

Neural induction and regionalisation by different subpopulations of cells in Hensen's node

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SUMMARY

Cell lineage analysis has revealed that the amniote organizer, Hensen's node, is subdivided into distinct regions, each containing a characteristic subpopulation of cells with defined fates. Here, we address the question of whether the inducing and regionalising ability of Hensen's node is associated with a specific subpopulation. Quail explants from Hensen's node are grafted into an extraembryonic site in a host chick embryo allowing host- and donor-derived cells to be distinguished. Cell-type- and region-specific markers are used to assess the fates of the mesodermal and neural cells that develop.

We find that neural inducing ability is localised in the epiblast layer and the mesendoderm (deep portion) of the

medial sector of the node. The deep portion of the posterolateral part of the node does not have neural inducing ability. Neural induction also correlates with the presence of particular prospective cell types in our grafts: chordamesoderm (notochord/head process), definitive (gut) endoderm or neural tissue. However, only grafts that include the epiblast layer of the node induce neural tissue expressing a complete range of anteroposterior characteristics, although prospective prechordal plate cells may also play a role in specification of the forebrain.

Key words: Hensen's node, induction, neural tissue, axial mesoderm, somite

INTRODUCTION

Seventy years ago, Spemann and Mangold (1924) demonstrated the organizing properties of a unique region of the amphibian embryo, the dorsal lip of the blastopore. Transplantation of this region to the ventral side of a host embryo results in the induction of new neural tissue, the dorsalisation of presumptive ventral mesoderm and the formation of a full range of structures along the anteroposterior axis. Inspired by this experiment, embryologists have striven to identify the cellular and molecular mechanisms that underlie this organizing ability.

Transplantation experiments carried out between rabbit and chick embryos (Waddington 1932, 1933) identified the tip of the primitive streak, Hensen's node (after Hensen, 1876) as the amniote equivalent of 'Spemann's organizer' (see also Gallera, 1971; Nieuwkoop et al., 1985; Dias and Schoenwolf, 1990; Storey et al., 1992; Beddington, 1994). This special region of the vertebrate embryo self-differentiates into a number of embryonic tissues (Spemann and Mangold, 1924; Smith and Slack, 1983; Dias and Schoenwolf, 1990; Selleck and Stern, 1991, 1992; Storey et al., 1992) including prechordal plate, notochord, somites, gut endoderm and neural tissue. The progenitor cells of these tissues have been mapped to specific subpopulations within the organizer region of amphibian (Keller,

1975, 1976, also see Shih and Keller, 1992) and chick embryos (Rosenquist, 1966; Gallera, 1971; Schoenwolf and Sheard, 1990; Selleck and Stern, 1991). These studies reveal that different cell fates are spatially organized in the dorsal lip of the blastopore and Hensen's node.

Fate maps of Hensen's node in the chick embryo show that medial and posterolateral epiblast contribute to neural tissue and notochord (Spratt, 1955; Rosenquist, 1966, 1983; Schoenwolf and Sheard, 1990; Selleck and Stern, 1991), while the mesendoderm layer contributes medially to notochord and laterally to notochord and somites (Selleck and Stern, 1991; see Fig. 1A). By interchanging medial and lateral sectors of the node, Selleck and Stern (1992) discovered that the medial sector contains cells committed to forming notochord, while the lateral sector can also contribute substantially to notochord when placed medially.

Here we have designed experiments to test whether different subpopulations of cells in the node differ in their neuralizing and/or regionalizing ability. We subdivide the node into medial and posterolateral sectors, and each sector is separated further into upper (epiblast) and lower (mesendoderm) layers. Quail grafts are placed against the extraembryonic epiblast in a chick host embryo and incubated for 24-30 hours. Each subpopulation is then assessed for its ability to induce and to self-differentiate into mesendoderm and neural tissue, using species-, cell

type- and region-specific markers. These experiments allow us to correlate neural inducing and regionalizing abilities with particular regions of the node and with the presence of specific cell types in our grafts.

MATERIALS AND METHODS

Dissection and grafting technique

Fertile hens' eggs (Warrens) were incubated at 38°C for 12 or 18 hours to give host embryos of stage 2⁺-3⁺ (Hamburger and Hamilton, 1951) or donor embryos at definitive primitive streak stage (stage 4). Host embryos were explanted and maintained ventral side up in New culture (New, 1955), modified as in Stern and Ireland (1981). Regions of Hensen's node were marked out using a glass micropipette and labelled with carmine powder prior to excision from donor embryos. Separation of the superficial epiblast from the deep mesendodermal layer was carried out in calcium- and magnesium-free (CMF) Tyrode's saline containing 0.12% Trypsin (Difco 1:250). Grafts were placed in host embryos against extraembryonic epiblast at the inner margin of the area opaca and cultured for a further 24-30 hours (Fig. 1C; except that those destined for *in situ* hybridization with the *gooseoid* probe were grown for only 12-15 hours). We chose this extraembryonic location because, while the epiblast of this region is competent to respond to neural inducing signals, the axes that form remain separate from the host embryo (Storey et al., 1992).

Criteria for identifying cell types

Prechordal plate was identified as the triangular region of mesoderm that contains *gooseoid*-expressing cells (revealed by *in situ* hybridization) at the rostral end of the notochord at stages 5-7.

Notochord was recognised using a notochord-specific antibody Not-1 in combination with its characteristic morphology: a rod-like structure containing large vacuolated cells.

Somites were identified morphologically as blocks of cells, each consisting of an epithelium around a small central lumen. Hox-c6 protein is not expressed in somites 1-5 but is expressed in regions posterior to somite 6 at stage 12. Two forms of this protein have been described: 'long' and 'short' (Oliver et al., 1988). Antibody 3N4 has been reported to recognise both forms and also labels cells in the neural tube (see below).

Neural tissue was identified by morphology (Dias and Schoenwolf, 1990; Storey et al., 1992) and by the expression of four markers: (a) a steroid-hormone receptor mRNA *tailless*, expressed in the forebrain (Yu et al., 1994) revealed by *in situ* hybridization; (b) Engrailed-2 protein, expressed in the metencephalic region, revealed with monoclonal antibody 4D9 (Patel et al., 1989); (c) Hox-c6 protein is expressed faintly in the hindbrain at the level of the otic vesicle and increases in intensity in more posterior CNS and was revealed using polyclonal antibody 3N4 (Oliver et al., 1988); (d) the L5 carbohydrate epitope (Streit et al., 1990; Roberts et al., 1992) is restricted to the entire elevating neural plate and neural tube between stages 7 and 12.

Definitive (gut) endoderm was identified in sections as a ring of cells, one layer thick, encircling a large central lumen.

Definition of sectors

The regions grafted are shown in Fig. 1B. The medial sector of the node used in these experiments is the same wedge of tissue as was defined by Selleck and Stern (1992). The apex of this wedge abuts the anterior lip of the primitive pit. The posterolateral sector grafted here is a square (75×75 µm), which excludes the groove, and whose anterior limit lies at the level of the primitive pit. This region lies posterior to the lateral sector defined by Selleck and Stern (1991, 1992).

Immunocytochemistry

Embryos were fixed for 1 hour in phosphate-buffered 4% formol saline (pH 7.0) for incubation with primary antibodies 4D9 (Patel et al., 1989; see also Gardner et al., 1988; Davis et al., 1991) and Not-1 (Yamada et al., 1991) or in Bouin's fixative for 1 hour for 3N4 antibody (kindly provided by Drs G. Oliver and E. M. De Robertis; Oliver et al., 1988; see also Storey et al., 1992). Immunolabelling of whole-mount embryos with antibodies 4D9 and Not-1 followed a standard protocol (see Storey et al., 1992). Briefly, following fixation, endogenous peroxidase activity was blocked with 0.25% hydrogen peroxide in phosphate-buffered saline (PBS), pH 7.4. Embryos were rinsed in PBS, then PBT (PBS containing 0.2% bovine serum albumin (BSA), 0.5% Triton X-100, 0.01% thimerosal and 5% heat-inactivated normal goat serum (NGS)). Supernatant was added 1:1 and embryos incubated overnight at 4°C. After extensive washing in PBT, embryos were incubated in peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch) overnight. Embryos were then washed in PBS and rinsed in 0.1 M Tris (pH 7.4) prior to conducting the HRP reaction with diaminobenzidine tetrahydrochloride (1 mg ml⁻¹ in 0.1 M Tris, pH 7.4) with H₂O₂ (0.001%). Embryos were then washed, dehydrated and cleared in oil of cedar wood.

Immunolabelling of whole embryos with antibody 3N4 has been described previously (Storey et al., 1992, after Oliver et al., 1988). Following Bouin's fixation, embryos were washed in 70% ethanol, hydrated, washed for 2 hours in TBS (50 mM Tris, 0.9% NaCl, pH 7.4) and then blocked in TBST (10 mM Tris, pH 7.4, 100 mM MgCl₂, 0.5% Tween-20, 1% BSA and 5% fetal calf serum) for 2 days at 4°C. They were then incubated in primary antibody (1:100 in TBST with 5% heat inactivated goat serum) for 2-3 days at 4°C. After washing in TBST embryos were placed in alkaline-phosphatase-coupled goat anti-rabbit IgG (BioRad; 1:1000) for 2-3 days, then washed in TBST. They were then rinsed in AP buffer (100 mM Tris, 100 mM NaCl, 50 mM MgCl₂, pH 9.5, 20°C) before reacting the alkaline phosphatase with 45 µl nitro blue tetrazolium (Sigma: 75 mg ml⁻¹ in 70% dimethylformamide) and 35 µl BCIP (5-bromo-4-chloro-3-indolyl phosphate, Sigma; 50 mg ml⁻¹ in dimethylformamide) in 10 ml of AP buffer in the dark for 10 minutes. Embryos were then washed in PBS and stored in 4% formol saline.

Embryos destined for labelling with the L5 antibody (Streit et al., 1990) were first fixed in methanol for 1 hour, endogenous peroxidase blocked in 0.25% H₂O₂, (1 hour), rinsed in PBS, followed by incubation in PBT + NGS (as described above) for 2 hours. Embryos were then incubated overnight at 4°C in primary antibody (1:50), washed extensively in PBS and reincubated overnight at 4°C in peroxidase-conjugated goat anti-rat IgM (Cappel, µ-chain specific; 1:1000 in PBT + NGS). Embryos were then washed in PBS and the peroxidase reaction carried out as above.

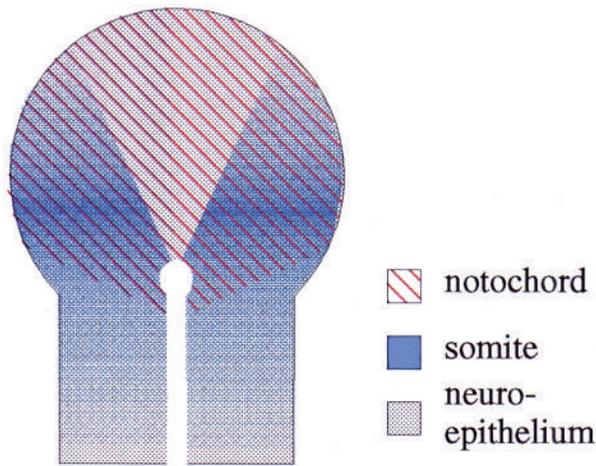
Whole-mount *in situ* hybridization

Embryos for whole-mount *in situ* hybridization were fixed in MEMFA (0.1 M MOPS pH 7.4, 2 mM EGTA, 1 mM MgSO₄, 10% formaldehyde solution) and hybridization performed with digoxigenin (DIG)-labelled riboprobes as described by Izpisua-Belmonte et al. (1993). Anti-DIG alkaline phosphatase-conjugated antibody (Boehringer) was visualised by the same chromogenic reaction as described above. Embryos were then washed twice in PBS and postfixed and stored in 4% paraformaldehyde. *gooseoid* cDNA probe has been described previously (Izpisua-Belmonte et al., 1993) and *tailless* cDNA was a generous gift of Ruth Yu, Kazuhiko Umesono and Ron Evans.

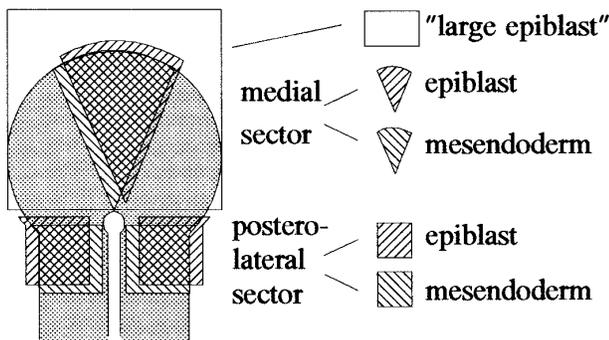
Quail nucleolar marker

Chick embryos containing quail grafts were fixed in Zenker's fixative (Drury and Wallington, 1967) for 3 hours, washed in tap water, dehydrated and cleared cedar wood oil and then in xylene prior to embedding in Paraplast and sectioning at 7 or 10 µm. Sections were

A summary fate map



B regions explanted



C grafting assay

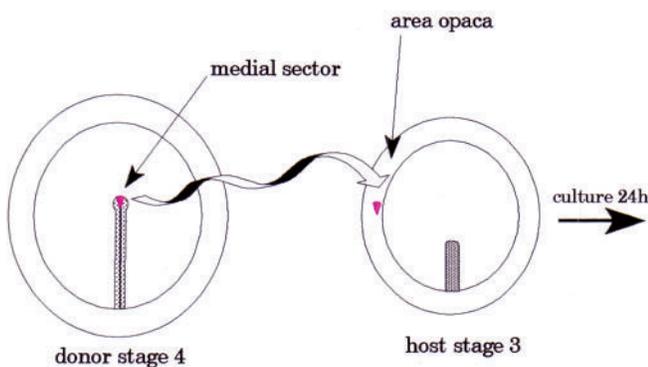


Fig. 1. (A) A summary fate map derived from the data in Selleck and Stern (1991). (B) Seven different subpopulations of cells from the organiser region were used in our grafting experiments: medial sector (consisting of both epiblast and mesendoderm layers), posterolateral sector (comprising both epiblast and mesendoderm layers), 'large epiblast' (including epiblast from the medial and lateral sectors and just anterior to the node), medial epiblast, medial mesendoderm, posterolateral epiblast and posterolateral mesendoderm. Width of streak = 150 μ m. (C) Grafts were apposed to the extraembryonic epiblast at the inner margin of the area opaca and maintained in New culture.

dewaxed in xylene, hydrated to 70% ethanol and passed through 0.5% iodine in 70% ETOH for 7 minutes, rinsed in water, placed in 2.5% sodium thiosulphate for 7 minutes and again washed in water. Sections were then placed in 5 N HCl for 20 minutes, washed thoroughly in water and stained with Harris's haematoxylin (Hutson and Donahoe, 1984). In some cases, embryos were fixed initially in formol saline for 1 hour prior to immunocytochemistry and then fixed secondarily overnight in Zenker's. Embryos were immunolabelled in whole mount, photographed and wax embedded, sectioned, dewaxed in xylene and hydrated prior to secondary fixation. Following Zenker's fixation, sections were washed in tap water and then underwent the Feulgen reaction consisting of an acid hydrolysis followed by staining in Schiff's reagent (Drury and Wallington, 1967).

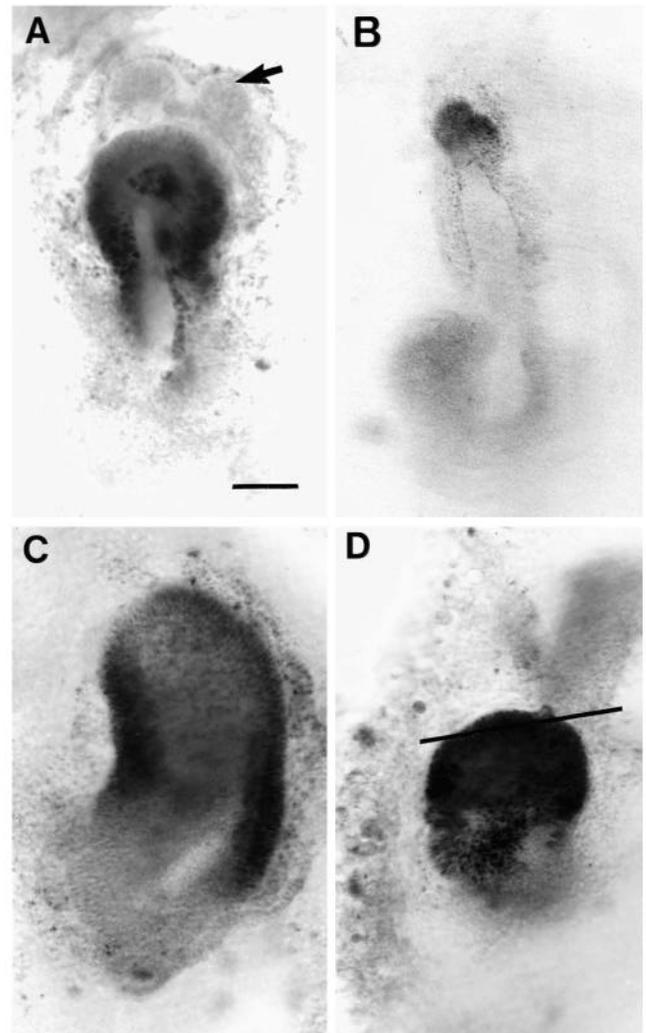


Fig. 2. Ectopic structures generated after transplantating different subpopulations of cells from the node. Engrailed-expressing cells after grafting: (A) posterolateral sector; this graft also gave rise to somite-like structures (arrowheads). This graft is shown in section in Fig. 4C; (B) medial sector; this axis starts anteriorly (top) with En-2-expressing cells; (C) medial mesendoderm, which gives rise to largely En-2-expressing neural tissue. This graft is shown in section in Fig. 4E, D. Large epiblast, also with an axis made up largely of En-2-expressing cells. Line marks section in Fig. 4G,H. Scale bar A,C,D = 30 μ m; B = 40 μ m.

Dil and DiO labelling

The use of DiI (1,1'-dioctadecyl-3,3,3',3' tetramethyl indocarbocyanineperchlorate, Molecular Probes), as a dye for fate mapping studies has been described in detail elsewhere (e.g. Wetts and Fraser, 1988; Honig and Hume, 1989; Stern, 1990; Selleck and Stern, 1991). In the present experiments, it was used to label as many cells as possible in a region of the node prior to grafting it to an ectopic site in a host embryo. Following incubation, all embryos containing DiI-labelled grafts were fixed in 4% buffered formol saline, pH 7.0. In some cases embryos were viewed, photographed and subsequently processed for immunocytochemistry after 1 hour fixation, while others were stored in fixative in the dark. All embryos were viewed by epifluorescence (peak excitation 484 nm) using an Olympus Vanox AHBT microscope.

RESULTS

A total of 438 grafts was made, of which 323 (74%) were used for analysis. Those that were not used include 20 secondary axes which merged with the host embryo; the remaining ones did not survive culture. Grafts were apposed to the extraembryonic epiblast at the inner margin of the area opaca of stage 2⁺-3⁺ host embryos and cultured for 24-30 hours (Fig. 1C).

Induction of neural tissue

Each region was assessed for its ability to induce neural tissue in the host epiblast by grafting quail explants into chick host embryos. Analysis of these chimaeric structures (see Fig. 2) is presented in Figs 3 and 4. Neural tissue is induced in the extraembryonic epiblast by grafts of both medial and posterolateral sectors of the node (both of which include all three germ layers) and by grafts of medial mesendoderm. Neural tissue is also induced by 'large epiblast' grafts (which include node epiblast and a small region of epiblast anterior to Hensen's node; see Fig. 1B). Posterolateral mesendoderm does not induce neural tissue (Fig. 3). The ability to induce neural tissue correlates with differentiation of particular cells types after grafting these regions (described in detail below).

Differentiation of graft-derived neural tissue

Medial and posterolateral epiblast grafts contain cells fated to contribute to neural tissue (Dias and Schoenwolf, 1990; Schoenwolf and Sheard, 1990; Selleck and Stern, 1991). Although 1/10 grafts of the full thickness of the medial sector contained donor-derived neural tissue,

transplants of the epiblast layer alone did not give rise to neural tissue. Medial and posterolateral epiblast do however, develop expression of the L5 epitope ($n=6$, Fig. 5). This antigen has been implicated in the initial response of the epiblast to neural inducing signals (Roberts et al., 1991). This suggests that isolated epiblast depends on continued interactions with the lower mesendoderm layer in order to differentiate into neural tissue.

Another possibility is that epiblast grafts do not form neural tissue because they are too small. To test this, we attempted to graft two medial epiblast explants; however, we found that the two pieces did not remain attached and another strategy was adopted. Instead, we grafted a large region of epiblast (Fig. 1B), including epiblast from the medial and lateral sectors, and from a small region anterior to Hensen's node (posterior half

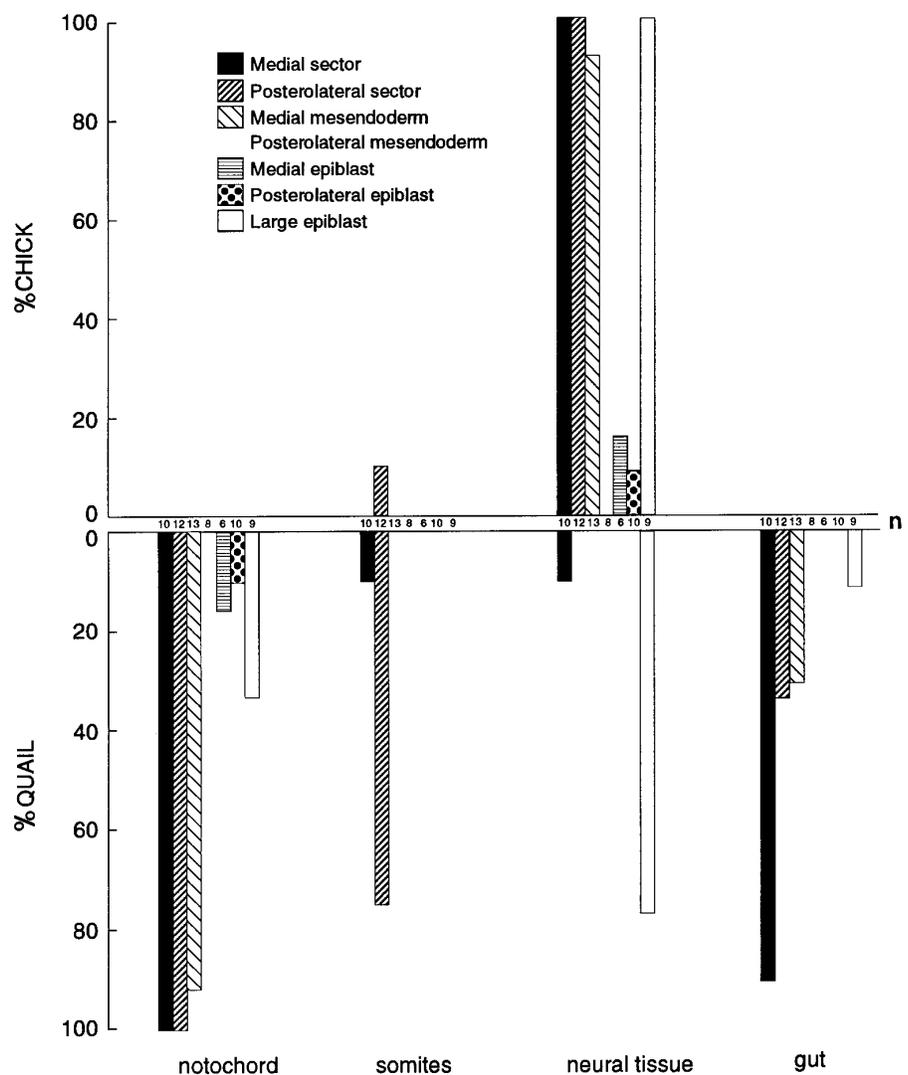


Fig. 3. Quail grafts were placed in chick host embryos to assess whether the cell types identified in ectopic structures are the result of self-differentiation of the graft (quail) or of induction of the host epiblast (chick). The histogram shows the number (n) of ectopic structures (generated by transplanting different regions of node) containing cells of chick or quail origin for each type of tissue (expressed as a % of the number of structures scored). A tissue was scored as containing quail cells if a group of more than five quail cells was identified in that tissue; in nearly all cases, however, tissues were not mixed and contained either chick or quail cells.

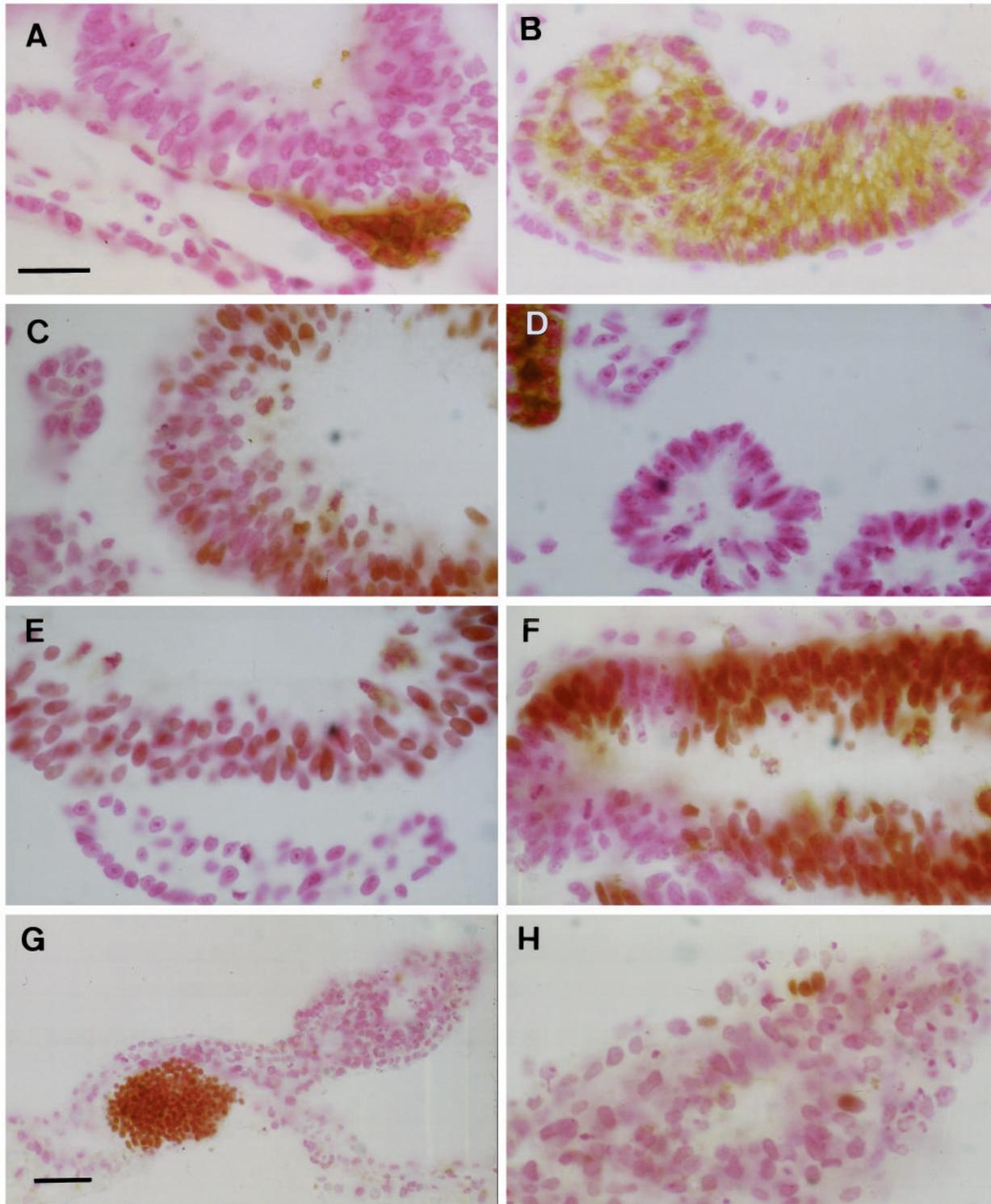


Fig. 4. Not-1 antigen or Engrailed expression revealed immunocytochemically together with quail nucleolar marker. (A) Graft of quail medial sector labelled with Not-1, showing quail notochord and gut and induced neural tissue of host (chick) origin. (B) Medial sector graft, showing expression of Not-1 in quail-derived notochord. (C) Posterolateral sector graft, expressing the En-2 antigen in the host-derived neural tube. This is a sagittal section through the ectopic axis shown in Fig. 2A; anterior is to the left. (D) Posterolateral sector graft, showing the Not-1 positive notochord and donor (quail) derived somites. (E) Graft of medial mesendoderm. En-2 is expressed in the host-derived neural tube, overlying the donor (quail) derived notochord. This is a transverse section through the ectopic axis shown in Fig. 2C. (F) Graft of 'large epiblast' showing quail derived neural tissue that also expresses En-2. This is a transverse section through the ectopic structure in Fig. 2D. (G) Another section through the embryo as in Figs. 2D and 4F, illustrating the relationship between donor-derived En-2 expressing cells and host-derived neural tissue. (H) Higher power view of part of the same section as in G, showing four En-2 expressing chick nuclei. Scale bar A-F and H = 20 μ m, G = 30 μ m.

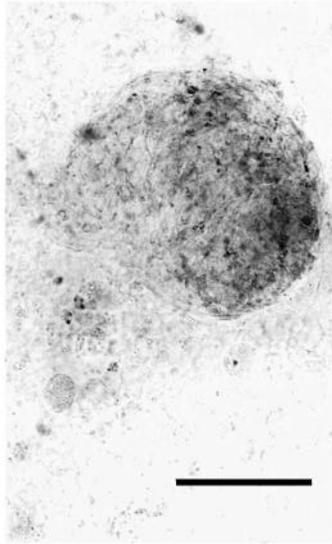
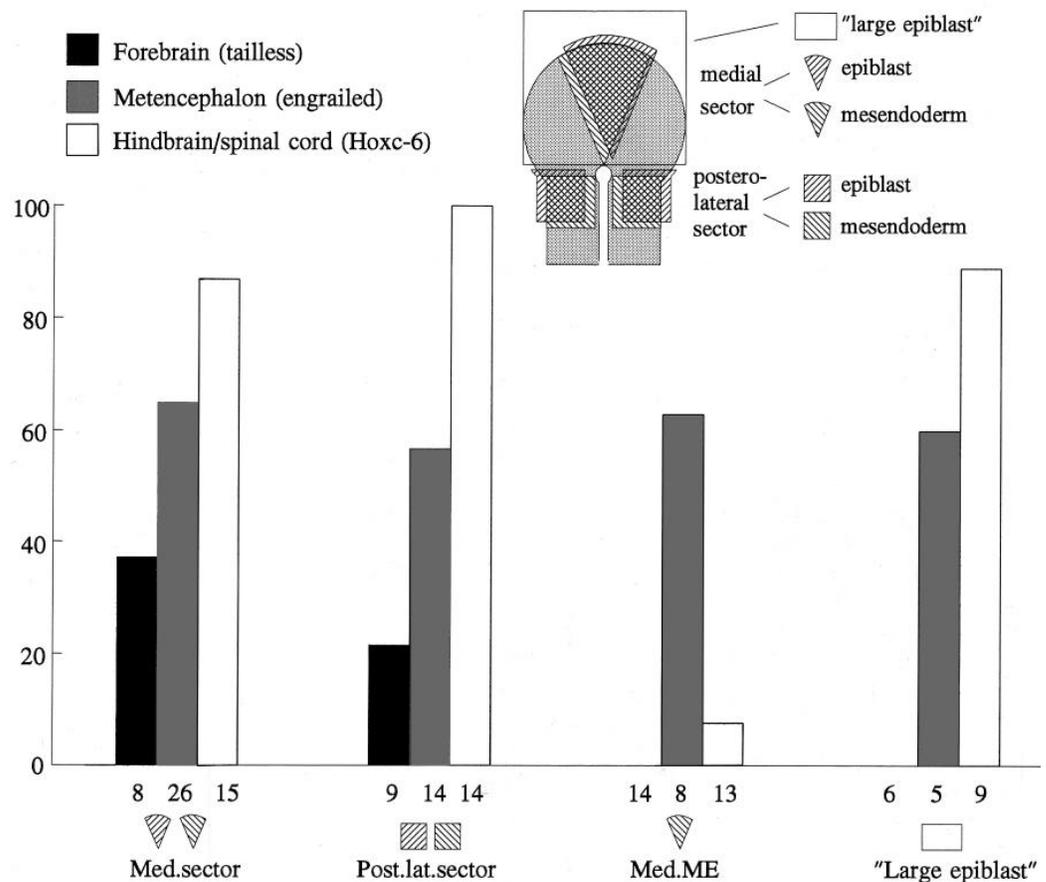


Fig. 5. Isolated epiblast from medial and posterolateral sectors does not differentiate into a neural tube, but does express the L5 epitope. Whole-mount immunoperoxidase. Scale bar = 50 μ m.

of Schoenwolf and Alvarez's, 1989 'region a'). In the majority of cases (7/9) this larger piece of epiblast did differentiate into neural tissue (Figs 3, 4F-H). Thus, the inability of grafts of medial and posterolateral epiblast to differentiate into neural tissue may be related to explant size.

Fig. 6. Histogram illustrating the percentage of grafts that led to expression of three region-specific neural markers (*tailless*, En-2 and Hoxc-6, expressed in forebrain, midbrain/hindbrain and hindbrain/spinal cord, respectively). The quail nucleolar marker was revealed in combination with only Engrailed-expressing neural tissue (as histology for the nucleolar marker is not compatible with techniques used to reveal *tailless* mRNA and the Hox c6 antigen). The host/donor origin of cells expressing these region-specific markers can be inferred from Fig. 3. This shows that in all but one case (1/10 medial sector grafts) only induced neural tissue is found in axes generated by medial, posterolateral and medial mesoderm grafts. In contrast 'large epiblast' axes may contain both chick- and quail-derived neural tissue. In one such case the induced neural tissue contained four Engrailed positive cells (Fig. 4G,H). In all other axes scored, 50 or more En2 positive cells were observed. Expression of *tailless* mRNA and the Hox c6 antigen was assessed in whole mount.



Anteroposterior regionalisation of neural tissue

The range of anteroposterior markers expressed by neural tissue induced by medial and posterolateral sectors (containing all three germ layers) is similar. Both sectors can elicit the formation of miniature axes expressing the region-specific neural markers *tailless*, Engrailed-2 and Hoxc-6, corresponding to prosencephalon, metencephalon and hindbrain-spinal cord respectively (Figs 2, 6-8). However, only a minority (5/17) of such axes include the most anterior, *tailless*-expressing region and some induced axes commence anteriorly with Engrailed-2 expressing cells (Fig. 2A,B). By combining histology for the quail nucleolar marker with an antibody to the Engrailed-2 protein, we were able to demonstrate that the metencephalic region of these ectopic axes is indeed derived from induced host epiblast (Fig. 4C). Thus, grafts that contain all three germ layers are able to induce neural tissue that can express a full range of anteroposterior characteristics.

Medial mesoderm induces a restricted region of neural tissue

Histology for the quail nucleolar marker combined with immunocytochemistry for Engrailed-2 also reveals that medial mesoderm grafts induce a small ectopic axis, which consists largely of Engrailed-2-expressing cells (Figs 2C, 4). In 1/13 cases a small region of cells, which express Hoxc-6 protein weakly, was also observed. Medial mesoderm, however, does not elicit expression of the forebrain marker

tailless (Fig. 6). Hence, in the absence of the overlying epiblast, a restricted region of neural tissue is induced.

Regional character of neural tissue developing after grafting 'large epiblast'

Grafts of node and adjacent epiblast ('large epiblast', see Fig. 1) form neural tissue that expresses both *Engrailed-2* and *Hoxc-6* but not the forebrain marker *tailless* (Figs 2, 6, 8). By combining histology for the quail nucleolar marker with the *Engrailed-2* antibody, we found that node epiblast differentiates into *Engrailed-2*-expressing neural tissue (3/5; Fig. 3). In 1/5 cases, the neural tissue induced by these grafts also included a few *Engrailed-2*-expressing cells (Fig. 4H). Therefore, grafts of node epiblast differentiate into neural tissue that does not express the most anterior CNS characteristics.

Mesodermal cell types

In order to understand better the different neural inducing and regionalizing abilities of each of the regions tested, we also assessed the different mesodermal cell types formed. These include prechordal plate, notochord and somites.

Medial and posterolateral sectors (all three germ layers)

When the medial sector of the node is grafted into the inner region of the area opaca, it gives rise to a small axis that includes prechordal plate and notochord (Figs 9, 10B-C, 11). In 20% (4/20) of these grafts 1 or 2 somite-like structures were also found. Transplanted posterolateral sectors also give rise to an ectopic axis comprising prechordal plate and notochord (Figs 10A, 11). However, in contrast with the medial sector, 90% (18/20) of posterolateral sector grafts form between 1 and 10 somites (Fig. 10A).

Isolated mesendoderm

Medial mesendoderm grafted alone to the area opaca gives rise to prechordal plate and notochord but does not form somites (Figs 10D-E, 11). Posterolateral mesendoderm did not generate *gooseoid*-expressing cells characteristic of prechordal plate and formed notochord in only 1/33 cases (Figs 11, 12A). Thus, in this assay, posterolateral mesendoderm cells do not differentiate into somites. This was observed in DiI-labelled grafts (Fig. 12A,B), in sectioned embryos assessed with the quail nucleolar marker (Fig. 3) and in embryos stained with the notochord-specific antibody Not-1. DiI labelling revealed that grafts of posterolateral mesendoderm remain as a clump ($n=11$) and in 6/11 cases also spread within the germ wall (deep layer) of the area opaca of the host (Fig. 12B).

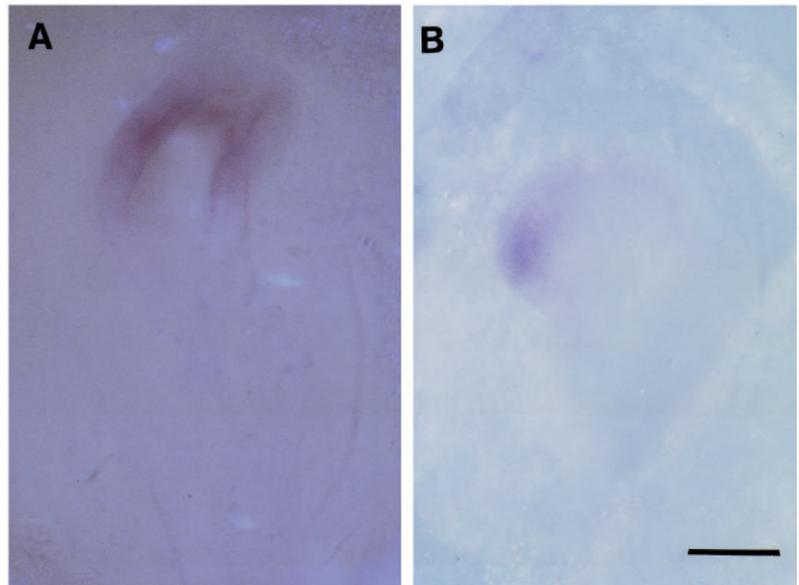


Fig. 7. Expression of *tailless* mRNA after grafting (A) posterolateral and (B) medial sectors. Scale bar = 75 μ m.

Thus, while medial mesendoderm differentiates into axial mesoderm (notochord and prechordal plate), posterolateral mesendoderm does not form these cell types. Neither region of mesendoderm gives rise to somites, even though the posterolateral mesendoderm contains cells that are fated to do so (Rosenquist, 1966; Selleck and Stern, 1991; Schoenwolf et al., 1992). These results support previous conclusions (Selleck and Stern, 1992) that the medial sector of the node contains cells committed to forming axial mesendoderm, while cells in more lateral regions are not yet committed.

Isolated epiblast

When 'large epiblast' is grafted into the area opaca of a host

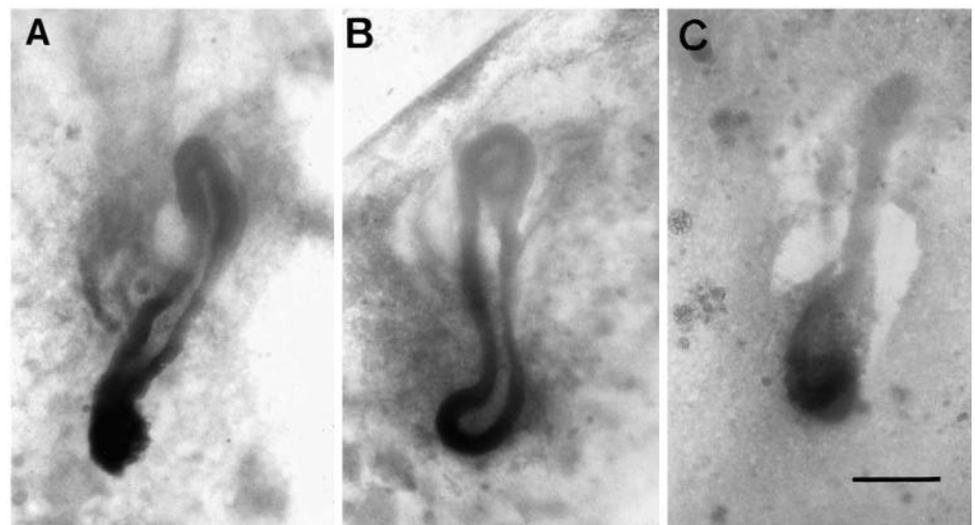


Fig. 8. *Hoxc-6*-expressing cells revealed immunocytochemically after grafting: (A) Posterolateral sector, including one somite-like structure; (B) medial sector; (C) large epiblast. Scale bar = 200 μ m.

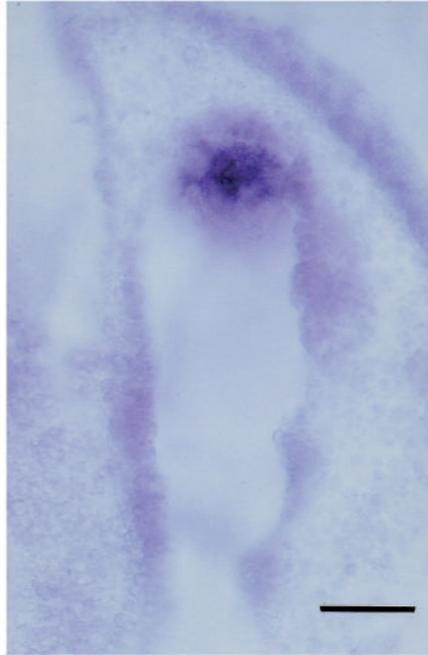


Fig. 9. Grafts containing all three germ layers express *goosecoid* mRNA. A graft of posterolateral sector is shown here. Scale bar = 75 μ m.

embryo, no *goosecoid*-expressing cells are generated, but notochord is produced in a third of cases (3/9). Medial and posterolateral epiblast each formed notochord in only one case (1/9 and 1/15) (Fig. 11). Somites were not found after grafting any region of the epiblast alone (Fig. 11). Thus, in the majority of cases isolated epiblast does not form mesodermal cell types.

Mesodermal tissue forms by self-differentiation and not induction of host epiblast

In order to establish whether the mesodermal tissue identified after grafting is the result of self-differentiation or of induction of the host extraembryonic epiblast, these tissues were scored in the embryos that received quail grafts (Fig. 3). Quail-derived notochord was present in structures generated by grafts of medial and posterolateral sectors and of medial mesendoderm. Notochord also formed in a small proportion of cases in grafts made by medial, posterolateral or 'large epiblast' and, in all cases, consisted of quail cells. Quail-derived somites were observed after transplantation of the posterolateral sector and, in one case, generated by a medial sector graft. One posterolateral sector graft (1/12) generated two somite-like structures, one of which contained chick cells (Figs 2A, 4C). However, in all other grafts containing somites or notochord, these struc-

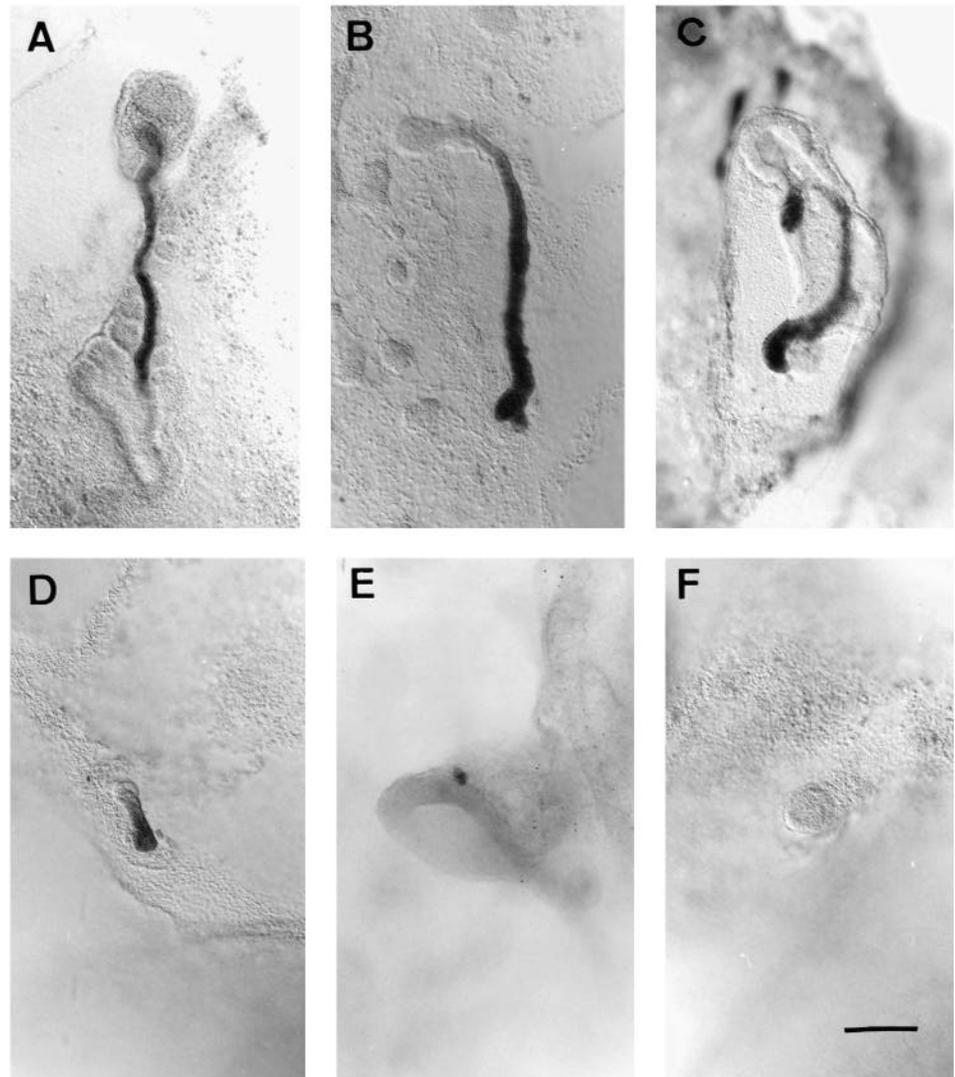


Fig. 10. Not-1-labelled grafts observed in whole mount. (A) Posterolateral sector graft, which led to the formation of somites, notochord and neural tube. (B) This graft of the medial sector was placed in the outer part of the area opaca, a region of epiblast that does not respond to neural inducing signals (Storey et al., 1992). The graft has formed notochord and perhaps a prechordal plate, but not somites or neural tube. (C) Graft of medial sector, containing notochord and neural tube, but no somites. (D) Medial mesendoderm graft; notochord and possibly prechordal plate have formed, but no neural plate. (E) Medial mesendoderm graft, which generated notochord and neural tissue. (F) Posterolateral epiblast graft, which does not produce Not-1-positive cells. Scale bar = 200 μ m.

tures were donor-derived and not induced from the host chick extraembryonic epiblast (Fig. 4). Gut endoderm was only identified in full thickness grafts of the medial and posterolateral sectors and in medial mesendoderm grafts.

DISCUSSION

Which are the neural inducing cells?

These experiments were designed to assess whether specific regions or cell populations in the node differ in their neural inducing abilities. One of the most striking conclusions from this study is that the mesendodermal layer requires the presence of the epiblast in the posterolateral, but not in the medial sector, in order to induce. This is despite the fact that grafts of posterolateral mesendoderm are slightly larger than those of medial mesendoderm. Therefore, the neural inducing ability of the deep cells differs between the medial and posterolateral sectors.

Epiblast isolated from either sector does not induce. But this inability may be due to the small number of cells grafted. For this reason, we tested a larger piece of epiblast (including both medial and lateral sectors), and found that this is able to induce new neural tissue. Thus, although we cannot exclude the possibility that the presence of another cell type in these larger grafts is responsible, this result indicates that at least some

isolated epiblast, in the absence of underlying mesendoderm, can indeed induce neural tissue.

These considerations point to the deep portion of the medial sector of the node and the epiblast of the whole node as the sites to seek expression of putative neural inducing molecules.

Does the presence of particular prospective cell types in our grafts correlate with neural inducing ability better than with the region of the node from which they are excised? We find that neural tissue is induced by grafts differentiating into chordamesoderm and gut endoderm (see also Gallera, 1966; Dias and Schoenwolf, 1990; Storey et al., 1992). This is consistent with the observed decline in neural inducing ability following the emergence of the first presumptive notochord cells/head process from the node (Dias and Schoenwolf, 1990; Storey et al., 1992). The early head process retains its neural inducing activity, as revealed by grafts to the area opaca (Izpisua-Belmonte et al., 1993). The endoderm seems to be in a different category. First, fate maps of the node have shown that by stage 4 this region no longer contributes cells to the definitive endoderm (Vakaet, 1970; Selleck and Stern, 1991). This suggests that the endodermal cells seen after grafting portions of the node from this stage in our experiments represent those that have already emerged as a layer. Second, grafts of other regions containing endoderm that has already emerged from the node do not induce (e. g. Waddington, 1933; Gallera, 1966; Hara, 1978), suggesting that if prospective endoderm cells are

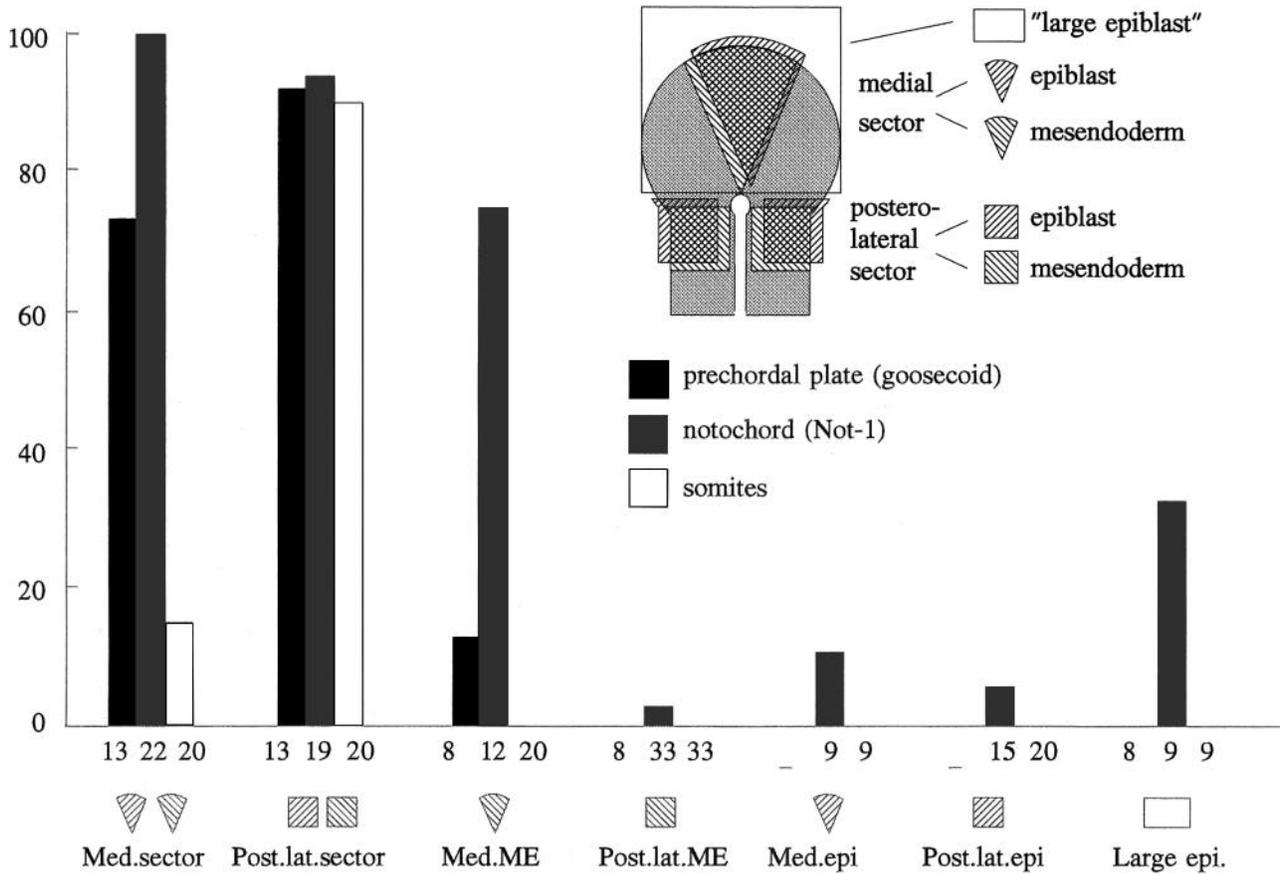


Fig. 11. Histogram showing the proportion of each type of graft that produced each of three mesodermal cell types: prechordal plate, notochord and somites. Notochord and somites were found to be graft derived (except one case in which a somite-like structure containing chick cells was observed, see Fig. 3). Prechordal plate was not identified by mophology alone.

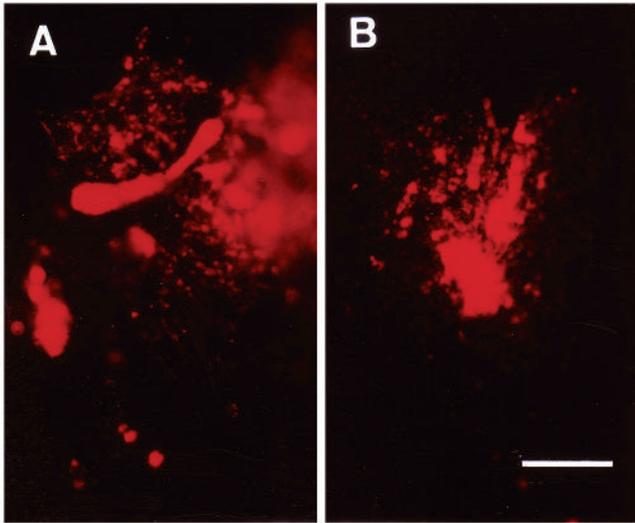


Fig. 12. Grafts of posterolateral mesendoderm did not lead to expression for any of the tissue-specific markers used. We therefore labelled some of the grafts with DiI prior to grafting. (A) A rod-like structure resembling a notochord was identified in 1/33 grafts of this region (1/11 DiI-labelled grafts). (B) A graft that generated a clump of cells within the germ wall of the host embryo. Scale bar = 200 μ m.

indeed inducers, they lose this ability as they emerge as a layer from the node.

In many cases, grafts of 'large epiblast', which self-differentiate into neural tissue, also induced the host epiblast. Dye labelling of single cells in the epiblast layer of the node (Selleck and Stern, 1991) shows that some epiblast cells contribute to both neural tissue and notochord. One possibility is that the epiblast could be the initial source of cells with neural inducing ability, some of which then go on to form neural tissue while others form the notochord. It is possible that the neural tissue from this region induces the host by homeogenetic induction (see below), but the epiblast could also exert its effects because it gives rise to some notochord/head process cells.

In conclusion, neural inducing ability is associated both with particular regions of the node and with the presence of particular prospective cell types in the graft.

Which are the cells responsible for regionalisation of the nervous system?

Neural tissue expressing a full range of anteroposterior characteristics was only found after grafting explants containing both epiblast and mesendoderm layers. Grafts of the mesendodermal layer of the medial sector do induce the expression of regional markers, but these correspond to a restricted anteroposterior region of the CNS (midbrain and hindbrain). These findings suggest that the epiblast is required for the establishment of a complete axis in our experiments. This conclusion predicts that grafts of epiblast alone should induce a more completely regionalised CNS. However, we were unable to assess the regionalising properties of epiblast from node sectors directly because they do not by themselves induce neural tissue in the host. And when we transplanted 'large epiblast', we were only able to study the expression of En-2 together with the quail nucleolar marker. In future experiments, it would be

interesting to transplant epiblast from various regions of the node and neuroepithelium to the neural plate of older host embryos (see Martinez et al., 1991) to test their ability to respecify the expression of anteroposterior markers independently of their ability to induce.

Planar vs. vertical interactions

The above discussion indicates that both the deep mesendodermal and the superficial epiblast layers play some role in neural induction and regionalisation. This is reminiscent of the considerations initiated by Spemann (1938) for amphibians, in which he distinguished between interactions occurring 'vertically' (between germ layers) and those that take place within the plane of the ectoderm ('planar'). There is some evidence for the idea that planar signals (Spemann, 1938; Kintner and Melton, 1987; Dixon and Kintner, 1989; Darnell et al., 1992; Doniach et al., 1992; Ruiz i Altaba, 1992, 1993) emanate from a specialised region of the ectoderm in amphibians, the notoplate (Jacobson and Gordon, 1976; Jessell et al., 1989; for review, see Ruiz i Altaba, 1993).

Our results could be interpreted by postulating that the mesendoderm of the medial sector of the node induces a region analogous to the amphibian notoplate in the overlying epiblast. This specialised region of epiblast could then mediate the spread of neural inducing signals. When medial mesendoderm alone is apposed to extraembryonic epiblast, it may first have to induce a notoplate, whereas grafts including node epiblast already contain notoplate tissue. Since induction of a notoplate might take some time, only a short period of competence might remain for the host epiblast to respond to neural inducing signals, which could explain why these grafts only give rise to a small neural axis. Planar signals may also mediate the induction of neural tissue by the 'large epiblast' grafts. However, as these grafts self-differentiate into neural tissue we cannot distinguish between signals emanating from the notoplate and homeogenetic signals produced by differentiating neural plate.

Our findings also provide evidence that vertical signals play a role. Grafts of medial mesendoderm (in the absence of epiblast) are able to induce neural tissue, as discussed above. But the prechordal plate may also emit vertical signals influencing specification of the forebrain: the only types of grafts that led to expression of *tailless* are those in which *gooseoid* was expressed most frequently. Our results therefore suggest that an interplay between planar and vertical cell interactions is responsible for the induction and regional specification of the CNS.

Mesodermal cell types differentiating from the grafts

In general, each region of the node tested, when grafted to the area opaca of a host embryo, contributes cells to the same structures as during normal development, but there are some exceptions. In particular, the deep (mesendodermal) layer of the posterolateral sector, which normally contains cells destined for somites as well as notochord at stage 4 (Selleck and Stern, 1991) does not form somites and did not differentiate into any of the cell types tested unless accompanied by the epiblast. It is also interesting that although the epiblast contains cells which normally contribute to notochord (Selleck and Stern, 1991), only a few grafts of isolated epiblast give rise to notochord. These results suggest that different subpopulations

of cells may interact within the node to generate or maintain the fates of the cells that normally arise from it. This will be the subject of a future study.

CONCLUSIONS

This study has allowed us to localise the source of neural inducing signals to two regions of Hensen's node: the epiblast layer and the deep portion (mesendoderm) of the medial sector of the node. The deep portion of the posterolateral part of the node does not induce. This difference may be independent of the prospective cell types contained in these regions. However, specific prospective cell types also seem to be associated with the ability to induce. These include the presumptive endoderm, notochord/head process and prechordal plate, as well as neural tissue. We also suggest that the epiblast layer is required for a complete neural axis to form, although prospective prechordal plate cells may also play a role in specification of the forebrain.

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