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Anterior mesendoderm induces mouse *Engrailed* genes in explant cultures

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SUMMARY

We have developed germ layer explant culture assays to study the role of mesoderm in anterior-posterior (A-P) patterning of the mouse neural plate. Using isolated explants of ectodermal tissue alone, we have demonstrated that the expression of *Engrailed-1* (*En-1*) and *En-2* genes in ectoderm is independent of mesoderm by the mid- to late streak stage, at least 12 hours before their onset of expression in the neural tube in vivo at the early somite stage. In recombination explants, anterior mesendoderm from headfold stage embryos induces the expression of *En-1* and *En-2* in pre- to early streak ectoderm and in posterior ectoderm from headfold stage embryos. In contrast, posterior mesendoderm from embryos of the same stage does not induce *En* genes in pre- to early streak ectoderm but is able to induce expression of a general neural marker, neurofilament 160×10^3 M_r. These results provide the first direct evidence for a role of mesendoderm in induction and regionalization of neural tissue in mouse.

Key words: neural induction, *Engrailed*, explant cultures, anterior-posterior axis, patterning, mouse

INTRODUCTION

The classical transplantation experiments of Spemann and Mangold (Spemann, 1938) demonstrated that the formation and patterning of the neural plate in amphibian embryos depends on interaction between the dorsal lip of the blastopore (DLB), the 'organizer', and overlying ectoderm. Similar experiments in chick involving transplants of Hensen's node tissue have implicated this structure at the anterior end of the primitive streak as critical for neural induction and axis formation in avian embryos (Waddington and Schmidt, 1933; Hara, 1978). The type of neural tissue induced depends on the age of the DLB or node used; early DLB or node tissue induces the expression of both anterior and posterior structures, while older tissue induces only posterior structures (Spemann, 1931; Tsung et al., 1965; Dias and Schoenwolf, 1990; Storey et al., 1992). There is evidence to suggest that the organizer and node can act at a distance to regionalize the neural ectoderm as well as by imparting regional inductive capacity to the dorsal mesoderm as it involutes. Several experiments in *Xenopus* have shown that induction of region-specific neural genes can occur after direct contact between appropriate axial mesoderm and uninduced ectoderm (Sharpe et al., 1989; Sharpe and Gurdon, 1990; Hemmati-Brivanlou et al., 1990; Saha and Grainger, 1992). However, exogastrula and Keller sandwich experiments have indicated that some regional neural markers can be induced in ectoderm at a considerable distance from the plane of interaction between mesoderm and ectoderm (Ruiz i Altaba, 1990, 1992; Doniach et al., 1992). This suggests that there may also be signalling through the ectoderm, so-called planar induction.

In the mouse, there is very little direct evidence on the cellular interactions involved in initial neural induction and A-P regionalization. It is generally assumed that the mechanisms will be similar to those described in chick and *Xenopus*, an assumption strengthened by the recent lineage studies showing that the fate map of the early streak mouse embryo is topologically similar to that of the other species (Lawson et al., 1991). A structure similar to Hensen's node is found at the anterior end of the mouse primitive streak and there is preliminary evidence that it also possesses organizer activity based on its ability to induce some new axial structures when transplanted into the *Xenopus* blastula (Blum et al., 1992). Apart from this experiment, the only other experimental information available relates more to the time of neural induction and regionalization than the inductive mechanism. Heterotopic grafts of [^{3}H]thymidine-labelled anterior ectodermal cells into embryos in culture have indicated that anterior ectoderm is restricted largely to the formation of the neurectoderm by the late streak stage of development (Beddington, 1982).

These experiments suggest that neural induction has occurred, at least in the anterior ectoderm, by the late streak stage. Evidence that A-P regionalization of the presumptive neural tissue has also begun slightly later at the headfold stage is provided by the expression patterns of a number of genes thought to be involved in regionalization along the

body axis. Several members of the Hox gene families, such as *Hox-2.8* and *Hox-2.9* (Wilkinson et al., 1989a; Frohman et al., 1990; Murphy and Hill, 1991) and the zinc finger gene, *Krox-20* (Wilkinson et al., 1989b), already show specific expression domains by the headfold stage, that will later correspond to the boundaries of specific hindbrain rhombomeres. The expression domains of genes such as the *Engrailed* genes, *En-1* and *En-2* (Davis et al., 1988a) and *Wnt-1* (Davis et al., 1988b; McMahon et al., 1992), which define the developing mid-hindbrain region, are established slightly later in development at the early somite stage.

It is not yet clear whether the different times of establishment of the different gene expression domains reflect ongoing inductive interactions between neural plate and mesoderm tissues, or whether there is a limited period of information flow between mesoderm and ectoderm, followed by interactions within the neurectoderm itself. We have developed germ layer explant culture assays in the mouse to investigate the timing of regionalization in the anterior neural plate and the regional inducing capacities of mesendoderm, using the *En-1* and *En-2* genes as regional neural markers. These studies have shown that expression of *En-1* and *En-2* is autonomous to the ectoderm by the mid- to late primitive streak stage, at least 12 hours before the normal onset of expression. We further demonstrate that anterior but not posterior mesendoderm of the headfold stage embryo can induce En expression in competent ectoderm. These studies suggest that ectodermal-mesendodermal interactions that take place between the mid-streak and headfold stages are critical for establishing regional identity in the anterior central nervous system (CNS).

MATERIALS AND METHODS

Mouse strains used

6.0-8.5 days p.c. mouse embryos were obtained from outbred CD1 mice (Charles River, Quebec), transgenic mouse line Tg5 (Logan et al., 1993), which expresses *lacZ* under the control of the *En-2* promoter on a CD1 background, or the genetrap mouse line C101 (S. Gasca, D. Hill and J. Rossant, unpublished), which carries an insertion of the genetrap vector pGT4.5a (Skarnes et al., 1992) on a 129/Sv background. In the latter two cases, animals heterozygous for the transgene or the gene trap insertion were obtained by mating homozygous males with CD1 females.

Embryo manipulations

Embryos were isolated from decidua and dissected into embryonic and extraembryonic parts. The embryonic part was subsequently incubated in 0.25% pancreatin, 0.5% trypsin in phosphate-buffered saline at 4°C for 10 minutes (pre-streak to late streak stages) or 15 minutes (headfold to early somite stages). Ectoderm was then separated from endoderm (pre-streak) or mesendoderm (later stages) with the aid of tungsten needles (Fig. 1A). Total pre-streak ectoderm or anterior half ectoderm (anterior to node) from later stages was then cultured alone in Dulbecco's modified Eagles medium (DME) plus 15% fetal calf serum (FCS) in bacteriological dishes for 2 and 4 days. In the recombination experiments, a single ectodermal piece was reaggregated with total mesendoderm or two pieces of lateral mesendoderm in small depression wells made in bacteriological dishes in a 20 μl drop of DME plus 15% FCS under mineral oil (Fig. 1B) and then cultured for 2 or 3 days (when headfold ectoderm was used) and 3 or 4 days for experiments using pre- to early streak ectoderm.

Survival and growth of pre- to early streak explants

Four parameters were used to determine the survival and growth of pre- to early streak explants, namely size, total cell number, percentage cell viability in each explant before and after 3 days culture and percentage of cells in mitosis in explants after 3 days culture. The size of explants was measured with a micrometer under the dissecting scope. Total cell number per explant was determined by dispersing each explant into a single cell suspension after incubation in 0.5% trypsin for 5 minutes at 37°C, followed by addition of DME plus 15% FCS, and then the cells were counted with a hemocytometer. Percentage cell viability was assessed by staining cells before counting with trypan blue (50:50). The percentage of cells in mitosis was determined by first fixing explants in methanol:acetic acid (3:1) for half an hour at room temperature and then dispersing cells of explant on a slide in a small drop of 60% acetic acid. The number of mitotic figures was counted after the cells have been stained with hematoxylin. 1000 cells were counted per explant. Each experiment was performed separately with three explants.

Whole-mount immunocytochemistry

Embryos were processed and analyzed for expression of En proteins using polyclonal rabbit antiserum, *a-Enhb1*, following the previously published procedure (Davis et al., 1991). The same procedure was used to study expression of the 160×10^3 M_r neurofilament protein using supernatant from mouse hybridoma 2H3 (1:5 dilution), obtained from the Developmental Studies Hybridoma bank, Iowa. In this case, 4% paraformaldehyde was used as a fixative, rather than dimethylsulfoxide:methanol (4:1) in the case of En proteins. Peroxidase-coupled goat anti-mouse (IgG) was used as secondary antibody (Boehringer Mannheim, 1:500).

Whole-mount RNA in situ hybridization

Whole-mount RNA in situ hybridization was performed on explants following the published protocol (Conlon and Rossant, 1992), except that protease K treatment was reduced to a 1 minute treatment at room temperature using a concentration of 20 μg/ml for the explants. The *En-1*-specific probe was a T7 polymerase transcript of a 184 basepairs (bp) 3′ untranslated *Sau*3A-*Eco*RI *En-1* cDNA fragment (Davis et al., 1988a). The *En-2*-specific probe was a T7 polymerase transcript of a 260 bp 3′ untranslated *Bgl*II/*Sst*I *En-2* cDNA fragment (Davis et al., 1988b). The *T* gene-specific probe was a 2 kbp cDNA fragment synthesized from clone pSK75 (Hermann et al., 1990) using T7 polymerase. As positive controls for the three RNA probes used, the normal expression pattern was detected in early somite embryos for *En* genes and in mid- to late streak and headfold embryos for the *T* gene (data not shown). The embryos were cleared for photography in 50% glycerol.

β-galactosidase staining of explants

Explants to be stained were fixed in 0.2% glutaraldehyde (Sigma) and stained with X-gal (Bethesda Research Laboratories) as described previously (Beddington et al., 1989).

RESULTS

Specification of *En* expression in ectodermal explants

Embryonic ectodermal tissues from 6.0-8.5 days p.c. mouse

embryos were separated from mesoderm and endoderm and cultured as explants in bacteriological dishes (Fig. 1A) to determine when *En* expression in ectoderm was independent of underlying tissues. After 2 and 4 days, the ectodermal explants were fixed and assayed initially for the expression of En proteins using the En-specific polyclonal rabbit antiserum, *a-Enhb1*.

With the particular light/dark cycle used in our animal colony, embryos of 6.0-8.5 days p.c. could be divided into four distinct stages that were easily distinguishable morphologically (Fig. 1C-F). These stages were pre- to early streak (6.0-6.5 days p.c.), mid- to late streak (7.0 days p.c.), headfold (7.5 days p.c.) and early somite (8.0-8.5 days p.c.) stages. Pre-streak embryos contain no mesoderm, while early streak embryos contain mesoderm only in the posterior part of the embryo. In mid- to late streak stages, mesoderm has migrated halfway (Fig. 1D, left) or fully (Fig. 1D, right) to the anterior end of the embryo, coincident with the formation of the amnion. The head process has begun to extend anteriorly by the late streak stage but is not visible in the diagram, which is slightly lateral to the midline. In headfold stage embryos, the elevated neural folds are clearly apparent in the anterior part of the embryo and the allantoic bud is visible at the posterior end (Fig. 1E). The headprocess continues to extend further anteriorly during this stage and forms the notochord. The early somite stage embryos contained between one and ten somites (Fig. 1F).

Ectoderm from early somite stage embryos, which already expresses En protein at the time of isolation, continued to show En expression after 2 and 4 days culture (Table 1). Interestingly, 16/32 explants from mid- to late streak stage (Fig. 2B) and all explants from headfold stage (Fig. 2C) also stained positive for En proteins after culture. These results demonstrate that *En* gene expression is autonomous to the ectoderm by the mid- to late streak stage of development. Only pre- to early streak ectoderm failed to express En proteins in culture (Fig. 2A), although the explants clearly increase in size from 0.05-0.15 mm to 0.40-0.55 mm over the culture period. The total number of cells before and after 3 days culture increased at least tenfold ranging from 1,500-1,800 to 18,000-43,600, respectively. Cell viablity was always more than 90%. Finally, the numbers of cells in mitosis was determined to be ranging from 1.4% to 2.1% after 3 days culture. Despite extensive growth in vitro, ectodermal explants of pre- to early streak embryos also failed to express a general neural marker, the 160×10^3 M_r neurofilament protein (NF160) (Fig. 4A) when assayed with monoclonal antibody 2H3 after 4 days culture. Headfold stage ectodermal explants all expressed the NF160 marker after 3 days culture (Fig. 4B), although embryos were negative for this marker when tissues were isolated. This result demonstrates that our culture conditions can support neural differentiation and suggest that anterior headfold ectoderm but not pre- to early streak ectoderm is already specified to develop into neural tissue.

Since the analysis with *aEnhb*-1 antiserum could not distinguish between expression of En-1 and En-2 proteins, we also studied specific expression of *En-1* and *En-2* RNA in early ectodermal explants after 2 days of culture by whole-mount nonradioactive in situ hybridization using *En-1* and *En-2*-specific RNA probes. 5/12 of the mid- to late streak explants expressed *En-1* RNA and 3/11 expressed *En-2*. In headfold explants, 14/16 explants showed some *En-1* RNA expression and *En-2* RNA was detected in 13/15 of these explants (Fig. 2D,E). Thus, the expression of both the *En-1* and *En-2* genes is autonomous to the ectoderm as early as the mid- to late streak stage, at least 12 hours earlier than the normal onset of *En* expression.

It was important to ensure that the ectodermal explants were free of contaminating or regenerating mesoderm. The enzyme treatment clearly removed the lateral mesodermal wings but primitive streak mesoderm and developing notochord lie in the midline and are closely apposed to the overlying ectoderm especially at the late streak and headfold stage. They are thus harder to separate. Even if separation is complete, the possibility exists that the ectoderm could regenerate axial mesoderm in vitro. To test for such contamination, mid- to late streak and headfold ectodermal explants were examined for expression of the *Brachyury* (*T*) gene after 2 days in culture (Hermann et al., 1990; Wilkinson et al., 1990) since its expression is restricted to these tissues at these stages (Hermann, 1991). 36/40 such explants did not express the *T* gene. The four positive headfold explants each contained a small isolated patch of *T*-expressing cells. The one with the largest patch of T expression is shown in Fig. 2F. As a second control for the presence of notochordal tissue and the node (Hensen's node equivalent), explants from a mouse strain carrying a *lacZ* gene trap vector that specifically marks these tissues at late streak and headfold stages (S. Gasca, D. Hill and J. Rossant, unpublished data) were stained for β-galactosidase activity. All 20 of mid- to late streak and headfold explants were negative. These results suggested that there was little contamination of the original cultures with midline streak or head process tissues and demonstrated that the isolated mid- to late streak and headfold ectodermal explants were not capable of differentiating into T-expressing or *lacZ*-positive axial mesodermal cells under our culture conditions. The small proportion of ectodermal explants with axial mesodermal contamination is insufficient to explain the *En* expression results.

Regional differences in the *En*-inducing ability of mesendoderm

Ectodermal explants from pre- to early streak embryos that do not have anterior mesoderm did not express *En* genes when cultured alone while explants from headfold and somite stage embryos that have anterior mesoderm did turn on *En* expression. These data are consistent with a role for anterior mesoderm from primitive streak stage embryos in inducing the later expression of the *En* genes. We tested this possibility by recombination of ectodermal and mesendodermal tissues in vitro. We first tested whether anterior mesendoderm from mid- to late streak and headfold stage embryos could induce *En* expression in pre- to early streak ectoderm, which does not express En proteins when cultured alone. After 2 days in culture, En proteins were induced in the recombination explants (Table 2; Fig. 3A), with headfold mesendoderm being more effective than mid- to late streak mesendoderm (74% versus 25%, respectively). Consequently, headfold mesendoderm was used for

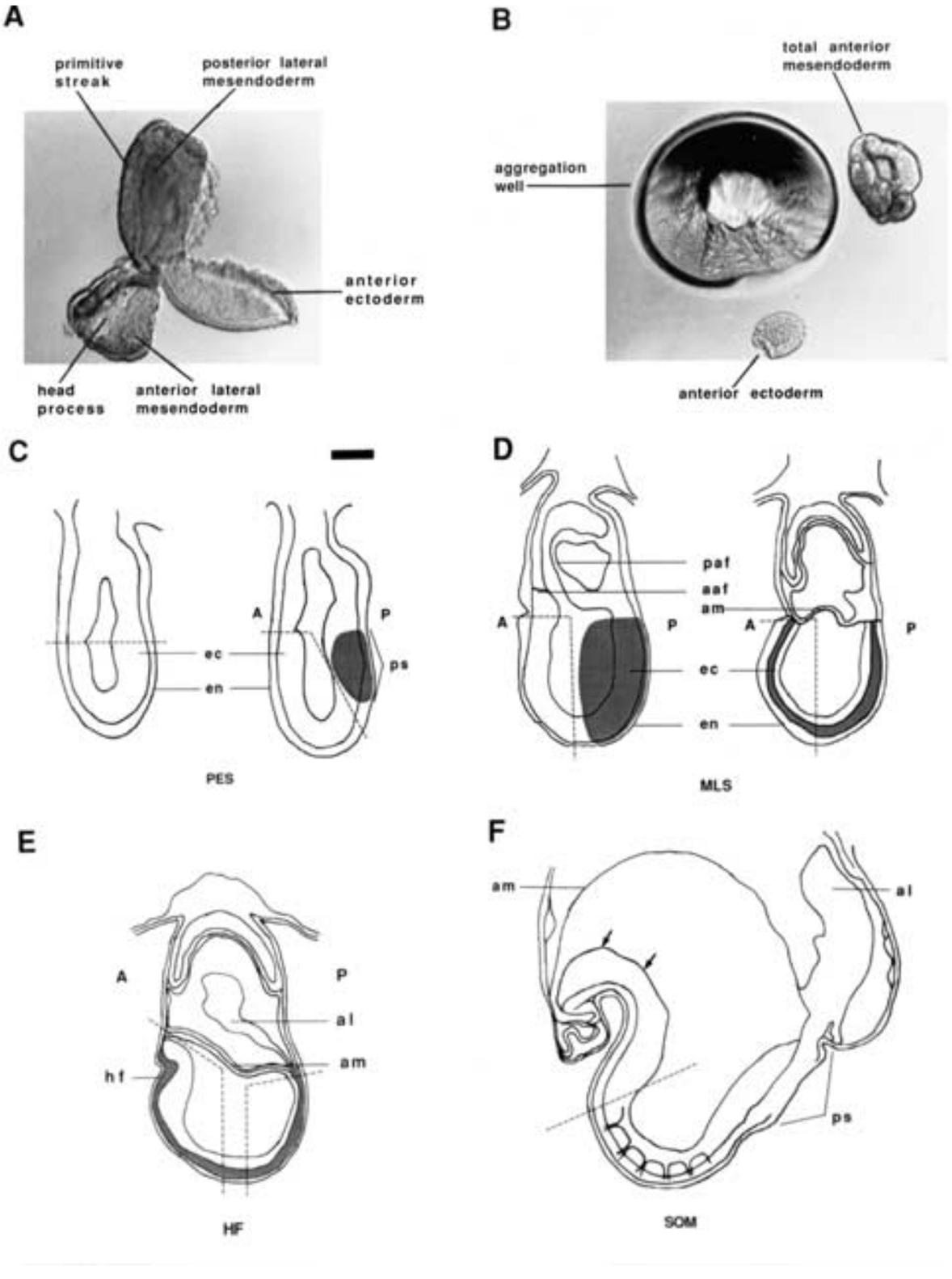
A
primitive streak
posterior lateral mesendoderm
anterior ectoderm
head process
anterior lateral mesendoderm
B
total anterior mesendoderm
aggregation well
anterior ectoderm
C
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Fig. 1.

Table 1. En protein or *LacZ* expression in wildtype or Tg5 ectodermal explants

Embryonic stage	En expression in vivo	En expression after 2 days culture No. expressing/ No. explants	En expression after 4 days culture No. expressing/ No. explants	*lacZ* expression after 2 days culture No. expressing/ No. explants
PES (6-6.5 d p.c.)	–	0/8	0/10	0/12
MLS (7.0 d p.c.)	–	14/23	2/9	7/10
HF (7.5 d p.c.)	–	15/15	7/7	12/12
SOM (8-8.5 d p.c.)	+	8/8	3/3	ND

Abbreviations used: PES, pre- to early streak; MLS, mid- to late streak; HF, headfold; SOM, early somites; No., number; ND, not determined.

all subsequent experiments. In control experiments, anterior mid- to late streak and headfold mesendoderm and pre- to early streak ectoderm did not express En proteins when cultured separately for 2 days or 3 days (Table 2 and data not shown).

In this initial experiment, the mesendodermal tissue used contained both lateral mesoderm as well as any presumptive notochordal tissue in the midline. When anterior lateral mesendoderm of headfold embryos (total anterior mesoderm minus midline head process; see Fig. 1A) was recombined with pre- to early streak ectoderm, it could also induce expression of *En* genes (Table 2). We were unable to test whether presumptive notochordal mesoderm alone could also induce *En* expression because this tissue is very thin and difficult to manipulate at these early stages of mouse development. Posterior lateral mesendoderm of headfold embryos (mesendoderm lateral to primitive streak area in posterior part of embryo; see Fig. 1A) failed to induce expression of En proteins in pre- to early streak ectoderm (Table 2), indicating that *En*-inducing ability is confined to a subset of mesodermal cells. Total posterior mesendoderm of the same stage was also unable to induce the expression of En proteins in pre- to early streak ectoderm (Table 2; Fig. 3B). We also showed that posterior mesendoderm (somites plus a portion of presomitic mesoderm and mesendoderm around them) from embryos with 2-5 somites could not induce expression of En proteins (Table 2). Thus both total anterior and anterior lateral mesendoderm from headfold stage embryos, but not posterior mesendoderm from headfold and early somite stage embryos have *En*-inducing ability.

The ability of both anterior and posterior mesendoderm to induce neural tissue was also tested by analyzing for expression of NF160 in recombination explants. Both total anterior and posterior lateral mesendoderm of headfold stage embryos were able to induce NF160 expression in pre- to early streak ectoderm after 4 days in culture in 7/17 and 8/17 of the explants respectively (Fig. 4C,D). The number of NF160-positive neuronal cell bodies found in ectoderm induced with either anterior or posterior mesendoderm was similar, ranging from 20 to 50; however, the neurons in the former case appear to have extended longer neurites. Thus the differences in *En*-inducing ability between anterior and posterior mesendoderm do not arise because posterior mesendoderm lacks the ability to induce any kind of neural tissue.

To demonstrate that *En* expression in recombination explants was due to induced expression in ectoderm rather than ectopic expression in mesendoderm, we made use of a transgenic mouse line, Tg5, that contains a construct encompassing 7.0 kb of *En-2* 5′ regulatory sequences driving *lacZ* followed by 2.5 kb of 3′ untranslated and 3′ flanking genomic DNA. The timing and spatial expression of *lacZ* in this transgenic line have been extensively analyzed and shown to be identical to the endogenous *En-2* gene (Logan et al., 1993). We first tested whether the timing of autonomous expression of the *En2-lacZ* construct in ectodermal explants was the same as that observed for the endogenous *En-2* gene. *lacZ* expression was autonomous in ectodermal explants of Tg5 embryos by the mid- to late streak stage, similar to results obtained with *En-1* and *En-2* genes (Table 1; Fig. 5A). Therefore, using Tg5 we can follow the behaviour of *En-2* expression simply by studying *lacZ* expression. In addition, the *lacZ* gene provides a lineage marker for the ectodermal tissue. When pre- to early streak ectoderm from Tg5 embryos was recombined with headfold anterior mesendoderm from wild-type embryos, *lacZ* expression was induced in 6/28 (21%) of the recombination explants (Fig. 5B), showing that *En-2* expression was induced in the ectoderm component. The difference in the ability of anterior mesendoderm from headfold stage embryos to induce *En* as assessed by *lacZ* expression using the *En2-lacZ* construct (21%) versus *En* expression using

Fig. 1. Illustration of the germ layer explant-recombination assay (A, B) and stages of mouse embryos used (C-F). (A) Germ layer separation of a headfold stage embryo showing the different tissue fragments. (B) Recombination of pre- to early streak ectoderm with headfold total anterior mesendoderm in an aggregation well. (C) Pre-streak to early streak stage (PES); 6-6.5 days p.c. Pre-streak embryos contain no mesoderm (left), while early streak embryos (right) contain mesoderm only in the posterior part of the embryo. (D) Mid-streak to late streak stage (MLS); 7.0 days p.c. In mid-streak stages, mesoderm has migrated halfway (left) or fully (right) to the anterior of the embryo, coincident with the formation of the amnion. The head process has begun to extend anteriorly by the late streak stage (right) but is not visible in the diagram which is slightly lateral to the midline. (E) Headfold stage (HF); 7.5 days p.c. The elevated neural folds are clearly apparent in the anterior part of the embryo. (F) Somite stage (SOM); 8.5 days p.c. The early somite stage embryos used contained 1-10 somites. Arrows mark the boundaries of *En* expression in the neural tube. In C-E, the anterior extent of migration of mesoderm is represented by the shaded area. Dashed lines show the cuts made to isolate the different tissue pieces. Abbreviations: A, anterior; P, posterior; ec, ectoderm; en, endoderm; ps, primitive streak; aaf, anterior amniotic fold; paf, posterior amniotic fold; am, amnion; hf, headfold; al, allantois. Scale bar in C represents 100 μm.

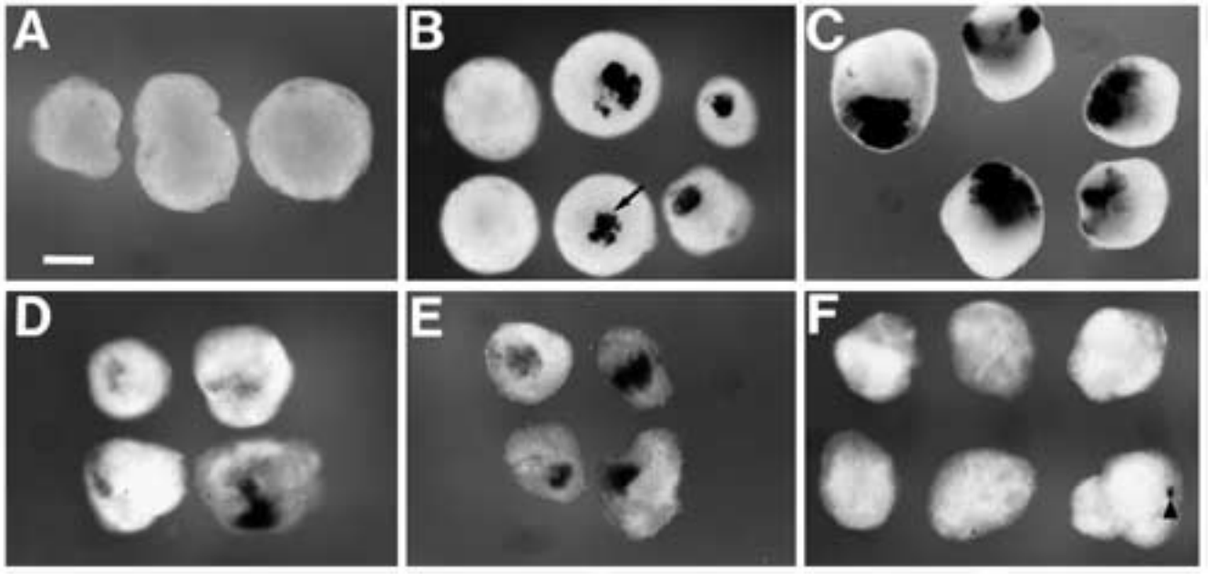

Fig. 2. Isolated ectodermal explants stained with *aEnhb*-1 antiserum (A-C) or by whole-mount RNA in situs (D-F). (A) Pre- to early streak ectodermal explants showing no En expression. (B) Mid- to late streak ectodermal explants. The arrow points to a discrete area of strong staining in one of the four positive mid- to late streak explants shown on the right, while two ectodermal explants of mid- to early streak stage illustrated on the left are clearly negative for En protein expression. (C) Headfold ectodermal explants expressing En proteins. (D) Whole-mount RNA in situ hybridization of *En-1* and (E) *En-2* RNAs in headfold ectodermal explants. Again, staining was confined to a discrete region of the explants. (F) Whole-mount RNA in situ hybridization with *T* probe on six headfold ectodermal explants. A rare (4/40) explant with T expression is indicated with the arrowhead. Scale bar represents 200 μm.

a-Enhb1 antiserum (74%, Table 2) may be due to the fact that in the former case only *En-2* expression was measured, while in the latter case both expression of *En-1* and *En-2* were measured.

Induction of *En-2* expression in both anterior and posterior headfold ectoderm

Having determined that mesendoderm is regionalized in its ability to induce *En* expression at the headfold stage, we tested whether ectoderm is also regionalized along the A-P axis in its ability to respond. It was not considered feasible to test the differential responsiveness of the ectoderm along the A-P axis at early gastrulation stages, since fate-map studies of pre- to early streak embryos do not allow clear delineation of regions that will give rise to anterior and posterior neurectoderm (Lawson et al., 1991). By the late streak stage onwards, however, the ectoderm from the posterior half of the embryo is fated to develop into structures posterior to the hindbrain (Tam, 1989). We, therefore, tested posterior ectoderm of embryos from headfold stage for its ability to respond to anterior mesendoderm.

Table 2. En protein expression in recombination explants

Type of explants	Number expressing / Number of explants (%)
PES ectoderm	0/15 (0)
HF anterior mesendoderm	0/20 (0)
PES ectoderm+MLS anterior mesendoderm (total)	4/16 (25)
PES ectoderm+HF anterior mesendoderm (total)	14/19 (74)
PES ectoderm+HF anterior mesendoderm (lateral)	6/12 (50)
PES ectoderm+HF posterior mesendoderm (total)	0/7 (0)
PES ectoderm+HF posterior mesendoderm (lateral)	0/6 (0)
PES ectoderm+SOM posterior mesendoderm (lateral)	0/11 (0)
HF total posterior half	0/13 (0)
HF posterior ectoderm	0/13 (0)
HF posterior ectoderm+HF anterior mesendoderm (total)	6/23 (26)
HF posterior ectoderm+HF anterior mesendoderm (lateral)	3/13 (23)
HF posterior ectoderm+HF posterior mesendoderm (lateral)	0/17 (0)

Same abbreviations are used as in Table 1.

Posterior lateral ectoderm from headfold stage embryos was recombined with anterior headfold mesendoderm and studied for En expression after 2 days in culture. Ectoderm from the posterior lateral region was used because separation of germ layers in the midline primitive streak area of headfold stage embryos is not feasible. When reaggregated with anterior lateral and anterior total mesendoderm, 23% and 26% of posterior lateral ectodermal explants of headfold stage embryos, respectively, were induced to express En proteins (Table 2; Fig. 3C). Therefore, both anterior and posterior ectoderm can be induced to express En proteins. Control total posterior half as well as posterior lateral ectodermal explants of headfold embryos never showed expression of En proteins after 2 days culture (Table 2), although posterior lateral ectoderm showed expression of NF160 proteins after 3 days culture (data not shown). Posterior lateral ectoderm reaggregated with posterior lateral mesendoderm also showed no En staining (Table 2; Fig. 3D), indicating that the enzymatic treatment did not artificially turn on

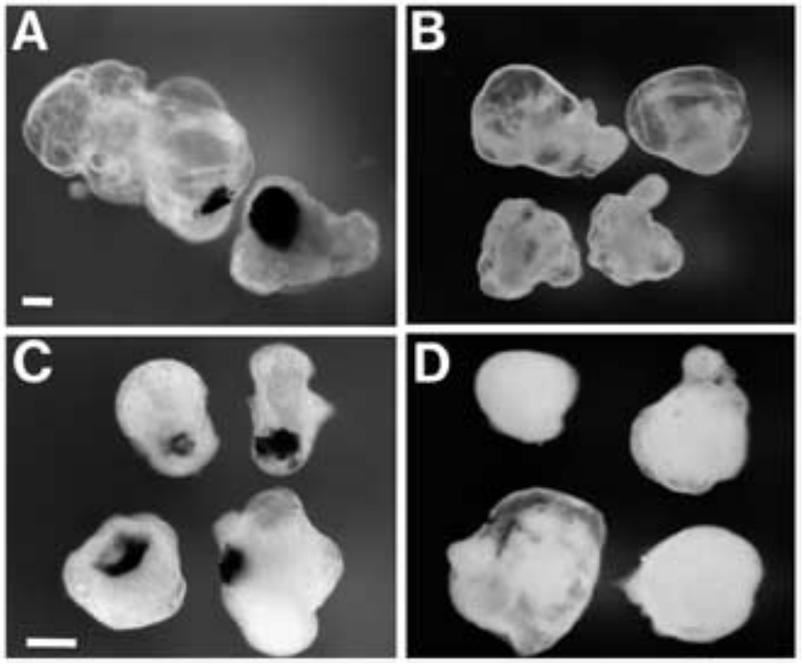

Fig. 3. Recombination explants of total pre- to early streak ectoderm and posterior headfold ectoderm with anterior or posterior headfold mesendoderm. (A) Pre- to early streak ectoderm recombined with total anterior mesendoderm showing induction of En expression, (B) Pre- to early streak ectoderm recombined with total posterior mesendoderm showing no En expression. (C) Headfold posterior ectoderm recombined with total anterior mesendoderm showing En expression. (D) Headfold posterior ectoderm plus lateral posterior mesendoderm showing no En induction. Scale bar represents 200 μm.

expression of *En* genes in posterior ectodermal cells. Again, using the Tg5 transgenic line, we showed that *En-2* expression was induced specifically in posterior ectoderm in 4/19 (19%) recombination explants of posterior ectoderm from headfold stage Tg5 embryos and anterior mesendoderm from wild-type embryos of the same stage (Fig. 5C). No expression of *lacZ* was observed in the reverse combination when mesendoderm from Tg5 embryos was recombined with ectoderm from wild-type embryos (Fig. 5D). These results demonstrate that induction of expression of En-2 occurs specifically in the ectoderm and not in the mesendoderm.

Competence of posterior ectoderm to respond to anterior mesendoderm

Having demonstrated that posterior ectoderm responded to anterior mesendoderm of headfold stage embryos by induction of En proteins, we decided to ask when posterior ectoderm loses its competence to respond. Posterior ectoderm of early somite stage Tg5 embryos was recombined with anterior mesendoderm from headfold stage wild-type embryos. All 18 of these recombination explants failed to induce *lacZ* expression. Therefore, posterior ectoderm is no longer competent to respond to *En-2*-inducing signals of anterior mesendoderm by the early somite stage.

DISCUSSION

By use of a germ layer explant-recombination assay, we have demonstrated a role for interactions between ectoderm and underlying mesendoderm in the induction of expression of the region-specific markers, *En-1* and *En-2*, in the anterior neural plate of the mouse embryo. When ectoderm tissue was grown in isolation, it was found that *En* expression developed autonomously in anterior but not posterior ectoderm by the mid- to late streak stage. Such ectoderm explants showed very little evidence of associated mesoderm tissue, either persisting as contamination or developing de novo in culture, as assessed by two axial mesodermal markers. These results indicated that, if any signal from mesendoderm tissue was required for induction, that signal must have passed to the ectoderm by the mid- to late streak stage, although *En* expression is not apparent in vivo until early somite stages. We then tested the ability of mesendoderm tissues from the mid- to late streak and headfold stage to induce *En* expression in non-expressing ectoderm tissues. Anterior mesendoderm from both stages induced *En* expression in pre- to early streak ectoderm, with headfold mesendoderm giving a higher inducing frequency. Confirmation that expression was induced in the ectoderm component and not ectopically in the mesendoderm was provided by recombination experiments of ectoderm derived from a transgenic mouse line expressing *lacZ* under the control of the *En-2* promoter, with non-transgenic mesendoderm. Anterior mesendoderm from headfold stage embryos was also able to induce *En* expression efficiently in posterior ectoderm from the same stage. Posterior mesendoderm from headfold and early somite stage embryos were not capable of inducing *En* expression, indicating that there was regional variation in the ability of mesendoderm to induce *En* expression. In contrast, both anterior and posterior mesendoderm from headfold stage embryos were also able to induce expression of a general neural marker, NF160, with similar efficiency. Together these experiments provide the first direct evidence for a role for mesendoderm in neural induction and early anterior patterning of the mammalian CNS.

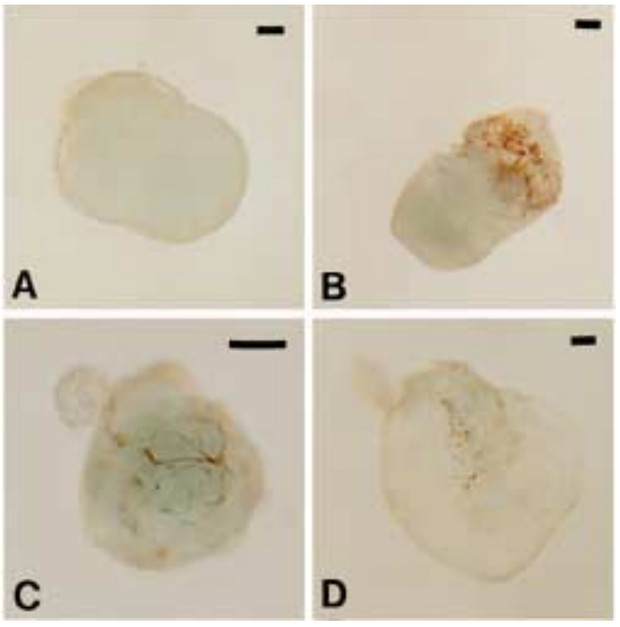

Fig. 4. Expression of NF160 in explants after culture for 3 or 4 days. (A) Pre- to early streak ectoderm showing no NF160 expression. (B) Anterior headfold ectoderm explants showing expression of NF160. (C) Pre- to early streak ectoderm recombined with total anterior headfold mesendoderm expressed NF160. (D) Pre- to early streak ectoderm recombined with posterior lateral headfold mesendoderm also showing expression of NF160. Scale bar represents 100 μm.

What is the predominant *En*-inducing tissue in vivo?

The experiments demonstrated that anterior lateral mesendoderm, which is the tissue that directly underlies the region of the neural plate that later expresses the *En* genes and is fated to develop into head mesoderm and hindbrain paraxial mesoderm (Tam and Beddington, 1992), is capable of inducing *En* expression in the absence of any midline tissue. In other species, however, strong *En*-inducing capacity has been shown to reside in anterior axial mesoderm. In chick, early Hensen's node tissue, which is fated to become notochord and other axial structures, can induce new *En*-expressing neurectoderm derivatives when grafted ectopically to extraembryonic sites (Storey et al., 1992). In *Xenopus*, anterior notochord from neurula stages was shown to induce *En* expression strongly when recombined with blastula stage ectoderm (Hemmati-Brivanlou et al., 1990). In our experiments, we were unable to test directly the inducing capacity of the notochord since it is impossible to isolate cleanly at the early stages used. Thus we cannot exclude a role for notochord in *En* induction, but we clearly demonstrate that it is not the only anterior mesoderm tissue capable of inducing anterior neural markers in the mouse. In *Xenopus*, *En-2* expression could also be induced by presumptive head mesoderm and anterior somites, albeit less efficiently than by anterior notochord (Hemmati-Brivanlou et al., 1990). In addition, induction of *En-2* expression can be observed in naive ectoderm after activin treatment, even in the absence of notochord tissue (Bolce et al., 1992), showing that other types of mesoderm may be involved in *En* induction in *Xenopus* also. Similarly, *En-2* expression is observed in the neural tube of chick embryos that have their cranial notochords removed (Darnell et al., 1992).

Both anterior lateral mesendoderm and anterior notochord underly the developing anterior neural plate and could pass signals by direct cell contact, as has been demonstrated in *Xenopus* (Sharpe et al., 1989; Sharpe and Gurdon, 1990; Hemmati-Brivanlou et al., 1990; Saha and Grainger, 1992). However, there is also evidence from exogastrula experiments in *Xenopus* that there can be planar induction of region-specific markers in ectoderm at a distance from the inducing mesoderm (Ruiz i Altaba, 1990, 1992; Doniach et al., 1992). In view of these findings, we can

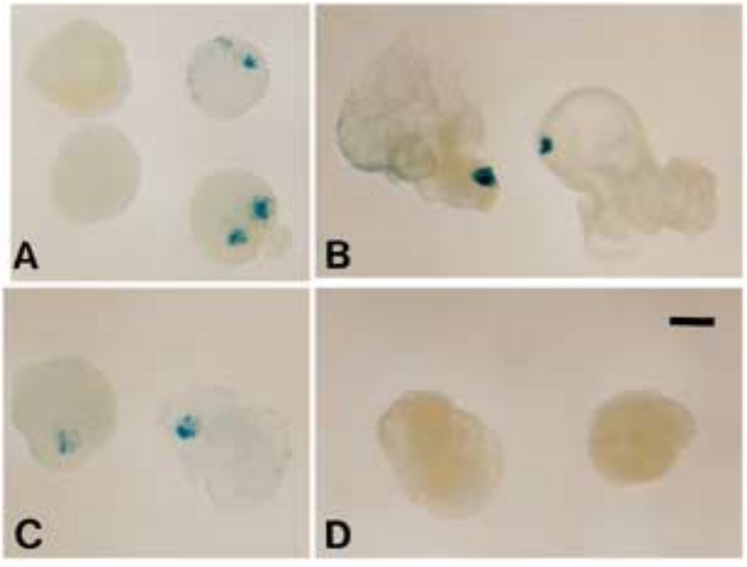

Fig. 5. *lacZ* expression in explants using Tg5 embryos. Histochemical analysis of β-galactosidase activity shows *lacZ* expression in two of the four ectodermal explants of mid- to late streak stage Tg5 embryos shown in A, in recombination explants of total anterior mesendoderm from headfold stage wild-type embryos with either (B) pre- to early streak ectoderm or (C) headfold posterior ectoderm from Tg5 embryos. No *lacZ* expression was detected in D when headfold posterior ectoderm from wild-type embryos was recombined with total anterior mesendoderm from Tg5 embryos. Scale bar represents 200 μm.

interpret in two ways our results showing that only 50% of the mid- to late streak explants go on to express En proteins after culture. Firstly, based on the model of direct cell contact, induction of *En* expression at this stage would correlate with the arrival of lateral and/or axial mesoderm underlying the presumptive *En*-expressing region in the ectoderm. Alternatively, according to the planar signal model, induction of *En* expression at the mid- to late streak stage would correlate with the acquisition of the planar signal, independently of the position of the underlying mesoderm. To address the first possibility, a detailed study of the position of mesoderm using relevant axial and lateral mesoderm markers and a refinement of the staging system of mouse embryos within the mid- to late streak stage will be necessary. In order to test the second hypothesis, a new assay similar to 'Keller' explants in *Xenopus* (Keller and Danilchik, 1988) must be developed in mouse because the design of our recombination assay does not allow us to specify the topological relationship between the interacting ectoderm and mesendoderm. If planar induction is to play a role in the intact embryo, then it seems likely that the source of the signal would be the node at the front of the streak. The equivalent structure in the chick is capable of neural induction and patterning when combined with *Xenopus* animal cap tissues, despite the lack of any further tissue development from the node under these suboptimal culture conditions (Kintner and Dodd, 1991). We have not tested directly the inducing capacity of node tissues but such experiments can now be envisaged, with the assay system in hand.

In this discussion, we have been assuming that it is the mesoderm component of the recombinants that possesses inducing capacity, by analogy with other vertebrate systems. However, since mesoderm plus underlying endoderm was actually used in the experiments, we cannot exclude an involvement of endoderm. It is worth noting in this regard that experiments on neural induction in Xenopus often also use mesendoderm as the inducing tissue (Saha and Grainger, 1992; Doniach et al., 1992). Having established the recombination assay, we may now be able to test the inducing capacity of mesoderm alone, although it is hard to obtain clean, intact pieces of mesoderm from the early stages used.

Regional restriction in *En*-inducing capacity of mesendoderm

Using the anti-neurofilament antibody 2H3, we were able to demonstrate that neural tissue was induced in the recombination explants with similar efficiency by both anterior or posterior mesendoderm from the headfold stage, showing that both areas have general neural-inducing capacity. However, using the same assays, posterior mesendoderm failed to induce *En* expression in either pre- to early streak ectoderm or posterior headfold ectoderm. This clearly cannot reflect its lack of general neural inducing ability and presumably indicates that there is regional restriction in the ability of mesendoderm to induce *En* expression. Since posterior mesendoderm from early somite stage embryos also failed to induce *En* expression, it is unlikely that this lack of *En*-inducing ability is due to a difference in timing of A-P maturation of the mesendoderm. It is more probable that the posterior mesendoderm does not induce *En* expression because it is already committed to induce more posterior neural markers. The availability of transgenic lines expressing the *lacZ* gene in the CNS under the control of posterior *Hox* gene products (for example, *Hox 2.6*; Whiting et al., 1991), should allow us to test this possibility directly in the future.

Gain and loss of ectodermal competence to respond to anterior mesendoderm

Ectoderm from the earliest stages analyzed was capable of being induced to express the *En* genes by anterior mesendoderm from the headfold stage of development. The proportion of the embryo destined to become ectoderm (Lawson et al., 1991) is too small at this stage to allow separation into regions fated to be anterior or posterior, so it was not possible to assess any intrinsic regional variation in ability to respond. However, given the results using posterior ectoderm from the later headfold stage, it seems likely that both anterior and posterior ectoderm are initially capable of responding to anterior mesendoderm by expressing En proteins, and the anterior restriction in in vivo *En* expression is initially due to an instructive interaction with anterior mesendodermal tissues. We have not assessed how long anterior mesendoderm retains *En*-inducing capacity, but we have demonstrated that posterior ectoderm from early somite stage embryos had completely lost the capacity to be induced to express *En* genes in our culture system. Thus, regionalization of *En* expression in the anterior neural tube may involve initially a localised signal from mesendoderm to a broadly competent ectoderm, followed by a progressive loss of responsiveness in the ectoderm.

Although the ectoderm may lose the ability to respond to inducing signals from the mesendoderm, there may still be interactions within the ectoderm itself that can play a role in determining the final domain of *En* expression. For example, transplantation of caudal mesencephalic *En*-expressing neurectoderm to the forebrain of early chick embryos results in induction of *En* expression in the surrounding forebrain, suggesting that there can be a form of homeogenetic induction within the neural tube (Martinez et al., 1991; Gardner and Barald, 1991). Also, maintenance of the complete domain of *En* expression seems to require the expression of the *Wnt-1* gene in the posterior mesencephalon, since mice homozygous for a null mutation in the *Wnt-1* gene fail to maintain the expression of the En genes in the metencephalon (McMahon et al., 1992).

A-P patterning in the neural plate

These experiments have demonstrated a role for ectodermal-mesendodermal interactions in the initial A-P patterning of the mammalian CNS. In terms of understanding the mechanisms of this interaction, they have provided clear evidence for a restriction in the ability of posterior ectoderm to respond by the early somite stage, and they have provided the first indication that there is regional variation in the inducing capacity of the mesendoderm. Perhaps the most surprising conclusion from the experiments is that the signals that result in *En* expression in the overlying ectoderm must have passed from the mesendoderm by the mid- to late streak, at least 12 hours before the onset of *En*

expression in the neural plate. Given that a number of genes, such as *Krox-20* (Wilkinson et al., 1989b), the homeobox gene *Otx2* (Simeone et al., 1992) and some of the anteriorly expressed *Hox* genes already have distinct anterior boundaries of expression within the neural plate by the headfold stage, this raises the possibility that the role of mesendoderm in establishing the A-P pattern of the anterior part of the CNS is restricted to the early neural plate stage and that all later interactions take place within the ectoderm itself. This hypothesis can be tested by determining the timing of onset of neurectoderm-autonomous expression of genes that define later domains of the anterior CNS. It does not seem likely that A-P patterning of the spinal cord is determined as early as the headfold stage, since this part of the CNS develops later as the embryo elongates along the A-P axis, but this can also be tested using suitable markers. Dorsal-ventral patterning in the CNS, which involves interactions between the notochord, the floor plate and the spinal cord (Placzek et al., 1990; Yamada et al., 1991), is also presumably a later event.

The availability of this in vitro assay for studying neural induction and regional patterning in the mammalian embryo should be useful in comparing the mechanisms of these events in different vertebrate embryos. It may also provide a useful assay for any putative neural-inducing factors, for which there are currently few candidates. However, perhaps the most important advantage of developing such an assay in mammals is that it can be used in association with the large numbers of existing developmental mutations (Green, 1989) and the rapidly increasing number of targeted and insertional mutations to provide new insights into the nature of the defects associated with such mutations.

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