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The prechordal region lacks neural inducing ability, but can confer anterior character to more posterior neuroepithelium

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SUMMARY

The avian equivalent of Spemann's organizer, Hensen's node, begins to lose its ability to induce a nervous system from area opaca epiblast cells at stage 4$^+$, immediately after the full primitive streak stage. From this stage, the node is no longer able to induce regions of the nervous system anterior to the hindbrain. Stage 4$^+$ is marked by the emergence from the node of a group of cells, the prechordal mesendoderm. Here we have investigated whether the prechordal region possesses the lost functions of the organizer, using quail-chick chimaeras to distinguish graft- and host-derived cells, together with several region-specific molecular markers. We find that the prechordal region does not have neural inducing ability, as it is unable to divert extraembryonic epiblast cells to a neural fate. However, it can confer more anterior character to prospective hindbrain cells of the host, making them acquire expression of the forebrain markers *tailless* and *Otx-2*. It can also rescue the expression of *Krox-20* and *Otx-2* from nervous system induced by an older (stage 5) node in extraembryonic epiblast. We show that these properties reflect a true change of fate of cells rather than recruitment from other regions. The competence of neuroectoderm to respond to anteriorizing signals declines by stages 7-9, but both posteriorizing signals and the ability of neuroectoderm to respond to them persist after this stage.

Key words: prechordal mesendoderm, neural induction, neural plate, regionalization, Hensen's node, forebrain, chick embryo, quail embryo

INTRODUCTION

The chick homologue of Spemann's organizer, Hensen's node, loses the ability to induce rostral portions of the central nervous system at the same time as it begins to lose its overall neural inducing strength. This occurs between Hamburger and Hamilton (1951) stages 4 and 4$^+$ (Dias and Schoenwolf, 1990; Kintner and Dodd, 1991; Storey et al., 1992), a period in development coincident with the emergence of the prechordal mesendoderm from Hensen's node (Hamburger and Hamilton, 1951).

Originally, the 'prechordal plate' was considered to be the axial mesendoderm that lies immediately in front of the forming notochord, visible in the chick embryo as a fan-shaped thickening at the rostral tip of the head process, comprising loose middle layer cells that are intimately associated with the underlying endoderm (Adelman, 1922, 1927; Meier, 1981; Izpisúa-Belmonte et al., 1993). Seifert et al. (1993) recently proposed that the term 'prechordal plate' should be applied only to a thickened endodermal component lying immediately rostral to the above-mentioned fan-shaped structure, and suggested that the mesodermal cells behind the plate should be referred to as 'prechordal mesoderm'. However, it is worth noting that several genes are expressed in both mesodermal and endodermal layers of the triangular structure (e.g. *goosecoid*, Izpisúa-Belmonte et al., 1993; Bally-Cuif et al., 1995; *Rpx/Hesx1*, Hermesz et al., 1996; Thomas and Beddington, 1996; *Otx2*, Ang et al., 1994; Bally-Cuif et al., 1995; Pannese et al. 1995; Simeone et al., 1995; Thomas and Beddington, 1996). For this reason, we have opted to use the term 'prechordal region' to define all three germ layers where this fan-shaped structure is found, and to reserve the term 'prechordal plate' to the thickened endoderm in front of it as defined by Seifert (1993).

The fate of the prechordal mesoderm appears to be to contribute to three extrinsic muscles of the eye (Adelman, 1927; Wachtler et al., 1984; Jacob et al., 1984; Couly et al. 1992; but see Johnston, 1979 and Noden, 1988). The endodermal component comes to form the pharyngeal lining adjacent and just caudal to ectoderm that will invaginate to form Rathke's pouch; the prechordal plate proper is directly adjacent to this pouch and to the future oropharyngeal region that will perforate to form the stomodaeum (Hamilton et al., 1962; Smith et al., 1994). It is believed, based mainly on circumstantial evidence, that these mesodermal, endodermal and ectodermal components interact at later stages of development, together with the neighbouring hypothalamic neuroectoderm, to give rise to elements of the pituitary gland.

The emergence of prechordal mesendoderm cells from the node is followed, between stages 4^+ and 5, by further forward movement of more midline mesoderm, forming the head process. The chordamesoderm caudal to the prechordal region gives rise to quite different structures. The fate of the head process itself has not been thoroughly investigated, but the notochord behind the otic vesicle is generally thought to contribute to the nucleus pulposus of the intervertebral disks and perhaps also to some extent to the vertebral centra (Hamilton et al., 1962). Despite these rather different fates, several genes are expressed in both structures (e.g. *Sonic hedgehog*; Riddle et al., 1993), and it has been proposed that teratogens such as LiCl and retinoic acid can convert amphibian prechordal cells into notochord and induce the movements of convergence and extension that accompany notochord formation during normal development (Yamada, 1994). A related finding is that overexpression of *Xnot2*, normally expressed in the chordamesoderm, causes expansion of the 'prechordal plate' as well as of the notochord (Gont et al., 1996). These findings suggest that despite the differences in fates and morphology and of the genes expressed by the chordamesoderm and of the prechordal region, these structures have at least some common properties.

In amphibians, both the chordamesoderm and prechordal mesendoderm appear to retain the neural inducing and patterning ability of the organizer from which they are derived (Holtfreter, 1933, 1936; for review see Takaya, 1978), but these two regions appear to induce nervous system with different regional character, a finding that led to the concept of separate 'head-' and 'tail-organizers'. However, these classical experiments in amphibians suffer from two problems: first, that it is impossible to distinguish precisely the limit between the prechordal mesendoderm and the axial chordamesoderm (notochord) – it is possible that only one of these structures has inducing and/or patterning ability. Second, the region into which most workers have placed their grafts to assess neural induction is very close to the edge of the prospective neural plate, and therefore it is formally impossible to distinguish between neuralizing and regionalizing signals from the grafted tissue.

The avian embryo offers an opportunity to overcome these problems, because the prechordal region can be distinguished clearly from the chordamesodermal rod behind it (and there are molecular markers for both), and also because the extraembryonic area opaca, which does not contain cells fated to form neural tissue, is competent to respond to both neuralizing and patterning signals and generate a complete nervous system. The only study to date in which the inducing and patterning properties of the prechordal mesendoderm and notochord/head process have been examined is that of Hara (1961), where isolated pieces of prospective neuroectoderm were cultured alone or together with prechordal or chordamesodermal cells in intracoelomic grafts. As in amphibians, he found that different axial regions induced nervous system with different character, and therefore concluded in favour of the concept of separate head- and tail-organizers. However, Hara's study did not use regional markers or any means to distinguish graft from host, used only prospective neuroectoderm as the responding tissue (where a 'maintenance' function of the mesoderm cannot be distinguished from inducing and patterning functions), and the findings were never published. We have therefore undertaken a detailed reexamination of this issue.

Here we examine the extent to which the prechordal region retains the functions lost by the organizer between stages 4 and 4^+. We used quail/chick chimaeras to distinguish graft- and host-derived cells and the molecular markers *tailless* and *Otx-2* (for forebrain: Yu et al., 1994; Bally-Cuif et al., 1995), *engrailed-2*, (for the midbrain/hindbrain border: Patel et al., 1989; Gardner and Barald, 1992), *Krox-20* (for hindbrain: Chavrier et al., 1988; Wilkinson et al., 1989) and *Sox-2* (a pan-neural marker at early stages; Uwanogho et al., 1995; Collignon et al., 1996; Streit et al., 1997). We show that prechordal tissue, when grafted alone in the extraembryonic epiblast, can neither induce the host to adopt a neural fate nor cause it to express regional neural markers. However, when an explant of prechordal tissue is placed adjacent to the presumptive hindbrain of the host embryo, cells that were fated to become hindbrain are induced to express forebrain markers. Moreover, when a stage 5 Hensen's node is grafted together with a prechordal explant into the area opaca of a host, the induced nervous system includes host cells that express rostral markers (*Krox-20* and *Otx-2*). The competence of the host neuroepithelium to respond to anteriorising signals from the prechordal region persists for only a short time, and is lost by stages 7-9. However, posteriorising signals that can affect both the host neuraxis and the neuroepithelium grafted with the prechordal explant persist to later stages. Together, these data suggest that the prechordal region does not possess the neural inducing properties that are lost by Hensen's node at stage 4^+. Instead, it can instruct cells to acquire more anterior fates. These experiments provide direct evidence for a patterning role of the prechordal region during early development of the central nervous system.

MATERIALS AND METHODS

Quails' eggs were obtained from Karasoulas Farm, CA. Hens' eggs (White Leghorn) were obtained from Spafas, CT. Both were staged according to Hamburger and Hamilton (1951), and incubated at 38°C for 10-22 hours to give embryos of stages 3^+-5.

Grafting technique

Chick embryos destined to be hosts were explanted at HH stages 3^+ to 4 and placed in modified New culture (New, 1955; Stern and Ireland, 1981). The region to be grafted was removed from the donor embryo, usually quail, using the tip of a 27G×½ inch needle. When a graft was taken from a chick donor, it was labelled with DiI (see below) prior to transfer to an unlabelled chick host. In some cases, the grafts were separated into ectodermal and mesendodermal layers by placing them in 0.05% trypsin in Pannet-Compton's solution (Stern, 1993) and gently separating the layers with fine steel needles. Some of the grafts were placed within the area pellucida by making a small hole in the hypoblast underlying the lateral part of the germinal crescent and placing the graft in the space between epiblast and hypoblast. In other cases, the graft was introduced adjacent to the extraembryonic epiblast of the inner third of the lateral area opaca, level with the host node. When possible, a flap of germ wall was used to cover the graft and anchor it in place. Following transplantation, embryos were cultured for a further 24-36 hours at 37°C.

DiI/ DiO labelling

DiI (1,1′-dioctadecyl 3,3,3′,3′-tetramethyl indocarbocyanine perchlorate) and DiO (3,3′-dioctadecyl oxacarbocyanine perchlorate; both from Molecular Probes) are lipophilic carbocyanine dyes that insert into cell membranes (see Honig and Hume, 1989). Injections were carried out as previously described (Stern, 1990; Selleck and Stern, 1991). Briefly, DiI is dissolved at 0.5% in absolute ethanol, DiO at 0.15% in ethanol, and diluted 1:9 with 0.3 M sucrose in distilled

water at 45-50°C. Microelectrodes were made using 50 μl borosilicate capillary glass (Fisher or Sigma) pulled with a vertical microelectrode puller (Kopf model 720). Electrodes were tip-filled with the dye stock and injections performed using air pressure. In most experiments, dye was applied to the region to be grafted several times to label as many cells as possible. The labelled region was then excised and transplanted to host embryos as described above. For time course experiments, small groups of cells in the presumptive forebrain and midbrain region of the host epiblast were labelled with either DiI or DiO.

Photo-oxidation of DiI with DAB

DiI-labelled grafts were photo-oxidised using the following protocol (Stern, 1990; Selleck and Stern, 1991). Embryos were fixed in 4% buffered formol saline (pH 7.0) containing 2 mM EGTA. Embryos were washed in 100 mM Tris-HCl (pH 7.4), then incubated in 500 μg/ml 3, 3′-diaminobenzidine (DAB) in Tris-HCl in the dark for 1-2 hours at room temperature. Embryos were transferred to a depression slide and epi-illuminated with rhodamine optics using an Olympus Vanox-T microscope fitted with a 200 W high-pressure mercury lamp until the fluorescence had been quenched. Photo-oxidised embryos were then rinsed in distilled water, postfixed for 1 hour in 4% buffered formol saline/EGTA, then placed in methanol overnight at −20°C prior to whole-mount in situ hybridisation.

Immunocytochemistry

Embryos to be stained with Not-1 antibody (to identify notochord; obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205 and the Department of Biological Sciences, University of Iowa, Iowa City 52242, under contract N01-HD-2-3144 from the NICHD) were fixed in 4% formol-saline for 1 hour and then incubated in 0.25% H_2O_2 in PBS for 2-3 hours. They were then washed for 1 hour in several changes of PBS, and then for at least 30 minutes in PBS containing 0.2% BSA and 0.5% Triton X-100 (PBT). Embryos were then blocked for at least 30 minutes in PBT containing 1% heat-inactivated goat serum. Not-1 antibody was added at a dilution of 1:1 in blocking buffer and incubated overnight at 4°C. Embryos were then rinsed extensively in PBT. Goat anti-mouse IgG-HRP (Jackson) was added to a final dilution of 1:2,500 and incubated overnight at 4°C. Finally, embryos were washed extensively in PBS, and rinsed twice in 100 mM Tris-HCl (pH 7.4). Embryos were then immersed for a few minutes in DAB (500 μg/ml in Tris-HCl), and then H_2O_2 added to a final dilution of 1:10,000 from a 30% stock. Staining was stopped by rinsing embryos several times in tap water.

The protocol for immunocytochemistry with monoclonal antibody QCPN (from the Developmental Studies Hybridoma Bank) was the same as for Not-1 except that the supernatant was used

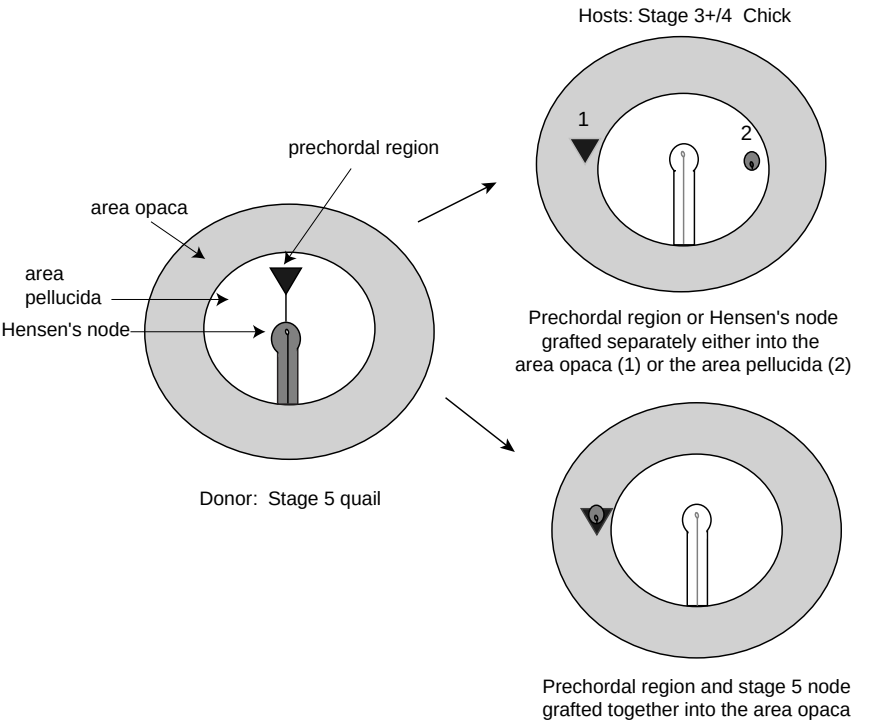

Fig. 1. Diagram showing the graft sources and the sites into which they were placed.

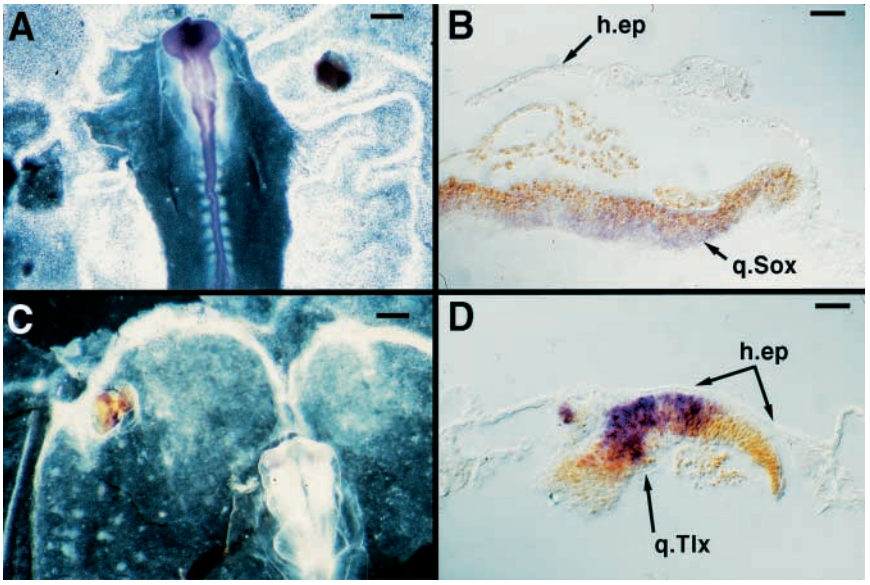

Fig. 2. Prechordal tissue is unable to induce nervous system from competent epiblast. (A) Prechordal explant grafted into the area opaca of a chick host, after hybridisation with the general neural marker, *Sox-2*. (B) In section, expression (purple) is seen only in cells derived from the quail graft (q.Sox; quail tissues stained brown with QCPN antibody). The epiblast of the host (h.ep) appears unaffected by the graft. (C) Prechordal explant grafted into the area opaca, showing expression of *tailless*. (D) In section, this expression (purple) is seen to be restricted to graft-derived quail (brown) cells (q.Tlx); the epiblast of the host (h.ep) appears unaffected. Scale bars: (A,C) 250 μm, (B,D) 50 μm.

at a dilution of 1:5. For staining with monoclonal antibody 4D9 (also from the Developmental Studies Hybridoma Bank), which recognises the engrailed-2 protein (Patel et al., 1989; Gardner and Barald, 1992) the procedure was the same as above except that the supernatant was diluted 1:10.

In situ hybridisation

Most embryos were assessed for the expression of regional neural markers by whole-mount in situ hybridisation using the protocol described by Théry et al. (1995). Following in situ hybridisation, embryos were postfixed in 4% formol saline, dehydrated for 5 minutes in methanol and 10 minutes in propan-2-ol, then cleared for 30 minutes in tetrahydronaphthalene prior to embedding in paraffin wax for sectioning.

The probes used were: *tailless* (a forebrain marker; Yu et al., 1994; kind gift from Dr R.T. Yu), *Otx-2* (another forebrain marker; Bally-Cuif et al., 1995; kind gift from Drs L. Bally-Cuif and E. Boncinelli), *engrailed-2* (probe kindly provided by Dr C. Logan and Prof. A. Lumsden), *Krox-20* (a marker for rhombomeres 3 and 5 of the hindbrain; Chavrier et al., 1988; Wilkinson et al., 1989; kind gift from Dr D. Wilkinson) and *Sox-2* (an early, pan-neural marker; Uwanogho et al., 1995; Collignon et al., 1996; Streit et al., 1997; probes generously provided by Drs R. Lovell-Badge and P. Scotting). All of them were cloned into pBluescript and transcribed with either T3 or T7 polymerase as appropriate.

RESULTS

Experimental design

The aim of this study is to determine the extent to which the prechordal region of the embryo possesses the neural inducing and/or regionalising functions lost by Hensen's node between HH stages 4 and 4^+. To test this, we made use of the fact that the outer, extraembryonic area opaca comprises epiblast that does not normally contribute to embryonic structures, yet it can respond to grafts of the organizer, Hensen's node, by generating a fully patterned nervous system (Waddington and Needham, 1936; Gallera, 1970, 1971; Storey et al., 1992; Streit et al., 1997). In order for such grafts to induce a complete nervous system, they must both divert the fate of the host epiblast to a neural one and impart regional character to this induced axis. By contrast, grafts placed in the anterior half of the area pellucida lie next to or within the prospective neural plate of the host (see Spratt, 1952; Rosenquist, 1966; Schoenwolf and Sheard, 1990; Bortier and Vakaet, 1992; García-Martínez et al., 1993) and can therefore either recruit host neural cells or impart them with regional character, without prior neural induction. Thus, grafts in the area opaca test for the ability of a graft both to induce and to regionalise, while those in the area pellucida test for regionalising ability alone (Fig. 1).

In most cases, operated embryos were cultured until the host had reached the 10 somite stage, or until the marker to be analysed is expressed in the host. Each type of graft was then assessed for the expression of regional neural markers in host cells by in situ hybridisation with digoxigenin-labelled probes. To distinguish host-derived from graft-derived cells, the QCPN antibody, which recognises all quail cells, was combined with whole-mount in situ hybridisation and the embryos were subsequently sectioned.

The prechordal region alone does not have neural inducing ability

To determine whether the prechordal region can induce a new nervous system in competent epiblast, an explant of stage 5 quail prechordal tissue (the visible triangle of prechordal mesendoderm together with the overlying epiblast) was grafted into the area opaca of a stage 3^+ chick host. At the time of fixation, the graft had not elongated. When these embryos were processed to assess the expression of regional markers, none showed expression of the hindbrain marker *Krox-20* (0/11; Table 1) or engrailed-2 (0/4), but they did express both the pan-neural marker *Sox-2* (5/5) (Fig. 2A,B) and the forebrain markers *tailless* (8/9) (Fig. 2C,D) and *Otx-2* (4/5; not shown). However, all cells expressing these markers were derived from the graft when examined histologically after QCPN staining (Fig. 2B,D).

These experiments show that grafts of the prechordal region will self-differentiate into *Sox-2/tailless/Otx-2*-expressing forebrain neuroepithelium, but are unable to induce extraembryonic cells of the host to become neural or to acquire regional markers.

Prechordal tissue causes ectopic expression of forebrain markers in area pellucida epiblast

The above experiment shows that the prechordal region cannot divert the fate of competent epiblast cells to neural. Can it alter the fates of cells that have already received a neural inducing signal? To investigate this, grafts of the prechordal region were placed within the area pellucida, at the edge of the prospective neural plate of stage 3^+-4 host chick embryos. Ectopic expression of the forebrain markers *tailless* (10/13; Fig. 3A,B; Table 1) and *Otx-2* (7/9) is seen in ectoderm cells of the host as well as in donor cells. Neither the hindbrain marker *Krox-20* (0/11; Fig. 3C) nor the midbrain-hindbrain marker engrailed-2 (0/5) is expressed in either donor- or host-derived epiblast. From these data we conclude that, unlike grafts placed far away from the prospective neural plate of the host, prechordal explants in the area pellucida can establish ectopic expression of forebrain, but not mid- or hindbrain markers.

Notochord cells are not required by prechordal tissue to elicit ectopic *tailless* expression

Since the prechordal region is intimately connected with the chordamesoderm, it is necessary to show that the effects observed in our grafts are not due to contamination of the donor tissue with head process/notochord cells. We used the monoclonal antibody Not-1, which recognises cells along the entire length of the notochord and head process but not the prechordal mesendoderm (Placzek et al., 1990; Yamada et al., 1991). Prechordal explants placed in the extraembryonic area opaca generally lack Not-1 immunoreactivity (1/8; Fig. 4A). The remaining embryo contained a small patch of Not-1-positive cells at the site of the graft (Fig. 4C). Prechordal explants placed in the area pellucida also lack Not-1 immunoreactivity (0/7). As a positive control, a stage 5 Hensen's node was grafted to the extraembryonic area opaca (n=5) or area pellucida (n=3). As expected, the node grafts, which contain notochord precursors (Rosenquist, 1966; Selleck and Stern, 1991), generated strong Not-1 staining at the graft site (Fig. 4B). Although these results show that in a minority of cases the prechordal explant may be contaminated with notochord cells, we are not as a matter of course transplanting presumptive notochord with these grafts. Furthermore, when isolated, the prechordal explant does not become chordamesoderm.

It has previously been reported that the emerging tip of the

head process/prechordal mesendoderm at stage 4^+ does have neural inducing ability when grafted to the area opaca of a host embryo (Izpisúa-Belmonte et al., 1993). Given the results presented above, we suspected that this could be due to the presence of prospective head process cells in this structure. We therefore performed several experiments to test this possibility directly. First, we labelled the emerging tip of the head process/prechordal mesendoderm (which forms a triangle with a rostrally facing apex at the rostral tip of Hensen's node at stage 4^+) with DiI (Fig. 4D) and incubated the embryos to stages 9-11. In 5/6 embryos, the labelled cells contributed to the prechordal region as well as to more caudal head process, as far as rhombomere 6 (Fig. 4E). Second, we grafted this tip (mesendodermal layer only) into the area opaca of a host embryo at stage 3^+ as done by Izpisúa-Belmonte et al. (1993) and assessed both the development of cells staining with Not-1 and the inducing ability of the graft on host ectoderm. In 5/7 cases the graft expressed Not-1 (Fig. 4F,I) and in 3/5 cases there was a region with clear neural plate morphology in the overlying host epiblast (Fig. 4I). Finally, we labelled the prechordal region at the tip of the head process that had already emerged at stage 5 with DiI (Fig. 4G). Upon incubation to stages 9-11, the labelled cells were found localized to the prechordal region only and did not extend into more caudal parts of the axis (6/6; Fig. 4H). These results suggest that although the structure at the tip of Hensen's node at stage 4^+ has inducing ability, this is correlated with the fact that this structure contributes both to prechordal and to chordal mesoderm. The visible prechordal region of embryos at stage 5 and beyond neither contributes to the head process nor can it induce neural tissue in the area opaca of a host.

The prechordal region alters cell fates rather than just recruiting host forebrain cells

It is possible that the ectopic *tailless* expression observed when prechordal tissue is grafted into the area pellucida results from recruitment of cells from the presumptive forebrain of the host. Alternatively, these grafts may change the fate of nearby neural cells to forebrain. Two experiments were done to distinguish between these possibilities.

The first experiment was designed to test for a change of fate of prospective hindbrain cells to forebrain. The location of the hindbrain territory at stage 4 in the fate maps published by Spratt (1952), Rosenquist (1966), Schoenwolf and Sheard (1990), Schoenwolf (1992), and García-Martínez et al. (1993) differs from that published by Bortier and Vakaet (1992). To confirm that the prospective hindbrain territory reported by Spratt, Rosenquist and Schoenwolf's group indeed contributes to hindbrain and not to more rostral structures, it was first labelled with DiI in normal embryos (n=30; Fig. 5A). In 22 cases, the labelled cells contributed to hindbrain (sometimes including spinal cord; Fig. 5B,F,H). In the remaining 8, only spinal cord was labelled, and no embryos showed labelling anterior to the hindbrain. In a similar experiment conducted in the prospective forebrain (which is similar in all available maps), 21 injections were conducted, of which 18 showed contributions exclusively to forebrain, 2 had contributions to fore- and midbrain and 1 to the entire length of the neuraxis. Having confirmed the accuracy of the maps, a graft of quail prechordal tissue was placed in direct contact with (ventral to) the labelled cells in the prospective hindbrain region of a chick host (Fig. 5E,G). Labelled cells now formed part of the ectopic neural plate associated with the graft (Fig. 5F,H), and expressed *tailless* (13/18; Fig. 5I,J). This experiment suggests that the prechordal region can induce the expression of forebrain markers in cells that would otherwise have given rise to hindbrain.

In the second experiment, the possibility that the presence of a graft of prechordal tissue can alter the movements of host forebrain cells ('recruitment') was tested by placing a mark of DiI in the presumptive forebrain and one of DiO in the pre-

Table 1. Summary of results obtained (excluding experiments using DiI, those analysed with Not-1 antibody, and grafts placed into older host embryos)

	Marker															
	Sox-2			*Krox-20*			engrailed-2			*tailless*			*Otx-2*			
Experiment	h.	gft.	**tot.**	h.	gft.	**tot.**	h.	gft.	**tot.**	h.	gft.	**tot.**	h.	gft.	**tot.**	**Total**
prech. AP				0	0	**11**	0	0	**5**	8	10	**13**	7	7	**9**	**38**
prech. AO	0	5	**5**	0	0	**11**	0	0	**4**	0	8	**9**	0	4	**5**	**34**
5 HN AP				9	0	**15**	0	0	**6**	0	0	**15**	0	1	**8**	**44**
5 HN AO				0	0	**13**	0	0	**4**	0	0	**9**	0	0	**8**	**34**
3+ HN AP				4	0	**6**				6	0	**13**				**19**
3+ HN AO				7	0	**9**	10	0	**12**	2	0	**4**	3	0	**3**	**28**
prech. ME AP										0	0	**18**				**18**
prech. Ec. AP										0	8	**14**				**14**
2× prech ME AP										1	0	**6**				**6**
2× prech Ec. AP										0	4	**4**				**4**
5 HN+prech.				8	2	**24**	0	0	**8**	0	8	**13**	2	6	**12**	**57**
5 HN+prech ME				2	1	**7**										**7**
5 HN+prech Ec.				0	0	**7**										**7**
2× 5 HN AO				0	0	**14**	1	0	**6**	0	0	**3**	0	0	**6**	**29**
2× prech. AO				0	0	**14**	0	0	**1**	0	9	**9**				**24**
Total			**5**			**131**			**46**			**130**			**51**	**363**

The rows correspond to the type of operation performed, and the columns show the markers used to analyse the results. The results have been separated according to whether expression was seen in the host (h.) and in the graft (gft), but those cases in which the host/graft site of expression was not determined or unclear are excluded. Abbreviations: prech., prechordal region; AP, area pellucida; AO, area opaca; 5 HN, stage 5 Hensen's node; 3^+ HN, stage 3^+-4 Hensen's node; ME, mesendoderm layer only; Ec., epiblast layer only; 2×, two grafts of the type shown.

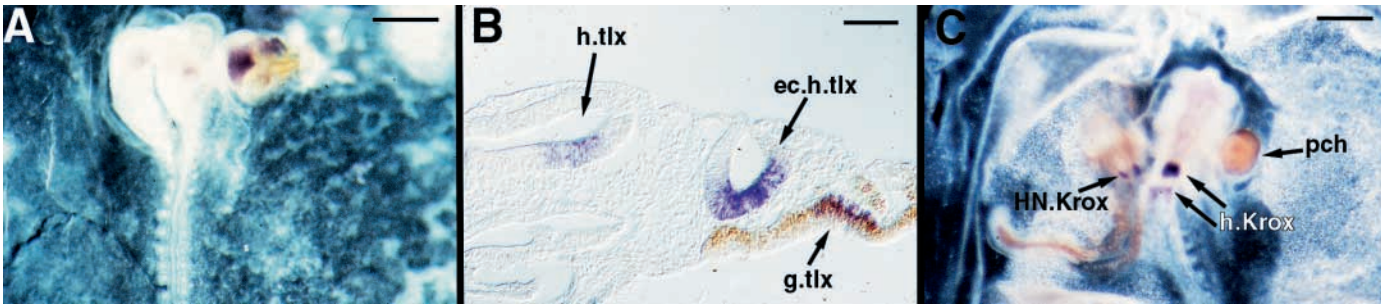

Fig. 3. Prechordal tissue can elicit ectopic expression of *tailless*, but not *Krox-20* in host prospective neuroectoderm. (A) Prechordal tissue grafted into the area pellucida of a host embryo shows expression of *tailless*. (B) A section through this embryo shows that some of the cells expressing *tailless* (purple) are derived from the host embryo (ec.h.tlx), while the graft itself (stained brown by QCPN) has formed a neural plate that also expresses *tailless* (g.tlx). (C) A similar graft to that in A. (pch), after in situ hybridisation with *Krox-20* (purple). No expression is seen associated with the graft (which is stained brown by QCPN). On the contralateral (left) side, a graft of a quail stage 4 Hensen's node has given rise to a complete axis that includes *Krox-20* expressing cells (HN.Krox). h.tlx, *tailless* expression in the host axis; h.Krox, *Krox-20* expression in the host axis. Scale bars: (A,C) 500 μm; (B) 100 μm.

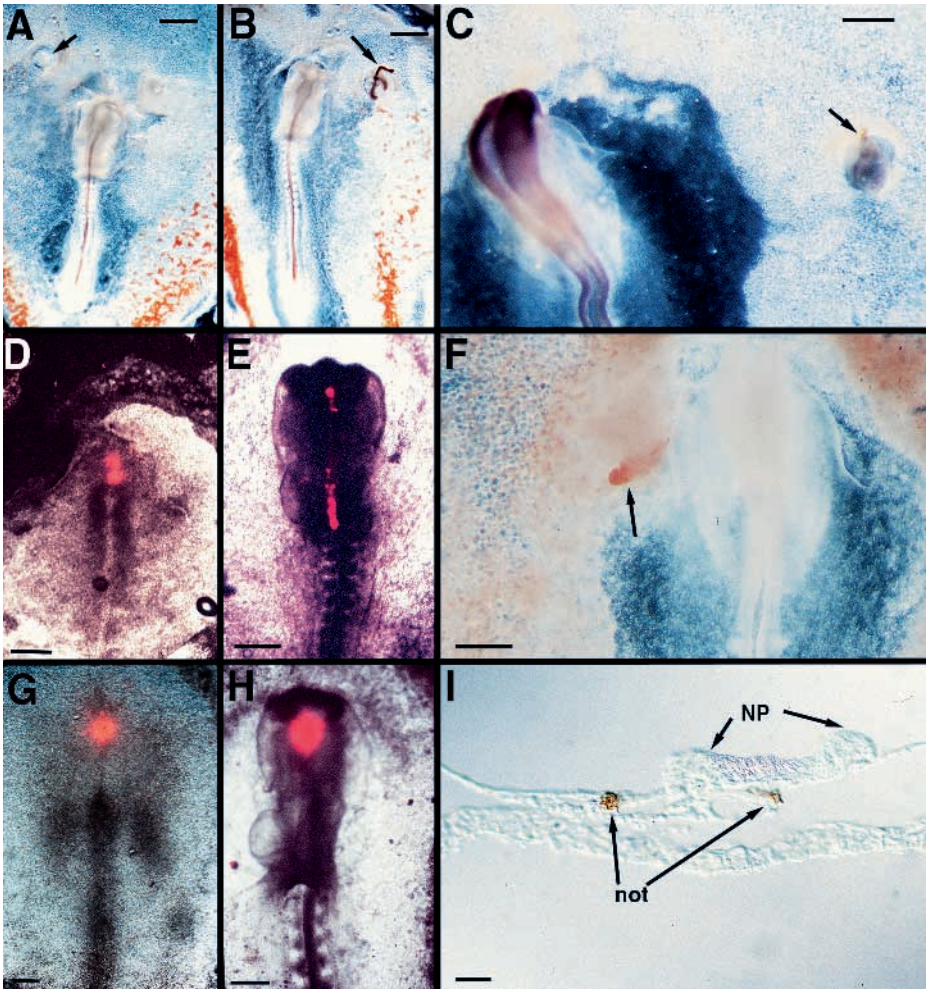

Fig. 4. Embryological relationships between the prechordal region and the head process. (A) Prechordal explant grafted into the area opaca of a host embryo (arrow), after staining with Not-1 antibody (brown). No staining is associated with the graft, revealing the absence of contamination with chordamesoderm cells in such explants. (B) When a stage 5 Hensen's node is grafted into the same region, a notochord-like, Not-1-positive structure is seen (arrow). (C) This is the only embryo in which Not-1-positive cells were detected after grafting a prechordal explant to the area opaca. A few Not-1-positive cells are present (which show up light brown; arrow). The notochord of the host embryo is out of the plane of focus but also expresses Not-1. *Sox-2* expression (purple) is also seen both in the host and associated with the graft. (D) Experiment to determine the extent to which the emerging tip of the chordamesoderm at stage 4^+ contributes to the axis of the head process. Embryo shown immediately after labelling the emerging tip of the head process/prechordal mesendoderm with DiI. (E) The same embryo after 24 hours incubation, showing that the labelled cells have not only contributed to the prechordal region but they also extend more posteriorly into the head process underlying the rostral hindbrain. (F) When the mesendodermal layer of the emerging tip of the head process of a stage 4^+ embryo is grafted into the area opaca of a host embryo, it develops into Not-1 expressing cells (brown) 24 hours later (arrow). (G,H) In contrast with labelling experiments of the tip of the head process at stage 4^+ (D-E above), when the prechordal region at the tip of the already emerged head process is labelled with DiI at stage 5 (G), labelled cells remain restricted to the prechordal region and do not extend more caudally (H). (I) Section through an embryo into which a stage 4^+ mesendoderm from the tip of the head process was grafted into the area opaca (as in F above). Not-1-positive cells (not, brown) are seen closely associated with a region where the host ectoderm has assumed a neural plate morphology (NP). Scale bars: (A,B) 500 μm; (C,E,F,H) 250 μm; (D,G) 100 μm; (I) 50 μm.

sumptive hindbrain of each side of a host embryo. In 6 experiments, a graft of prechordal tissue was then placed between the two labels on one side of the embryo (Fig. 5C). Photographs of the position of labelled cells were taken at 4-6 hour intervals following grafting. No differences in the patterns of movement were observed between experimental and control sides of the embryos. At the end of the 24 hour incubation period, the ectopic structures formed by the graft did not contain labelled cells derived from either injection site (Fig. 5D). This experiment suggests that the graft does not significantly alter the patterns of cell movements within the host neural plate, and that it does not recruit cells from the prospective forebrain territory of the host.

Together, both of the above experiments suggest that prechordal tissue can respecify hindbrain to become forebrain, rather than recruit forebrain cells.

The prechordal region can rescue the ability of stage 5 Hensen's node to induce anterior neural markers

The experiments described above show that although the prechordal region does not have the ability to induce neural tissue from competent epiblast cells, it can alter the fate of prospective hindbrain cells and cause them to acquire forebrain markers. Since Hensen's node loses its ability to induce forebrain between stages 4 and 5 (reviewed by Gallera, 1971; Dias and Schoenwolf, 1990; Kintner and Dodd, 1991; Storey et al., 1992), at the same time as the prospective prechordal cells emerge from the node, we wished to determine whether a graft of prechordal region together with a stage 5 node can rescue the ability of the older node to induce more anterior structures.

First, to confirm and extend the published findings with the use of molecular markers, we compared the expression of regional markers elicited by quail Hensen's nodes from stages 3^+-4 (positive control) and 5 in host chick area opaca epiblast at stages 3^+-4. As expected, grafts of stage 3^+-4 Hensen's nodes induce expression of *tailless* (2/4) (Fig. 6A,B), *Otx-2* (3/3), engrailed-2 (10/12) and *Krox-20* (7/9) (Fig. 6C,D) in the area opaca, and the same result is obtained in the area pellucida (6/13 for *tailless*, 4/6 for *Krox-20*). By contrast, stage 5 Hensen's nodes induce neither *tailless* (0/9), nor *Otx-2* (0/8), nor engrailed-2 (0/4), nor *Krox-20* (0/13) in host cells of the area opaca, but do induce a neural plate expressing the more caudal

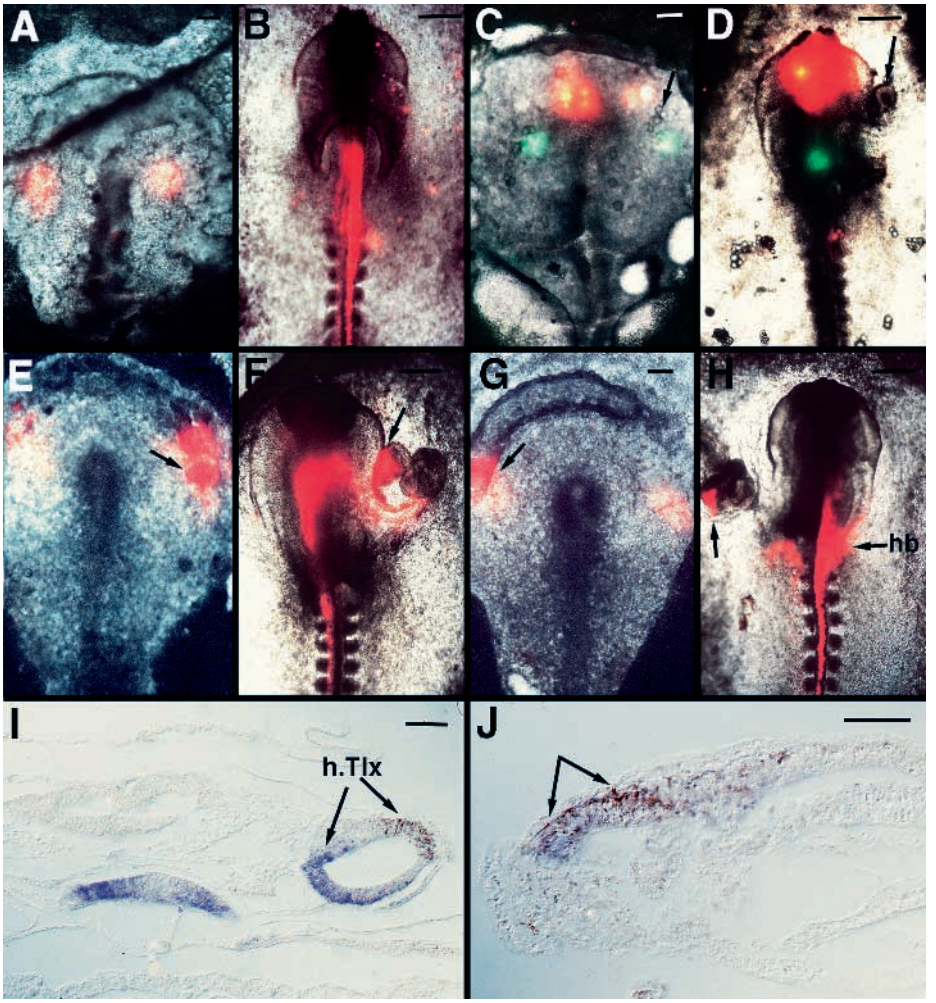

Fig. 5. Prechordal tissue respecifies hindbrain to become forebrain, but does not alter the movements of host neuroepithelial cells. (A) Stage 3^+ embryo in which a spot of DiI was placed on each side, in the presumptive hindbrain territory, shown immediately after labelling. (B) The same embryo, 24 hours later, confirms that the labelled cells have contributed to hindbrain and spinal cord, but not to more rostral regions of the neuroectoderm. (C) Embryo immediately after being labelled bilaterally with DiO (green) in the future hindbrain territory and bilaterally with DiI (red) in the prospective forebrain. A prechordal graft was then placed between the two marks on the right, at the position shown by the arrow. (D) After 24 hours, on both sides, the two marked areas have contributed to regions according to their fates, and the ectopic structure that developed (arrow) contains no cells derived from either labelled territory. (E) Embryo labelled as in A, but a graft of prechordal tissue was placed in direct contact with (ventral to) the labelled prospective hindbrain territory on the right (arrow). (F) The same embryo after 24 hours shows that while on the non-grafted side (left) the labelled cells have contributed to hindbrain and spinal cord according to their fate, labelled cells on the right are now located within an ectopic neural structure (arrow). (G) The prospective hindbrain of this embryo was labelled bilaterally with DiI (red), and a prechordal graft was placed in contact with the DiI labelled territory on the left (arrow). (H) After incubation, the DiI labelled cells have contributed to hindbrain (hb) and spinal cord according to their fate on the right, while on the left they have become incorporated into an ectopic neural structure (arrow) that remained separate from the host neural tube. (I) Section through a similar embryo to that in F, showing *tailless* expression (purple) in an ectopic host-derived neural tube (labelled brown by photooxidation of DiI) (h.Tlx). (J) Section through a similar embryo to that in H above, showing *tailless* expression (purple) in an ectopic neural plate of host origin (arrows; stained brown by photoconversion of DiI). Scale bars: (A,C,E,G,I,J) 100 μm; (B,D,F,H) 250 μm.

marker *Hoxc-6* in about 50% of cases (Storey et al., 1992 and data not shown). However, when grafted into the area pellucida, stage 5 nodes do generate ectopic expression of *Krox-20* (9/15; Fig. 6E,F) (but not *tailless*, 0/15, *Otx-2*, 1/8 or engrailed-2, 0/6) in host cells. These results confirm that Hensen's node loses the ability to induce anterior (forebrain-midbrain) structures between stages 4 and 5.

To determine whether the ability of the stage 5 node to induce rostral tissues can be rescued by prechordal cells, we grafted it together with a prechordal explant into the area opaca. When quail prechordal tissue is grafted together with a stage 5 quail Hensen's node into the extraembryonic area opaca of a chick host, a neural plate was induced in host cells in 23/38 (61%) cases analysed. This is comparable to the inducing ability of stage 5 nodes grafted alone, indicating that the presence of the prechordal explant does not enhance neural inducing ability. However, host cells expressed *Krox-20* in most (8/14; 57%) of the embryos in which a neural plate had been induced (Fig. 7A,B), and *Otx-2* was induced in 2/4 cases with an ectopic neural plate (Fig. 7C,D). Other markers (*tailless*, 0/13 and engrailed-2, 0/8) were not induced.

It is possible that the expression of *Krox-20* and *Otx-2* in this experiment are merely due to an increase in signalling arising from the doubly large grafts. To test this, either two prechordal regions or two stage 5 Hensen's nodes were transplanted together into the area opaca of a host embryo. Neither double prechordal grafts (0/14) nor double node grafts (0/14) induce *Krox-20* expression. Double grafts of stage 5 Hensen's node also failed to induce *tailless* (0/3) and *Otx-2* (0/6), although expression was seen in graft- derived cells. In 1/6 cases, double node grafts did induce engrailed expression but this may be due to contamination with cells from the head process, which can induce engrailed expression by themselves (A. Rowan, C. D. Stern and K. G. Storey, unpublished data). These results suggest that the prechordal region has limited ability to recover some of the lost patterning properties of the stage 5 Hensen's node. However, the anteriorising effect of the prechordal region when grafted together with a stage 5 node is weaker than when the former is grafted alone, adjacent to prospective hindbrain cells of the host.

Do anteriorising signals come from a particular germ layer?

All of the above experiments were conducted with prechordal regions that included all three germ layers. To determine if the regionalising properties of the prechordal region in the area pellucida are due to signals from a particular germ layer, the prechordal graft was subdivided into ectodermal and mesendodermal layers and each grafted separately to the area pellucida of a host embryo. Most of the ectodermal grafts (8/14) self-differentiated and expressed *tailless*; however, none induced ectopic expression in host cells (Fig. 8A,B). Grafts of the mesendodermal layer alone failed to show ectopic *tailless* expression (0/18; Fig. 8A).

The failure of both the ectodermal and mesendodermal layers of the prechordal region to elicit ectopic *tailless* expression might be due to the smaller size of each graft. To test this, either two quail prechordal mesendoderms or two quail prechordal ectoderms were combined before grafting. As before, double-ectoderm grafts self-differentiated and expressed *tailless* but had no influence on the cells of the host (0/4). Double-mesendoderm also generally failed to generate ectopic expression of *tailless* (1/6). In the one embryo in which *tailless* expression was seen, the grafts were in very close association with the host axis. These results show that neither layer alone can elicit the ectopic *tailless* expression seen when the whole thickness of the prechordal region is transplanted, and that this failure is not due to the reduced amount of tissue grafted.

We also investigated whether the ability of the prechordal region to recover induction of *Krox-20* by grafts of stage 5 Hensen's node in the area opaca is associated with a particular cell layer. A stage 5 quail node was grafted together with either the mesendodermal or epiblast component of the prechordal region of a quail embryo. In recombinants with only the mesendodermal portion, as with grafts of stage 5 Hensen's node alone, an ectopic neural plate was induced from host cells in 3/7 (43%) cases. Two of these 3 embryos showed expression of *Krox-20* in host cells. By contrast, in recombinants with only the ectodermal portion of the prechordal region none of the embryos (0/7) showed expression of *Krox-20* in host cells. This experiment suggests that prechordal mesendoderm may be sufficient to anteriorise the neural plate induced by a stage 5 node.

Host neuroepithelium loses competence to respond to signals from the prechordal region by stages 7-9, except near the otic placode and optic region

Since the experiments described above suggest that the prechordal region can respecify hindbrain to become forebrain, we tested whether this ability extends to later stages of development. A stage 5 quail prechordal graft was placed in contact with the neural tube of an older host (stages 7-9; n=27). In 15 of these, the graft was placed in the midbrain/forebrain region, and in 12 it was placed adjacent to the hindbrain of the host. In 3 of the latter, the graft was very close to the otic vesicle of the host, and differentiated into ectopic neural structures that expressed *tailless* in cells of both graft and host origin (3/3; Fig. 9B) but not engrailed-2 or *Krox-20*. In the remaining embryos, the grafts neither expressed *tailless* themselves nor caused the host to acquire ectopic expression unless the graft had found itself near the eye region (n=6), in which case it developed into an ectopic eye-like vesicle (3/6) or into ectopic neural structures (2/6) that contained both host and graft cells and expressed *tailless* (3/6). In section, the vesicles had a double-layered retina-like structure but no lens was present (Fig. 9A).

These findings suggest that older (stage 7-9) neuroepithelium can no longer respond to regionalizing signals from the prechordal region, except in placodal regions. However, there seems to be an effect of the older host neuroepithelium on the expression of *tailless* by the ectoderm of the younger graft. This further suggests that the period during which more posterior regions of the neural plate can be respecified to become forebrain may end before stage 7.

DISCUSSION

Induction and patterning by the organizer and its descendants

One of the properties that link the prechordal region to the chordamesoderm, at least in avian embryos, is their embryonic origin: both are derived from Hensen's node (see Spratt, 1955; Rosenquist, 1966, 1983; Selleck and Stern, 1991). It has been known since the 1930s (Waddington, 1932, 1933, 1934, 1936,

1940) that Hensen's node is the avian and mammalian functional homologue of the amphibian organizer. However, there has been considerable controversy concerning the extent to which some of the derivatives of the organizer possess inducing and/or patterning roles at different stages of development. Nieuwkoop and Nigtevecht (1954), Eyal-Giladi (1954), and others (Dalcq and Pasteels, 1937; Damas, 1947; Gallera, 1947, 1948, 1952; Blitz and Cho, 1995), have proposed that the prechordal mesendoderm is a strong neural inducer ('activator') but rather weaker at regionalising ('transforming') the neural plate in amphibians. These authors and others (e.g. Mangold, 1929; Holtfreter, 1933, 1936; see Nakamura and Toivonen, 1978) have shown that in amphibians, the entire length of the chordamesoderm is capable of inducing an ectopic neural plate. These findings have led to the general assumption (e.g. Ruiz i Altaba, 1993; Doniach, 1993; Doniach et al., 1992; Lemaire et al., 1997) that the organizer and its midline descendants, the prechordal plate, head process and notochord, all share the ability both to induce and to pattern the nervous system.

However, several lines of evidence cast doubt on this interpretation. Zebrafish mutants such as *floating head* lack a notochord (but not the prechordal region) yet have a nervous system with normal rostrocaudal organisation (Talbot et al., 1995), and comparable findings were made by surgical ablation of the teleost shield by some workers (Nicholas and Oppenheimer, 1942; Shih and Fraser, 1996). Similarly, chick embryos lacking a notochord as a result of extirpation of Hensen's node have normal rostrocaudal pattern in the nervous system (Darnell et al., 1992; see also Fukushima et al., 1996). Lastly, a mouse transgenic model in which the transcription factor *HNF-3β* was inactivated lacks both a morphological node and its descendants, yet has apparently normal rostrocaudal pattern in its nervous system (Ang and Rossant, 1994; Weinstein et al., 1994). These findings all suggest that if the chordamesoderm and/or prechordal tissue play a role in neural induction and regionalisation, they are dispensable for generating a grossly normal rostrocaudal pattern in the central nervous system. However, classical studies on twinned embryos by Clavert (1978) and Ulshafer and Clavert (1979) suggest that the prechordal region is required for induction of the forebrain and optic vesicles, and that the presence of hindbrain is dependent upon both prechordal and more posterior chordamesodermal cells. Furthermore, Thomas and Beddington (1996) have shown that when an anterior region of the mouse endoderm is ablated, the forebrain does not form normally, a finding strengthened by the observation of Varlet et al. (1997) that expression of the *nodal* gene is required in the endodermal layer for development of the rostral brain.

While there is general agreement that development of a normal nervous system depends on inductive interactions between different groups of cells, these studies present a confusing view of the sources of such inductive signals. One of the problems is that in amphibian and fish embryos, as well as in much of the earlier work using chick, transplantation operations have generally been conducted very close to the neural plate of the host embryo, which may already have received some neural inducing and/or patterning signals prior to the operation (see Streit et al., 1997). For this reason we decided to exploit the fact that the extraembryonic region (area opaca) of the avian embryo does not normally contribute to any embryonic structures, yet it is able to respond to signals from Hensen's node by giving rise to a fully patterned neural plate that is organised along the rostrocaudal as well as the dorsoventral axis (Waddington and Needham, 1936; Gallera, 1970, 1971; Storey et al., 1992, 1995; Streit et al., 1995, 1997). We have shown that grafts of prechordal region are unable to give rise to any type of nervous system from this extraembryonic epiblast. This suggests that, if a 'head-organizer' capable of both neural induction and patterning of the anterior nervous system exists in the chick (see Introduction), it is unlikely to reside in the prechordal region.

Anteriorisation and posteriorisation

One classical view (Nieuwkoop et al., 1952; Nieuwkoop and Nigtevecht, 1954; Eyal-Giladi, 1954) that has received increasing support recently from workers using *Xenopus* is that early steps in neural induction ('activation') result in a nervous system with anterior character (cement gland and forebrain), while interactions with other tissues like the notochord, organizer or other caudal tissues can 'transform' the anterior neuroepithelium into more posterior regions. Two reasons why this view has gained recent favour are the findings that three neuralising factors, noggin, chordin and follistatin, all give rise to anterior nervous system (Lamb et al., 1993; Hemmati-Brivanlou et al., 1994; Sasai et al., 1995), and that three factors, retinoic acid, fibroblast growth factors and Wnts, can mimic caudal tissues in generating posterior nervous system from more anterior regions (Yamada 1994; Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995; McGrew et al., 1995; Launay et al., 1996). Other experiments (e.g. Itasaki et al., 1991, 1996; Martínez et al. 1991; Bally-Cuif et al., 1992; Nakamura et al., 1994; Grapin-Botton et al., 1995) have shown that translocations of caudal neuroepithelium to more rostral regions can induce the expression of caudal markers and traits in neighbouring neuroepithelium, and that the competence of the neural tube to respond to these signals continues to stages in development (stage 10) long after neural induction has ended. Therefore, at least some rostrocaudal patterning signals can be separated experimentally from neural induction. It is also apparent from the above studies that it is easier to posteriorise the nervous system than it is to force it to acquire more rostral character, supporting the model of Nieuwkoop et al. (1952), Nieuwkoop and Nigtevecht (1954) and Eyal-Giladi (1954).

Nevertheless, a few scattered observations suggest that it is indeed possible to 'anteriorise' epiblast from regions destined to give rise to more posterior structures. The most convincing of these studies are those of Ang and Rossant (1993), who found that anterior mesendoderm can induce the expression of *engrailed* from posterior epiblast of head-fold stage mouse embryos. Anterior mesendoderm can also stabilise the anterior domain of expression of *Otx-2* in explant combinations (Ang et al., 1994). Here we have confirmed their observation directly, by showing that the prechordal region can induce the forebrain markers *tailless* and *Otx-2* from cells destined to be hindbrain. We also show that in some cases, the mesendoderm of this region is sufficient for this effect, although inclusion of the overlying ectoderm strengthens the signals. Furthermore, the neural tube induced from area opaca cells by a stage 5 node together with prechordal tissue is more anterior in character (including the *Krox-20* domain and sometimes *Otx-2*) than when the older node is grafted alone. Our findings provide direct evidence for the existence of anteriorising signals emanating from the prechordal region during the early stages of neural development.

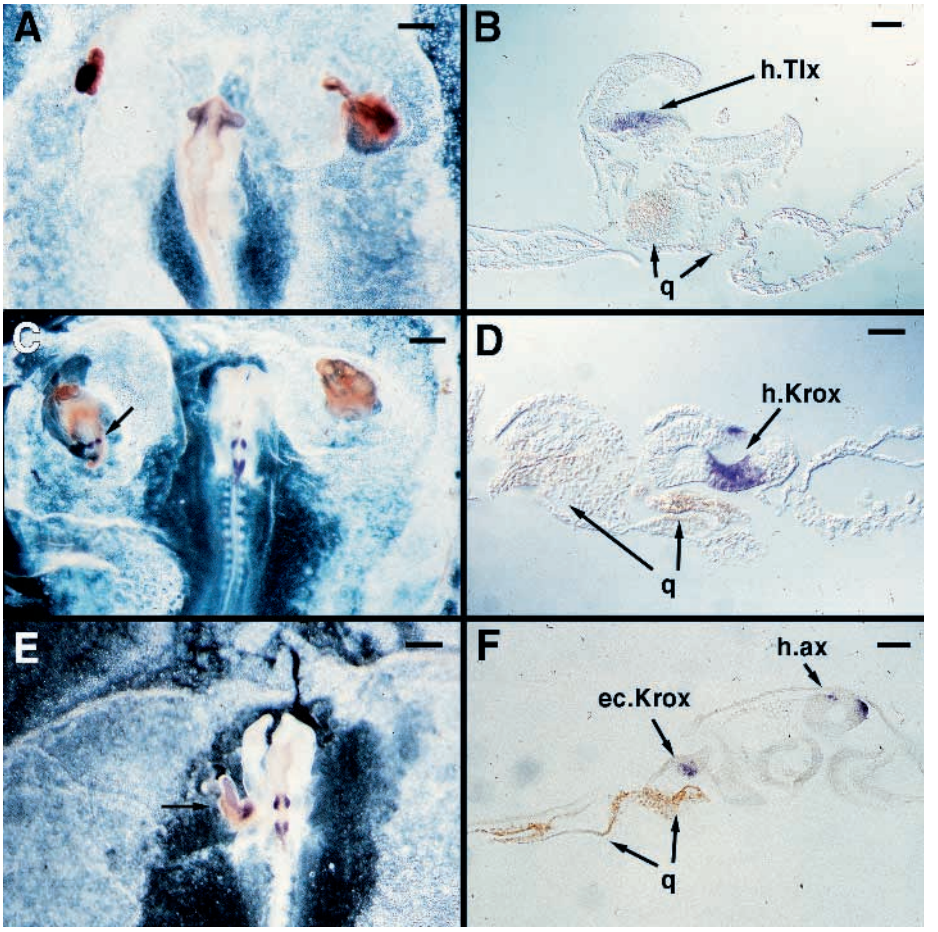

Fig. 6. Comparison of the inducing ability of Hensen's node of different stages and prechordal tissue placed in the area opaca or area pellucida. (A) Embryo that had received a graft of quail stage 4⁻ Hensen's node on the right and a quail prechordal explant on the left. After incubation to stage 11 and in situ hybridisation with *tailless* (purple), expression is seen associated with both grafts as well as in the host. In situ hybridisation was followed by QCPN staining to reveal quail donor cells (brown). (B) Section through the Hensen's node graft of the embryo shown in A. *tailless* expression is seen in host (chick) cells (h.Tlx). (C) Embryo grafted with a quail stage 4 Hensen's node on the left and a quail prechordal explant on the right. *Krox-20* expression (arrow) is associated only with the node graft. (D) Section through the Hensen's node graft in a similar embryo to that in C, showing *Krox-20* expression (purple) in host (chick) cells (h.Krox). (E) Graft of a quail stage 5 Hensen's node into the area pellucida of a chick host. An ectopic neural tube has developed (arrow). In section (F), *Krox-20* expression (ec.Krox) is seen in host cells. q, quail graft. h.ax, host axis. Scale bars: (A,C,E) 250 μm; (B,D,F) 50 μm.

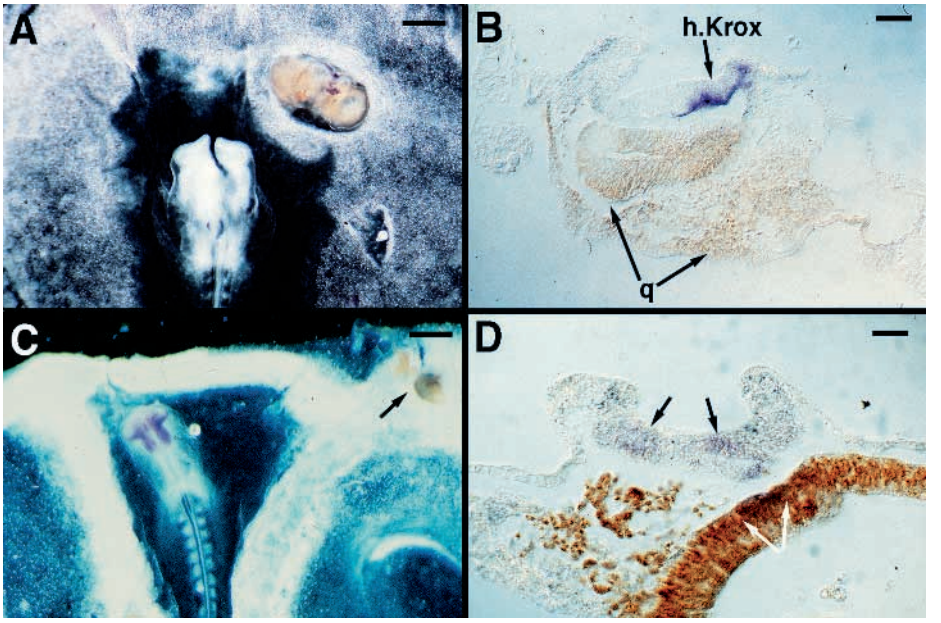

Fig. 7. Prechordal tissue can rescue the expression of *Krox-20* and *Otx-2* in neural tissue induced by stage 5 Hensen's node. (A) Graft of a quail stage 5 Hensen's node and a quail prechordal explant together into the area opaca of a host chick embryo, hybridised with *Krox-20*. (B) In section, expression (h.Krox, purple) is seen in host (chick) cells. Quail cells (q) are stained brown with QCPN antibody. (C) After a similar operation to that described in A, the embryo was hybridised with *Otx-2*. The site of the graft is marked by an arrow. (D) In section, host cells have formed a neural plate that contains *Otx-2* expressing cells (black arrows). A region of *Otx-2* expression (white arrows) is also seen in graft-derived quail (brown) cells. Scale bars: (A,C) 250 μm; (B,D) 50 μm.

When do anteriorising and posteriorising signals act?

One line of evidence suggests that the mesendoderm may influence the regional differentiation of rostral brain at very early stages of development: when the anterior domain of endoderm that expresses *Hesx1/Rpx* is removed at early- or mid-streak stages of mouse development, severe abnormalities of the forebrain ensue (Thomas and Beddington, 1996). These results suggest that in the mouse, the endoderm could begin to exert its influence on the future anterior neuroectoderm before the end of

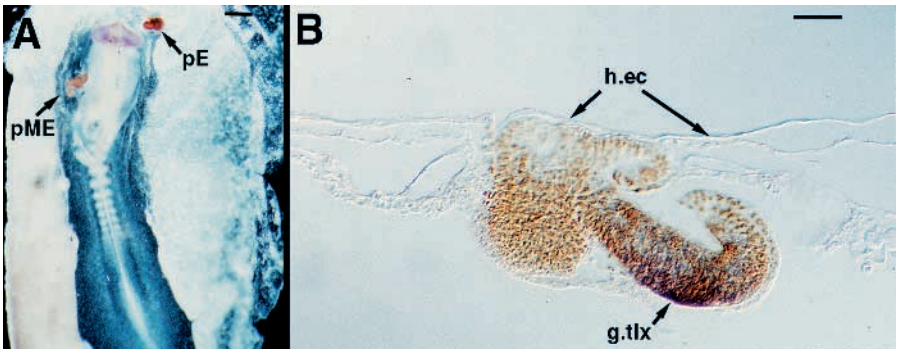

Fig. 8. Neither the ectoderm nor the mesendoderm of the prechordal region can elicit ectopic expression of *tailless* in host neuroectoderm. (A) Prechordal mesendoderm (pME) and prechordal ectoderm (pE) grafted into the area pellucida of a host embryo, stained with QCPN (brown) and *tailless* (purple). The ectoderm graft expresses *tailless* (which shows up dark brown because of the overlap of the two colours in the whole mount) but the mesendoderm does not. (B) Section through the ectoderm graft in the embryo in A. above, showing that the expression of *tailless* (purple) associated with the grafted prechordal ectoderm (g.tlx) is entirely contained within graft-derived (brown) cells. The host ectoderm (h.ec) above the graft does not express *tailless* and its morphology is unaltered. Scale bars: (A) 500 μm; (B) 50 μm.

gastrulation. In mammalian embryos the prechordal region appears to be specified by the early streak stage (see Tuchman-Duplessis, 1974; Hogan et al., 1994; Sulik et al., 1994; Viebahn et al., 1995; Hermesz et al., 1996; Thomas and Beddington, 1996; Varlet et al., 1997), but there is no evidence at present that this is the case in the chick. The fact that a chick node from the mid- to late-primitive streak stage (stages 3^{+}-4) can induce a complete nervous system, including forebrain (Storey et al., 1992, 1995; Streit et al., 1997 and the present paper) outside the region fated to do so, suggests that if a forebrain bias is established by tissues other than the organiser early during gastrulation, this is not absolutely essential for the formation of a normal forebrain in the chick.

When a graft of prechordal tissue is placed adjacent to the neuroectoderm of an older (stage 7-9) host, it is unable to cause ectopic expression of anterior markers in more posterior regions of the nervous system, even if the graft includes forebrain neuroepithelium. However, in these grafts, the prospective neuroepithelium of the graft itself is affected by the host environment. The graft will only express forebrain markers if it is placed in the forebrain region or adjacent to the otic vesicle of the host. This finding supports the discussion above: the competence of neuroepithelium to respond to anteriorising signals from the prechordal region is lost by stages 7-9, but the ability to respond to posteriorising signals is retained for much longer (at least stage 10; see Martínez et al., 1991; Grapin-Botton et al., 1995; Itasaki et al., 1996).

One interpretation of this finding is that that planar signals from within the neural plate can continue to act as weak anteriorising influences at later stages in development, but the neuroepithelium is no longer competent to respond to vertical signals from prechordal mesendoderm. These findings further suggest that there are different kinds of regionalising signals, each operating at different times in development and each with its own window of competence in the responding neural plate (see Storey et al., 1992). The existence of such multiple signals for rostrocaudal patterning of the neural plate could explain the finding that notochordless and/or nodeless embryos can develop with apparently normal rostrocaudal pattern in their nervous system (see above), yet the chordamesoderm and prechordal region do have the ability to pattern a neural plate when transplanted to appropriate locations.

Differences in patterning by grafts placed into the area pellucida or area opaca

A stage 5 Hensen's node, when grafted alone into the area opaca of a host embryo, sometimes (in about 50% of cases) induces a nervous system from the host, but this excludes the preotic head region (*Krox-20*, en-2, *Otx-2* and *Tailless* expression domains). When combined with a graft of prechordal tissue, the induced nervous system now includes one *Krox-20* expression domain, and sometimes *Otx-2* expressing cells. By contrast, when the prechordal graft is placed adjacent to the prospective hindbrain of a host embryo, it is able to anteriorise this region more strongly, and can also elicit expression of *tailless* in host cells. The difference between these two sets of results is difficult to interpret. Some possible reasons are:

(a) the prechordal graft quickly loses its patterning ability after stage 5. When grafted together with a node, which causes

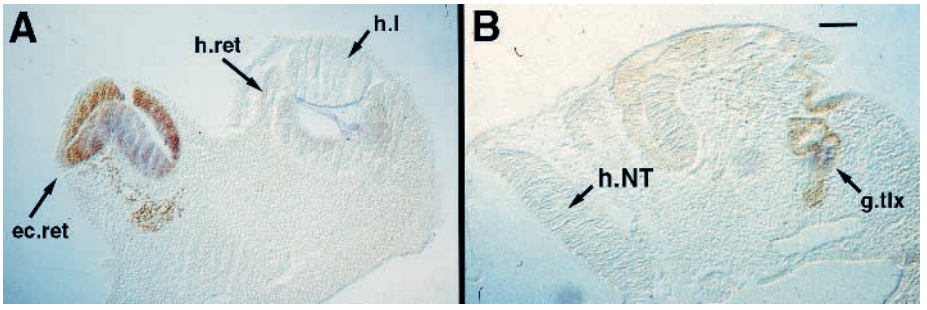

Fig. 9. Grafts of prechordal tissue placed into later embryos self-differentiate according to their fate but fail to respecify host neuroepithelium. (A) Prechordal explant placed in the forebrain region of a stage 8 embryo. 48 hours later it was hybridised with *tailless* (purple) and stained with QCPN (brown). In section, a retina-like structure (ec.ret) made up of both donor and host cells, and expression of *tailless* is seen. (B) Prechordal tissue placed in the hindbrain region of a host embryo at stage 8, processed as above. Ectopic host neural structures are seen nearby (h.NT), but *tailless* expression is seen only in donor-derived (brown) cells (g.tlx), albeit faintly. h.ret, host retina; h.l, host lens. Scale bar 50 μm.

a nervous system to be induced anew, there is insufficient time for the prechordal tissue to complete its anteriorising influence before it loses its patterning strength;

(b) the prospective neural plate region might receive patterning signals that begin very early in development, which confer an anterior bias to much of the neural plate (see Thomas and Beddington, 1996; Streit et al., 1997; Varlet et al., 1997 for discussion of this issue). Continuing influences from the node and/or more posterior neural plate are required to posteriorise portions of it, and the prechordal plate can antagonise these influences. The area opaca has not received the early anterior biassing signals, and stronger patterning signals are therefore required;

(c) the environment of the area opaca somehow alters the character of the prechordal graft and causes it to lose some of its patterning strength. This includes the possibility that the area opaca lacks maintenance factors present in the area pellucida;

(d) the chordamesoderm of the head process (which is missing in the combined grafts of prechordal region and stage 5 nodes) might also be required to generate more anterior regions of the nervous system. Evidence for this will be provided elsewhere (A. Rowan, C.D. Stern and K.G. Storey, in preparation);

(e) the rostral neuroepithelium generated in the area opaca may be only ventral in character, because *Otx-2* and *Krox-20* are expressed at all dorsoventral levels, while engrailed-2 and *tailless* are exclusively dorsal (Darnell et al., 1992; Yu et al., 1994; Monaghan et al., 1995). This might arise if the area opaca lacks signals (such as BMPs; Liem et al., 1995) required to produce dorsal neural structures.

Some evidence exists supporting each of these possibilities, and it seems likely that all of them contribute to some extent to the results obtained here.

Are placode fields special?

In our transplants of prechordal tissue to older (stages 7-9) hosts, we have found that the graft itself is influenced by the location of the host axis at which it is placed. When placed close to the future forebrain or otic region of the host, it maintains its expression of *tailless*. In the former region, it can also self- differentiate into a retina-like structure and recruit host cells into this ectopic partial eye, where they continue to express *tailless*. When placed in other regions, however, the graft both loses its expression of this gene and it does not differentiate into recognisable eye structures. These findings suggest that the otic region of the hindbrain and the optic region of the forebrain both possess signals that can support forebrain differentiation or alternatively, that they lack strong posteriorising influences present in the rest of the axis. Placode fields may therefore be different from the rest of the axis. Consistent with this hypothesis, Oliver et al. (1996) recently demonstrated that a lens can be induced in the otic (but not in other) regions of fish embryos by ectopic expression of *Six3*, a mouse homologue of the Drosophila *sine oculis* homeobox gene. In our own experiments, lenses were never associated with the ectopic retinae produced by the grafted prechordal tissue, even in the forebrain of the host. Since the results of Oliver et al. show that the ectoderm of the otic region is competent to form a lens at least when *Six3* is expressed, these results suggest that several signals are involved in the induction of elements of the eye and ear, and that some of these are common to both.

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