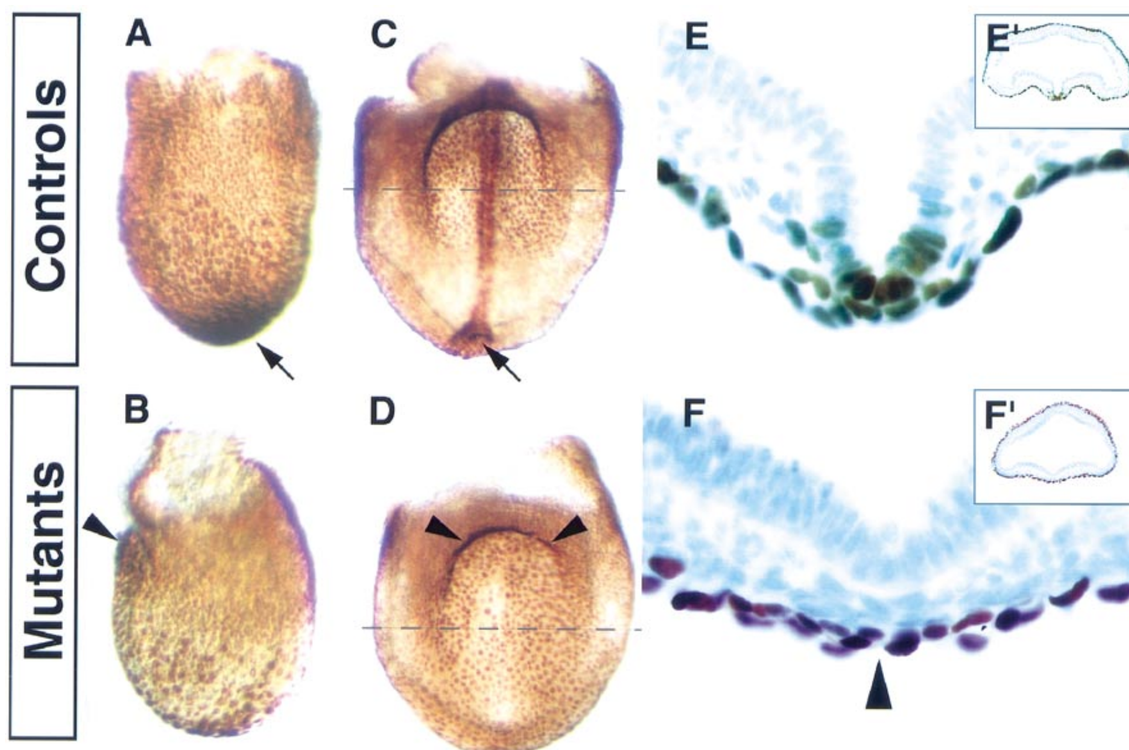


**FIG. 2.** Absence of morphologically identifiable axial mesendoderm in Foxa2 mutant embryos at 7.5 dpc. (A–D) Whole-mount immunocytochemistry staining of Foxa2 in brown. (A, B) Late bud embryos; lateral views; anterior is to the left. (C, D) Headfold embryos; frontal views; anterior is to the top. (E, F) Transverse sections of the embryos shown in (C) and (D), respectively. (E'', F') Low-power magnification of the section enlarged in (E) and (F), respectively. Mutant and control embryos look similar prior to headfold stages (compare A and B). In conditional mutants, Foxa2 expression is absent from the anterior primitive streak (B) and the node (D) and from any axial structure (compare C and D), but is expressed in the AVE (arrowhead in B) and in the anterior definitive endoderm (arrowheads in D). Arrows in (A) and (C) point to the anterior primitive streak and node, respectively. Histological examination (E, F) confirms the absence of morphologically recognizable axial mesendoderm (F) and of any axial structure expressing Foxa2. Moreover, the lateral mesoderm mass is fused in the midline in conditional mutants (arrowhead in F).

*al.,* 1992), was severely affected or absent in conditional mutants (Fig. 6B). Similarly, *Shh* transcripts are normally detected in the axial mesendoderm at 7.75 dpc (Fig. 6C; Echelard *et al.,* 1993), and expression was strongly reduced or absent in conditional mutants beginning at the headfold stage (Fig. 6D).

The anterior axial mesendoderm was thus severely affected or totally absent in conditional mutants from 7.5 dpc onward.

### *The Anterior Definitive Endoderm Is Formed but Fails to Maintain Its Specification in Conditional Mutants*

It has recently been suggested that forebrain formation depends on the anterior definitive endoderm, a region where Foxa2 is expressed (for a review, see Martinez-Barbera and Beddington, 2001; Martinez-Barbera *et al.,* 2000; Withington *et al.,* 2000). We therefore tested whether the anterior definitive endoderm was affected by the conditional inactivation of *Foxa2.*

In headfold-stage control embryos, the level of Foxa2 protein is higher in the axial mesendoderm than in more lateral regions of the endoderm (see Fig. 2C). In contrast, the level of Foxa2 protein was as low in the midline as in more lateral endoderm regions of *Foxa2$^{flox}$*; *Nestin::Cre* conditional mutant embryos at the headfold stage (Fig. 2D). This observation was consistent with the detection of lacZ activity only in axial mesendoderm of *lacZ$^{flox}$*; *Nestin::Cre* compound embryos at the headfold stage (see Figs. 1A and 1B) and suggested that only the axial mesendoderm expression of Foxa2 was altered in conditional mutants. In contrast, Foxa2 expression persists in the anterior definitive endoderm, consistent with the absence of βgal activity in descendants of the anterior definitive endoderm in the lateral and ventral foregut of *lacZ$^{flox}$*; *Nestin::Cre* compound embryos.

*SII6* is a putative *Lim1* target gene expressed in the

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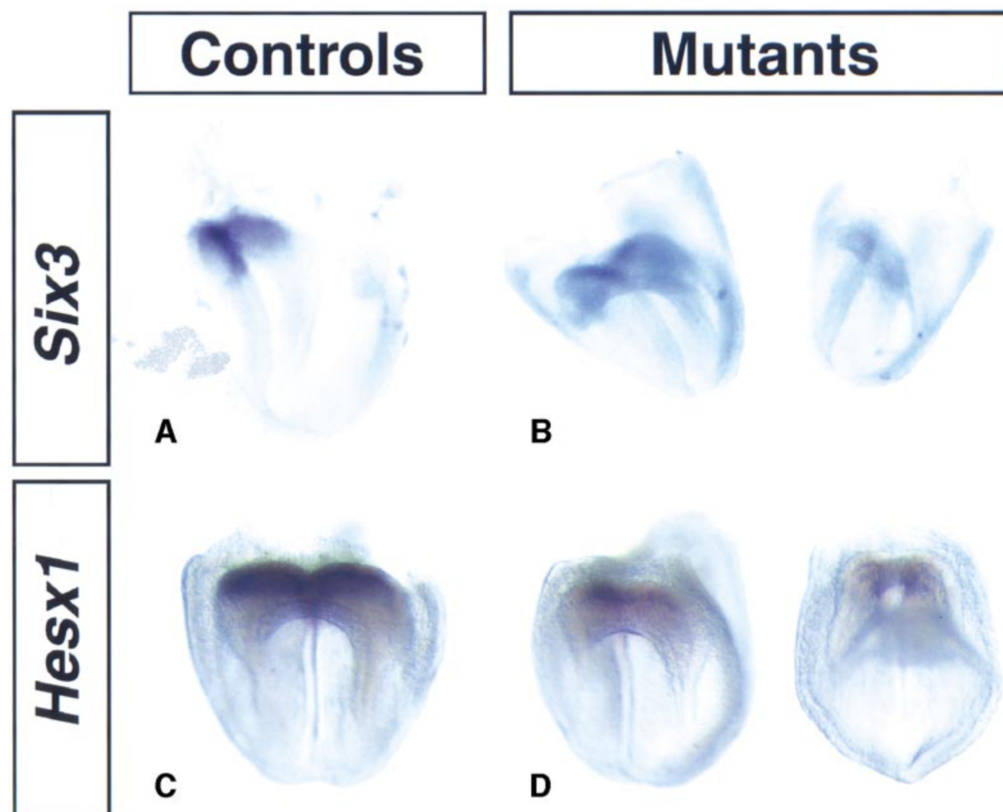


**FIG. 3.** The anterior neural folds are specified in conditional Foxa2 mutant embryos. Whole-mount *in situ* hybridization (purple) to *Six3* (A, B) and *Hesx1* (C, D) on headfold-stage control (A, C) and *Foxa2* conditional mutant (B, D) embryos. (A) Control embryo, showing *Six3* expression in the anterior neural plate. (B) *Six3* is expressed in the anterior neural plate of conditional mutant embryos, but the territory of expression is reduced to various extents (see also D). (C) Control embryo, showing *Hesx1* expression in the anterior neural plate. (D) *Hesx1* is expressed in the anterior neural plate of conditional mutant embryos. Similarly to that of *Six3*, the territory of expression of *Hesx1* is reduced. Induction and specification of the most anterior neural plate thus occurred in conditional mutants, although the reduced size of the territories suggests improper growth of the conditional mutant anterior neural plate.

anterior definitive endoderm (Shimono and Behringer, 1999). *SII6* was similarly expressed in the anterior definitive endoderm of conditional mutants and control embryos at the headfold stage (Fig. 7). Thus, anterior definitive endoderm was accordingly generated in conditional mutant embryos, most likely because Foxa2 is not inactivated in these cells.

The mouse *cerberus-like* (*Cerl*) gene encodes a secreted protein acting as a potent neuralizing factor that induces expression of forebrain- and endoderm-specific genes (Belo *et al.,* 1997; Biben *et al.,* 1998). *Cerl* expression in the AVE and definitive endoderm of conditional mutants was similar to that of wild-type embryos at the midstreak and late-streak stages (data not shown). In contrast, *Cerl* expression was absent from the anterior definitive endoderm in conditional mutant embryos at the headfold stage (Figs. 8D–8F and 8F′), while expression in the anterior mesoderm was observed in control and mutant embryos. Since the endoderm of these mutants is still expressing Foxa2, it is unlikely that this tissue was primarily affected by the *Foxa2* inactivation. In summary, these results indicate that the anterior definitive endoderm is not properly specified as the expression of *Cerl* is not maintained in conditional mutant embryos.

## DISCUSSION

The purpose of the present investigation was to study the role of axial mesendoderm in forebrain specification by using conditional mutants of Foxa2. Previous studies already had demonstrated that a null mutation of *Foxa2* in mice results in embryonic lethality (Ang *et al.,* 1994b; Weinstein *et al.,* 1994) and that *Foxa2* is required cell autonomously in the embryonic epiblast at the node level for the specification of axial mesendoderm precursors (Dufort *et al.,* 1998). To address later requirements for Foxa2 function, we inactivated the gene after the onset of gastru-

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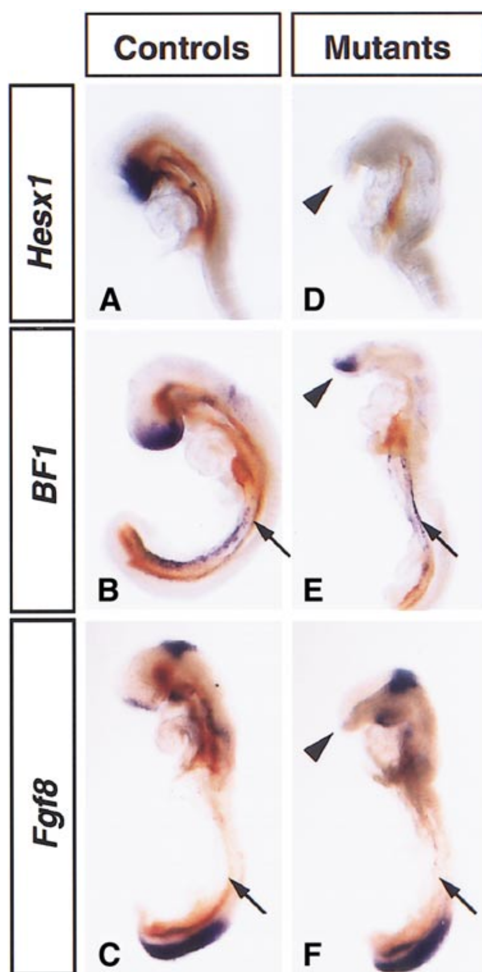


**FIG. 4.** The expression of anterior markers is reduced or missing in Foxa2 mutant embryos at 8.5 dpc. Whole-mount *in situ* hybridization to *Hesx1* (A, D), *BF1* (B, E), and *Fgf8* (C, F) on 8.5-dpc control (A–C) and conditional mutant embryos (D–F). Foxa2 protein is stained in brown by whole-mount immunocytochemistry. Morphologically, the medial fusion of the telencephalon results in holoprosencephaly in *Foxa2* mutant embryos. At 8.5 dpc, the expression of *Hesx1*, *BF1*, and *Fgf8* was reduced (arrowhead in E) and sometimes absent from the most anterior neural structures (arrowhead in D and F), indicative of the instability of neural plate specification in these mutants. At the trunk level, the primitive streak elongates, but axial structures, including the floor plate and notochord, which should express Foxa2 (arrow in B and C), are absent at the trunk level (arrow in E and F).

lation by crossing *Foxa2*$^{flox}$ with *Nestin::Cre* transgenic mice, in which the *Cre* recombinase is expressed efficiently in embryonic but not in extraembryonic derivatives (Betz *et al.,* 1996). The *Foxa2* conditional mutants provide for the first time a model system in which anterior definitive endoderm is present in the absence of axial mesoderm. This allowed us to uncover evidence for a interaction between these tissues that is required to maintain the specification of the endoderm. Despite the presence of anterior definitive endoderm, the forebrain is not maintained in the conditional mutants, indicating an absolute requirement for anterior axial mesendoderm for its maintenance.

### *The Anterior Neural Plate Is Prepatterned in the Absence of Axial Mesendoderm*

In *Foxa2* conditional mutants, the constriction between embryonic and extraembryonic structures observed in null mutants was absent. Moreover, the expression of anterior neural markers, including *Hesx1/Rpx* and *Six3*, which depends on signals from the AVE (Rhinn *et al.,* 1998; Acampora *et al.,* 1998), is observed in conditional mutant embryos (see Figs. 3 and 4). Altogether, these observations strongly suggest that the AVE is normal in *Foxa2*$^{flox}$; *Nestin::Cre* conditional mutants so that head induction and early gastrulation events are unaffected. This is consistent with the situation in mouse chimeric embryos composed of *Foxa2*$^{-/-}$ embryonic tissues and wild-type extraembryonic derivatives (Dufort *et al.,* 1998), where specification of the anterior neural plate was rescued, presumably under the control of signals provided by the AVE (Thomas and Beddington, 1996). However, we have found that the anterior specification in conditional mutants is unstable such that all of the forebrain markers were affected in our mutants. These results strongly suggest that Foxa2 is required in the axial mesendoderm to maintain the forebrain. This observation is consistent with the conclusion that the specification of the anterior neural plate is labile and depends on Lim1 activity in the axial mesendoderm (Shawlot *et al.,* 1999).

### *Specification of the Anterior Definitive Endoderm Is Not Maintained in Conditional Mutants*

Cell lineage analyses indicate that the anterior definitive endoderm and the axial mesendoderm share a common origin in the epiblast at the anterior level of the early primitive streak. Anterior definitive endoderm cells reach their final anterior destination by the neural plate stage (Lawson *et al.,* 1986, 1991; Lawson and Pedersen, 1987). These studies also demonstrated that the earlier the axial embryonic endoderm is generated the more anterior its position in the embryo. Endoderm located at, and anterior to, the distal tip of the midstreak embryo, and extending more anteriorly at late streak/neural plate stages, is presumed to emerge from primitive ectoderm at the beginning of gastrulation and contributes to the ventral foregut (Lawson *et al.,* 1986; Lawson and Pedersen, 1987). Foxa2 is expressed in the anterior definitive endoderm in *Foxa2*$^{flox}$*; Nestin::Cre* mutant embryos at the headfold stage. In addition, this region did not show any lacZ activity in *lacZ*$^{flox}$*; Nestin::Cre* embryos at the headfold stage (see Figs. 1A and 1B), suggesting that the *Nestin* promoter is silent before the late-streak/early headfold stage in node precursors at the

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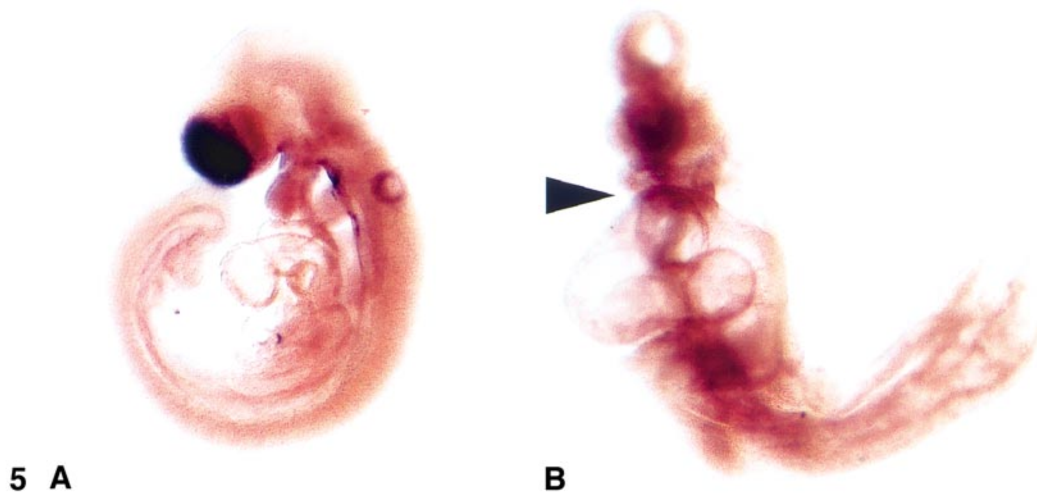


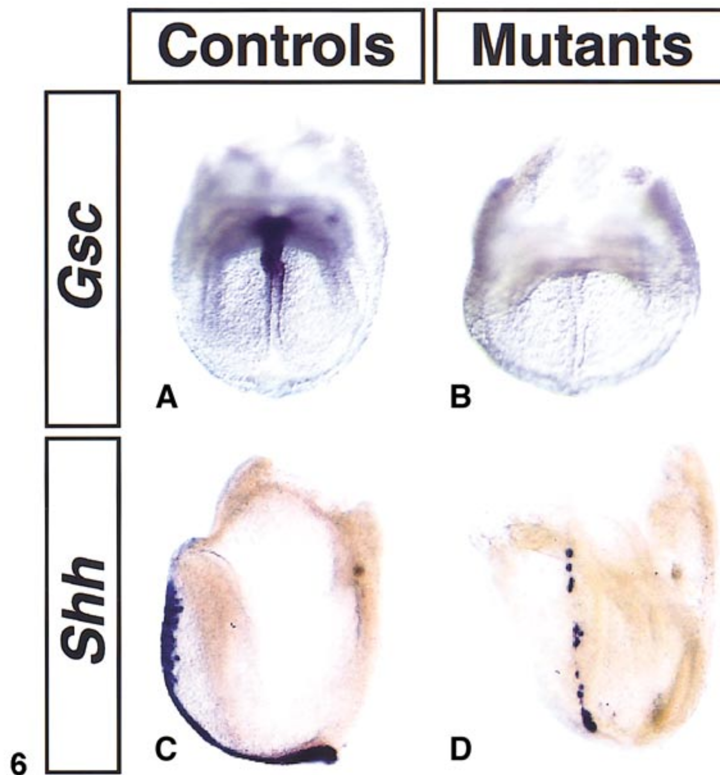


**FIG. 5.** The anterior head and brain are truncated in Foxa2 mutant embryos at 9.5 dpc. Whole-mount *in situ* hybridization to *BF1* on 9.5-dpc control (A, lateral view) and conditional mutant embryos (B, frontal view). (A) Control embryo, showing *BF1* expression in the telencephalon. (B) The absence of expression of the telencephalic marker *BF1* and the lack of telencephalic bulging (arrowhead in B) indicate that the anterior brain is truncated in *Foxa2* conditional mutants.

**FIG. 6.** The anterior axial mesendoderm is severely affected in Foxa2 mutant embryos. Whole-mount *in situ* hybridization to *Gsc* (A, B; ventral views) and *Shh* (C, D; lateral views), of control (A, C) and mutant (B, D) embryos, at the headfold stage. *Gsc* expression, which is specific for the most anterior axial mesendoderm (A), including the prechordal plate, was severely affected or absent in the conditional mutant (B). *Shh* expression, which is specific for the axial mesendoderm (C), is strongly affected or absent in conditional mutants (D).

time they generate the most anterior definitive endoderm. Consistent with this observation, *SII6*, which is expressed in the anterior definitive endoderm, is observed in *Foxa2* conditional mutants, suggesting that the most anterior foregut is produced in the *Foxa2$^{flox}$*; *Nestin::Cre* conditional mutant embryos.

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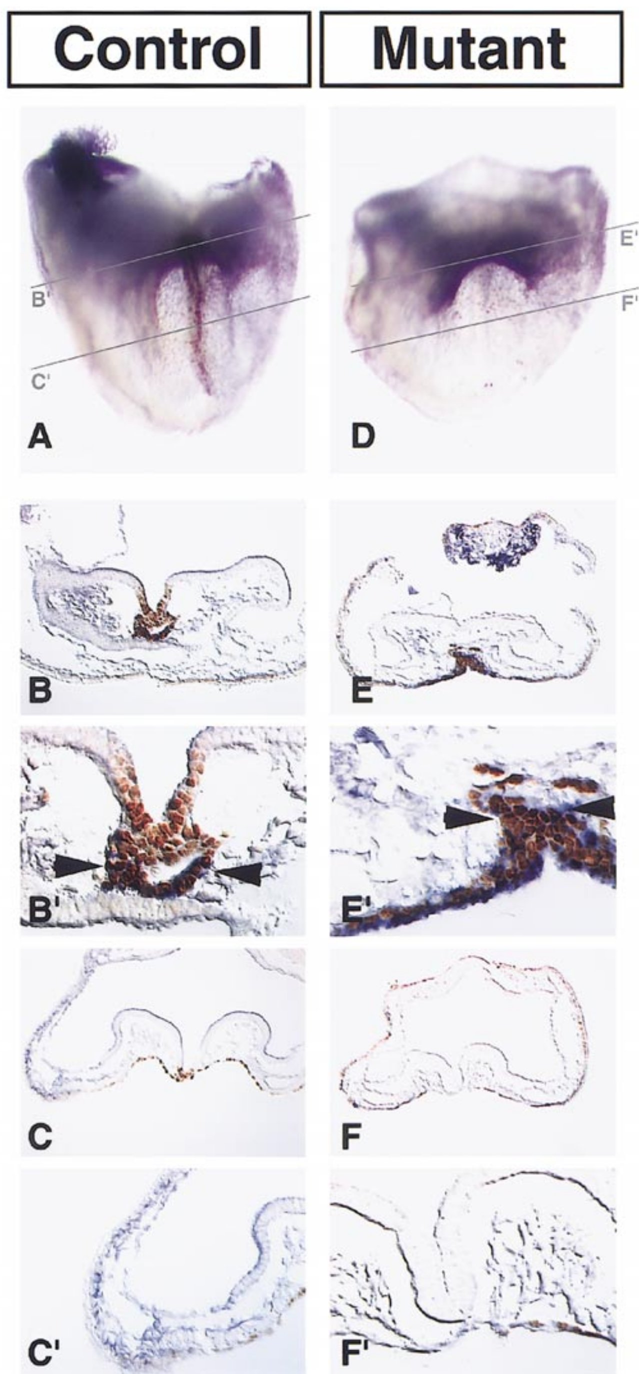


**FIG. 7.** The anterior endoderm is produced in Foxa2 conditional mutant. (A–C′) Control embryo. (D–F′) Conditional mutant embryo. Whole-mount *in situ* hybridization (purple) to *SII6* and antibody staining (brown) to Foxa2 (A, D) and sections (B–C′, D–F′) of the corresponding control (A) and conditional mutant embryo (D) at 7.75 dpc. (B′, C′, E′, F′) Higher magnification of the view respectively shown in (B), (C), (E), and (F). (A–C′) At the headfold stage (A), *SII6* is expressed with a rostral (B, B′) to caudal decreasing gradient in control embryos (C, C′). The highest level of expression is located at the level of the presumptive ventral foregut (B, arrowheads in B′). (D–F′) Despite the lack of axial structures (D), *SII6* is expressed in the anterior definitive endoderm with the same rostral (E, E′) to caudal (F, F′) decreasing gradient as in control embryos (see B–C′). Anterior definitive endoderm cells that do not undergo morphogenesis to form the ventral foregut coexpress *SII6* and Foxa2 in the conditional mutant embryo (E, arrowheads in E′), like in control embryos (B, arrowheads in B′). The anterior definitive endoderm cells are accordingly generated in *Foxa2* conditional mutant embryos.

However, *Cerl* expression was missing from the presumptive gut in *Foxa2*$^{flox}$; *Nestin::Cre* conditional mutant embryos (see Figs. 8D–8F and 8F′). Given that Cre activity was not detected in this region of the embryo and that Foxa2 remains expressed at that level, the deficient specification of the anterior definitive endoderm must not result from a direct effect of the conditional inactivation of Foxa2. One possibility is that the absence of *Cerl* expression results from a lack of signals normally produced in midline structures that are absent in *Foxa2* conditional mutant embryos. The foregut endoderm is generated but its specification subsequently is not maintained in *Foxa2*$^{flox}$; *Nestin::Cre* conditional mutant embryos.

### *Axial Mesendoderm Maintains Anterior Neural Plate and Foregut Specification*

The structures affected in our conditional mutant include tissues where *Foxa2* is not normally expressed (see Figs. 8D–8F), raising the possibility of nonautonomous mechanisms regulating the specification of the endoderm in the conditional mutants.

Patterning of the anterolateral neural plate (telencephalon) may be regulated by FGF8 produced in the anterior neural ridge (for a review, see Rubenstein *et al.,* 1998; Shimamura and Rubenstein, 1997). The reduction in *Fgf8* expression that we observed in the anterior neural ridge (see Fig. 4F) might therefore partly account for the forebrain defects of *Foxa2*$^{flox}$; *Nestin::Cre* conditional mutant embryos (Rubenstein *et al.,* 1998; Shimamura and Rubenstein, 1997). This hypothesis would suggest that the reduction of *Fgf8* expression could partly result from defects in ventral midline signaling. The molecular mechanisms of this reduction remain to be determined.

The absence of axial mesendoderm in *Foxa2* conditional mutants may also result in defects in axial signals, such as *Mdkk1* (Mukhopadhyay *et al.,* in press) and *Shh*, which normally participate in the maintenance and/or the refinement of the specification of adjacent structures, including the foregut and the anterior neural plate. Cooperation of BMP7 and SHH in the induction of forebrain ventral midline cells by prechordal mesoderm has already been demonstrated (Dale *et al.,* 1997). Similar to our results, ablation experiments in the mouse, involving the removal of the rostral anterior midline mesendoderm, including the pre-

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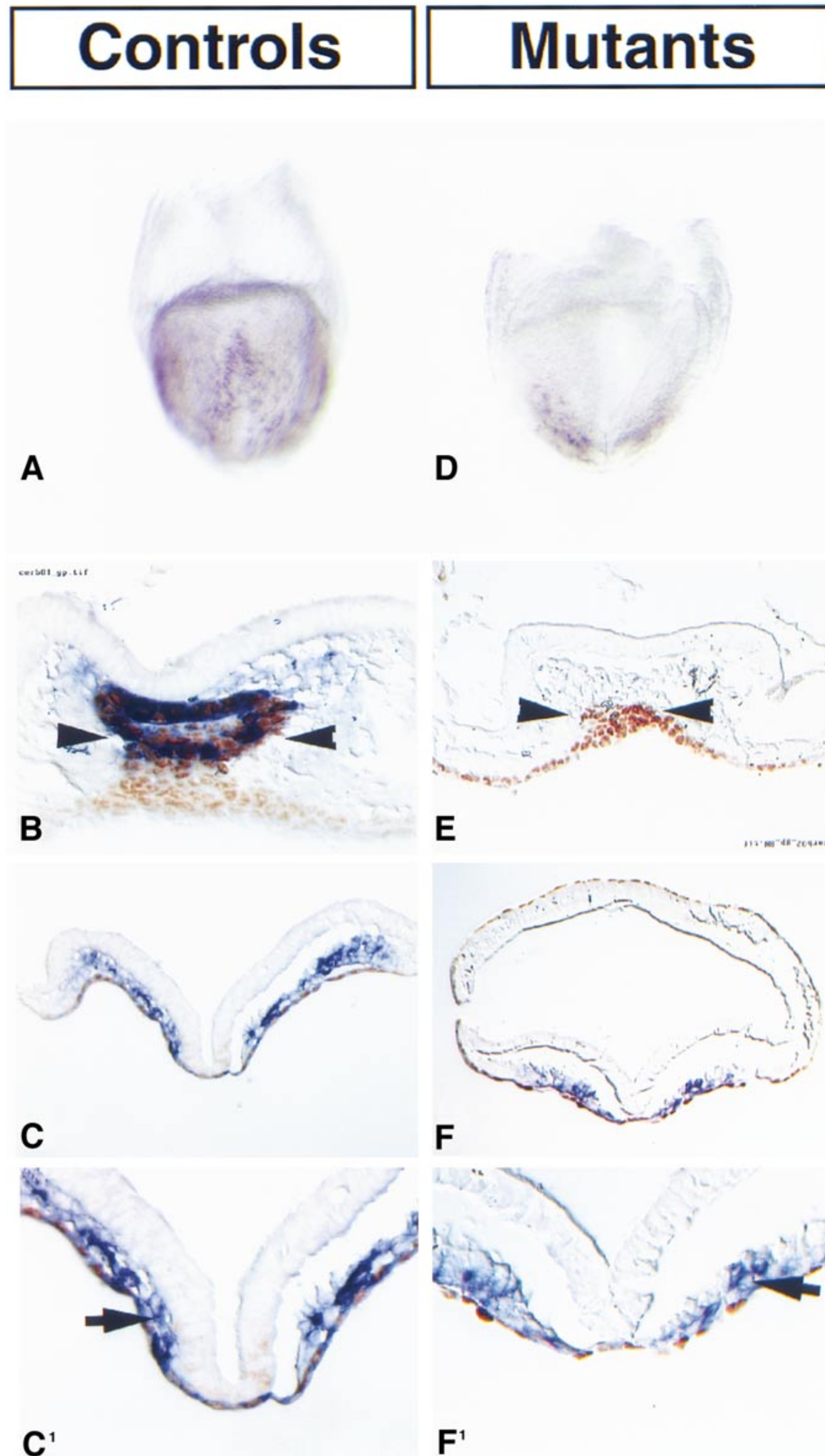


**FIG. 8.** Cerl expression is affected in the anterior definitive endoderm of Foxa2 mutant embryos at 7.75 dpc. (A–C′) Control embryo. (D–F′) Conditional mutant embryo. Whole-mount *in situ* hybridization (purple) to *Cerl* and antibody staining (brown) to Foxa2 (A, D) and sections (B–C′ and E–F′) of the corresponding control (A) and conditional mutant embryo (D) at 7.75 dpc. (C′, F′) Higher magnification of the view respectively shown in (C) and (F). At the headfold stage, *Cerl* is coexpressed with Foxa2 in the endoderm of the whole foregut pocket in control embryos (B). In *Foxa2* conditional mutants, the foregut pocket does not form (E) and *Cerl* is not expressed in the Foxa2-positive anterior definitive endoderm cells (arrowheads in E). In contrast, *Cerl* is expressed in the anterior mesoderm (arrows) of mutant (C, C′) and wild-type embryos (F, F′).

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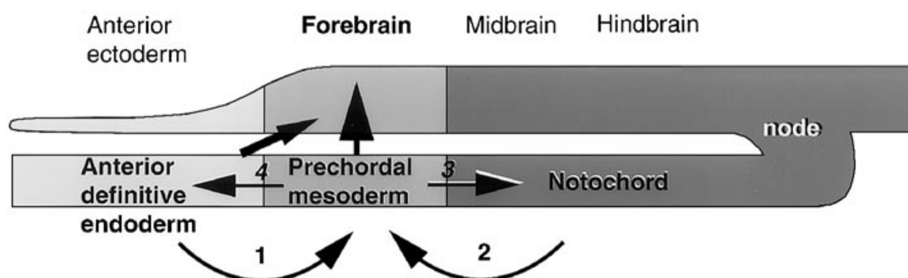


**FIG. 9.** Schematic summary of the putative signals exchanged by the prechordal mesoderm with the neighboring posterior axial mesendoderm caudally and with the endoderm anteriorly. The character of the early extending axial mesoderm cells may be defined through TGF$\beta$s signals from anterior endoderm tissues (arrow 1). Maintenance and function of the prechordal mesoderm, specifically expressing Gsc and acting on the anterior neural plate (vertical arrow), requires inductive signals emanating from the posterior axial mesendoderm, the presumptive notochord (arrow 2). Conversely, signals emanating from the anterior mesendoderm, the presumptive prechordal mesoderm, restrict the extent of the posterior mesendoderm (arrow 3). Our results suggest, in addition, that the maintenance of the specification of the anterior endoderm, specifically expressing Cerl at the headfold stage, requires inductive signals from the anterior axial mesendoderm, the presumptive prechordal mesoderm (arrow 4).

chordal plate, resulted in a truncation of the head accompanied by the loss of several forebrain markers (Camus *et al.,* 2000). It is therefore likely that the head truncation observed in *Foxa2* conditional mutant embryos results from the absence of prechordal axial mesendoderm. Similarly, such a regulatory mechanism has also been suggested from the functional study of various mutations in the mouse. For example, *Lim1* is expressed in the AVE, the anterior mesendoderm, and the mesoderm wings (Barnes *et al.,* 1994; Belo *et al.,* 1997; Shawlot and Behringer, 1995; Shimono and Behringer, 1999). Chimeric embryos derived from $Lim1^{-/-}$ ES cells display a range of head defects similar to those observed in Foxa2 conditional mutants, suggesting that similar regulatory mechanisms are affected. In the case of *Lim1* mutants, explant culture experiments demonstrated that $Lim1^{-/-}$ cells contributing to the anterior mesendoderm were deficient in anterior neural signaling (Shawlot *et al.,* 1999). In addition, *Gsc* also participates in the differentiation of the ventral forebrain synergistically with *Foxa2* (Filosa *et al.,* 1997).

Taken together, these results support the idea that the axial mesendoderm is a signaling structure required for anterior head formation by maintaining the specification of both the anterior foregut and the anterior neural plate.

### *Reciprocal Tissue Interactions Are Required to Maintain Anterior Specification*

Following Spemann's hypothesis (Hamburger, 1988) and Nieuwkoop's analysis (Stern, 2001), a sequential-step model for anterior neural induction in the mouse has been proposed (Foley *et al.,* 2000; Shawlot *et al.,* 1999; Stern, 2001; Thomas and Beddington, 1996). This model suggests that the anterior region of the embryo is first specified under the regulation of the AVE and the anterior primitive streak (Beddington and Robertson, 1999). Subsequently, this specification is maintained and/or reinforced by the primitive streak-derived anterior mesendoderm (for reviews, see Kiecker and Niehrs, 2001; Stern, 2001). Our results provide additional support for the second phase of this model according to which anterior primitive streak-derived axial mesendoderm replaces the AVE at the late-streak stage and maintains and/or reinforces anterior neural plate specification. This second phase would depend on an essential role of *Foxa2* in the formation of the anterior mesendoderm.

Studies of the homeobox gene, Hesx1, suggest that the anterior definitive endoderm is also required for the formation of the forebrain (Martinez-Barbera and Beddington, 2001), raising the question of how and whether the anterior axial mesendoderm and the anterior endoderm interact. In the chick, *in vitro* culture studies, together with *in vivo* grafting experiments, revealed that the differentiation of early extending axial mesoderm cells is labile and defined through TGF$\beta$ signals from anterior endoderm. Anterior endoderm would elicit aspects of prechordal mesoderm identity in extending axial mesoderm by repressing notochord characteristics, briefly maintaining *Gsc* expression and inducing *Bmp7* expression. These results suggest that, *in vivo*, signaling by anterior endoderm may determine the extent of prechordal mesoderm (Vesque *et al.,* 2000).

Ablation experiments in the mouse, involving the removal of either the rostral or the posterior anterior midline mesendoderm (AML) demonstrated that maintenance and function of the rostral AML, a region expressing *Gsc*, requires inductive signals emanating from the caudal AML (Camus *et al.,* 2000). After the ablation of the rostral AML, Gsc expression was recovered, indicating that caudal AML could form rostral AML (Camus *et al.,* 2000). This observation therefore suggests that signals emanating from the rostral AML restrict the extent of the caudal AML. We therefore suggest that the prechordal mesoderm receives signals from both the more posterior axial mesendoderm caudally and from the adjacent endoderm anteriorly. The

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prechordal mesoderm would interact with the anterior endoderm for the maintenance of *Cerl* expression in the anterior endoderm and with the posterior axial mesendoderm to restrict the extent of the notochord (Fig. 9).

In summary, in the present study, we have demonstrated that *Foxa2* is required for the formation of the anterior axial mesendoderm, itself secondarily required to relieve the function of the AVE by maintaining the primary specification of the anterior neural plate and foregut. Further studies are now required to dissect the molecular regulatory cascades involved in the interaction between the various compartments of the axial mesendoderm, and between the prechordal plate and the anterior neuroepithelium.

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