Evidence has recently accumulated that in mammalian development a separate site, distinct from the classical organiser before gastrulation, provides anteriormost axial patterning signals (mouse data reviewed by Beddington and Robertson, 1999, and see Knoetgen et al., 1999 for the evidence from rabbit). This site, the anterior visceral endoderm (AVE) is of purely extra-embryonic fate, being initially the lower layer at the distal tip of the pregastrulation mouse egg cylinder. It moves to define the future anterior side of the egg cylinder, opposite the site where the streak will form, and is finally replaced as a layer by definitive foregut endoderm as the head process forms (the phase of gastrulation equivalent to node regression in chick). Coordination of signals from the AVE with those from the node and its derivatives is thought to be required, to achieve full anterior neural pattern (see for example, Shawlot et al., 1999).

Bird development involves pregastrulation movements, streak formation and then lower layer replacements in the blastoderm that closely resemble those in the mouse egg cylinder. Definitive endoderm forms during gastrulation from an expanding pool of anterior streak- and node-derived cells that insert themselves into the earlier lower layer or hypoblast, displacing this to peripheral, extra-embryonic positions. Current work suggests that signals from that pregastrulation lower layer are not required for complete anterior development in chick, as they are in mouse (Knoetgen et al., 1999; S. Withington and J. Cooke, unpublished data). Studies in Xenopus had led to a model whereby neurally induced tissue is initially of anterior character, and is modulated only by progressively posteriorising signals for production of the complete neuraxial pattern in normal development (Niewkoop 1977; Nieuwkoop et al., 1985; Knecht et al., 1995; Holowacz and Sokol, 1999). However more recent results, especially...
those from other vertebrates, suggest that a default neural specification may correspond only with a generalised forebrain (prosencephalic) state, with separate posteriorising and anteriorising signal systems required to generate the territories of a complete neural rudiment. Thus the chick head process, anterior-most definitive embryonic mesoderm + endoderm that emerges from the node or organiser during gastrulation, is indeed important for completion of anterior neural pattern (Perach and Kessel, 1997; Foley et al., 1997; Dale et al., 1997). In bird gastrulation, unlike in the mouse, axial mesoderm and definitive endoderm appear to form defined layers as they first emerge from the node (Sanders et al., 1978; Stern and Ireland, 1981). This process of definitive endoderm emigration in bird gastrulation has been considered by most investigators to begin prior to the formation of axial mesoderm, such that in the region anterior to the prechordal mesoderm, definitive endoderm that will eventually line the anterior foregut directly underlies anterior neural and non-neural ectoderm. At these stages (4+ to 5; Hamburger and Hamilton, 1951), we find that chick endoderm is cleanly separable as an intact epithelium-like layer, without disturbing the integrity of the prechordal mesoderm. However, the endoderm is appreciably thickened just anterior to the limit of the prechordal mesoderm, so that after endoderm removal the exposed prechordal mesoderm appears narrower in plan view than the transilluminated intact structure. By stage 6, after about only 2 hours of further development, this clean separation is impossible to make without either disrupting the anterior-most prechordal mesoderm structure, or leaving in place cells that appear integrated with it, so that small gaps are created in the removed cell layer. Seifert et al. (Seifert et al., 1993) similarly describe the later chick 'prechordal plate', a region where definitive endoderm and mesoderm layers cannot be resolved that lies just ahead of most prechordal mesoderm. Dil evidence (not shown) indicates that a minority of cells from the lowest epithelium-like layer beneath Hensen's node at stage 4 may become intercalated into the central part of the prechordal plate by stage 6. Thus the clearly epithelial, early lower layer is substantially of definitive endodermal fate, but may include some prospective (though not necessarily prespecified) prechordal mesodermal cells.

To investigate whether developmental information for neural patterning is carried in the chick anterior definitive endoderm, we have studied the results of removing it at stages between 4, the full length streak just before node regression begins, and 6, the head process just before headfold initiation.

During headfold stages 7 or 8, during which anterior neural patterning is believed to become stabilised, we have examined expression of various genes of known or putative signalling function, both in exposed axial mesoderm and in relation to the region of lower layer that is removed.

We discuss the results in terms of current ideas about the roles of the genes mentioned, and about steps in vertebrate neural induction and anterior patterning.

**MATERIALS AND METHODS**

**Embryo culture and operations**

Operations were performed in modified New (New, 1955) ring culture, where blastoderm are developing inverted so that the lower layer (endoderm) is accessible. Cultures were set up at stages 4-5 and brought to a temporary condition with the vitelline membranes stretched on the rings but slightly convex from pressure of albumen medium from beneath, and the blastoderm just covered with a 1:1 mixture of Liebovitz air-buffered TCM (GIBCO/BRL): Hanks BSS (buffered salt solution; made up with 1/10 the normal concentration, i.e. 0.1 mM, of Ca²⁺ and Mg²⁺). Such preparations were incubated (38°C) for a further 2 or more hours, whereupon development continues. Following addition of a little more TCM:BSS, these warmed embryos were in the condition most suited for selective removal of the lower layer from an anterior sector that includes Hensen's node and extends to the periphery of the area opaca. In a smaller sample of embryos, this layer and also the entire emerged axial and prechordal mesoderm were removed. Fig. 1A-C show diagrammatically the versions of the lower layer operation performed, indicating the extents of emerged head process, while D-F show, in sagittal section, the cellular anatomy of the head process stages. Following operations, the space above the membrane is drained and the medium beneath replenished to give slight convexity for onward culture. Too much convexity encourages incomplete dorsal closure, especially in neural tubes of reduced pattern, in embryos without foregut tunnels. Following heterotopic lower layer replacements however, convexity and thorough draining of fluid was absolutely required for good contact and healing of the flattened graft.

**In situ hybridisation and histological procedures**

Operated and synchronous control embryos were inspected, and fixed either at later head process and headfold stages (up to 8 hours further culture), or at the 10- to 15-somite stages (18-24 hours further culture). Fixation, after washing in BSS, was either in 4% paraformaldehyde in PBS for whole-mount in situ hybridisation (Nieto et al., 1995), or in 75% ethanol: 23% formaldehyde (36%w/v): 2% acetic acid, for embedding in Fibrowax, sectioning at 7 μm and staining with iron Haematoxylin/Eosin. Gene expression signal was examined and photographed in whole mount, and after vibratome sectioning at 50 μm.

**Dil labelling**

Dil (Molecular Probes) from a 0.5% stock ethanolic solution was diluted 1:10 with 0.3 M sucrose. With the embryo stretched in culture (see Embryo Culture), small groups of superficial cells were labelled by ejecting pulses of dye from a micropipette tip of approx. 10 μm diameter, onto the surface of the cells. A Picospitzter II (General Valve Corporation, USA) delivering a square pulse of air pressure was used, with empirical adjustment of the pressure head (approx. 10 psi) and pulse length (approx. 50 milliseconds) to give suitable sized labelled cell groups. Following further development for various time periods, embryos were fixed in 4% paraformaldehyde and examined in a Zeiss Axioptoph microscope under phase and epifluorescence optics.

**RESULTS**

**Region of endoderm removed**

Fig. 1 shows the lower layer region removed. This includes the most anterolateral definitive endoderm, which emits inductive signals inducing cardiac development in mesoderm (Schulteiss et al., 1995). The bilateral precardiac mesoderms ultimately migrate across this region, with both layers undergoing rostrocaudal sequence inversion and convergent extension, as the foregut and associated heart tube form. We have not mapped in detail any relative movements between endo- and mesodermal layers within head process during its emergence, as has been done for axial mesoderm and
neurectoderm (Dale et al., 1999). However, DiI fate mapping in normal development (not shown) indicates that endoderm may continue to slide forward and outward relative to other layers as the head process advances (see also Bellairs, 1953a; Bellairs, 1953b). The removal operation is most safely carried out without puncturing ectoderm if resection of the lower layer extends right to the anterior periphery of the area pellucida as shown. However, removals that are confined to the prospective area that will lie around and ahead of prechordal mesoderm at headfold stages, i.e. to the limit defined by dashed line in Fig. 1B (8 cases), give equivalent results. This indicates that the forebrain patterning signals we are studying reside there rather than more peripherally, which maps to extra-embryonic definitive endoderm + displaced hypoblast.

Anterior neural pattern defects depend upon stage of host at operation

Following stage 4 operations, the window in lower layer quickly compresses anteriorly and closes up, so that all axial mesoderm emerging from the regressing node is covered by lower layer as normal (Fig. 2A-D). DiI labelling of the node region immediately after endoderm removal at stage 4, shows that the window is filled in by additional cells ingressing through the node and moving anteriorly (Fig. 2H-K). This new lower layer forms a normal substratum for cardiac induction and migration, and forms normal foregut. Thus, it either is, or has reguatively acquired the properties of, the endoderm that would normally have underlain the head process. We have been unable to ascertain to what extent this result occurs because node cells are normally still emigrating to form definitive foregut endoderm at this stage, or because a regulatory extension of this emigration takes place in response to endoderm removal.

Following later endoderm removals (stage 4+ onwards), the window of endoderm does not close up. Therefore all head process mesoderm that has left the node prior to the operation remains permanently uncovered (Fig. 2E-G). Middle layer tissue denuded of the endodermal epithelium can sometimes collapse its intercellular spaces, superficially re-assuming an epithelial appearance. This can be seen in the hours following operation (Fig. 6E,F), and at more advanced stages (Fig. 3B,C), but is quite distinct from genuine re-covering by endodermal lateral migration. Performance of the operation at head process stages 4+ or 5 leads to loss of forebrain pattern in the majority of embryos (Fig. 1B,H). Since, despite Tables of Normal Stages, development is continuous, the correlation between embryonic stage recorded at operation and the anatomical outcome is imperfect. However, this correlation is highly significant. When performed at stage 4, the operation usually leads to normal later development (Fig. 1A,G; 166/221 essentially normal cases). From operations performed at stages 4+ and 5, there were only 64/235 normal cases. In addition to the forebrain patterning defect, the normally induced heart tissue is unable to migrate to the midline, and differentiates as two separated lateral halves. Presumably due to lack of the mechanical influence of foregut tunnel formation, the neuraxis remains stretched out in a line rather than ventrally flexed and raised from the general blastoderm.

Following similar endoderm removal operations attempted at even later stages, from 5+ and especially at stage 6, the final
forebrain pattern tends once again towards being complete, although neuraxaxes are nevertheless unfamiliar in appearance in the continued absence of ventral flexure, foregut formation and heart migration (Fig. 1C,1). At the latter stage, it is not possible (either surgically or in light microscopy, see Fig. 1F) clearly to resolve an epithelial covering from prechordal mesoderm at its anterior extreme. Thus, lower layer at these stages can never be removed entire, without gaps corresponding to cells that have been left behind. (We do, however, make every effort to remove these remaining cells as well.) Results of these later operations are more variable, with the anteriormost gene expressions variably restored as described below, and small or normal sized optic vesicles appearing anterolaterally. The period during which removal of endoderm alone can be performed successfully, and cause defects in subsequent forebrain regionalisation, is therefore essentially restricted to stages 4+ and 5.

Fig. 3 shows sagittal and anterior transverse sectional views, at the 12- to 15-somite stages, of the structure of control embryos (stage 4 operated, or with peripheral cutting but no resection of endoderm layer), in comparison with typical pattern-reduced experimental ones. Examples are also shown of experimental embryos that nevertheless have normally regionalised forebrains, following ‘late’ attempts at endoderm removal. Following the effectively timed endoderm removals, the dorsal forebrain territory remains as a simple-looking vesicle, rather than becoming subdivided into anterior telencephalon, and diencephalon associated with lateral optic rudiments, However, this vesicle is bulbous, rather than the distinctive, narrow proboscis-like shape (minimal growth) that is associated with loss of Sonic hedgehog (Shh) gene function in both mouse (Chiang et al., 1996) and chick (Towers et al., 1999). In addition, the early morphological appearance of the infundibular region remains as a keel-shaped depression, in the ventral midline of the neural cross-section associated with the anteriormost axial mesoderm (Fig. 3K,L). This neural ventral midline morphology is suppressed following disruption of Shh function as such (Chiang et al., 1996; Towers et al., 1999). Interestingly, following late and only partially effective endoderm removals, the small eye cups that develop are situated wide apart at lateral extremes of the expanded brain vesicle (Fig. 3F), whereas the small eye cups of threshold Shh deficiency are very ‘ventral’ and close together near the midline of the narrowed forebrain, giving a morphological series from cyclopia to semi-synopthalmia (Incardona et al., 1998; Towers et al., 1999).

**Loss of forebrain-specific gene expressions following endoderm removal**

Forebrain pattern was further assessed at the 12- to 15-somite stage by in situ hybridisation for expressions of anterior neural marker genes. Fig. 4 shows typical results. The telencephalic region, although still small at the 12-somite stage, will ultimately undergo the greatest growth of all brain parts. Its
Foregut endoderm requirement in neural patterning

absence in our experimental embryos was demonstrated by the use of several marker genes. **BF1** (Alvarez et al., 1998) a winged helix transcription factor gene with normal early expression restricted to telencephalon and anteromedial eyecup regions (Fig. 4E), and essential for their development in mice (Dou et al., 1999), is not expressed (4F, 8/15 cases) or shows small, asymmetrical remnants of expression (5/15). **GANF**, the chick homologue of the mouse homeobox gene **Hesx1**, is also normally expressed in the telencephalon at the 10- to 12-somite stage, but with a ventral extension beyond the **BF1** domain and back to prospective infundibular level (Fig. 4I-J). The pattern-reduced brains usually show no neural **GANF** expression (4K, 16/25 cases), or only a small ventral patch remains. Forebrain expression of **FGF8** is also specifically lost (12/16 cases), while all other aspects of its expression remain normal (Fig. 4G,H). The retained **FGF8** expression marking the isthmus (midbrain-hindbrain boundary) at the rear of two simple vesicles, together with the homogeneous dorsal **Pax-6** expression throughout the more anterior of these vesicles (Fig. 4D, 11 cases), strongly suggests a general prosencephalic identity for the latter (Pera and Kessel, 1997). However, subregionalisation of this vesicle does not occur, since the absence in our experimental embryos was demonstrated by the use of several marker genes. **BF1** (Alvarez et al., 1998) a winged helix transcription factor gene with normal early expression restricted to telencephalon and anteromedial eyecup regions (Fig. 4E), and essential for their development in mice (Dou et al., 1999), is not expressed (4F, 8/15 cases) or shows small, asymmetrical remnants of expression (5/15). **GANF**, the chick homologue of the mouse homeobox gene **Hesx1**, is also normally expressed in the telencephalon at the 10- to 12-somite stage, but with a ventral extension beyond the **BF1** domain and back to prospective infundibular level (Fig. 4I-J). The pattern-reduced brains usually show no neural **GANF** expression (4K, 16/25 cases), or only a small ventral patch remains. Forebrain expression of **FGF8** is also specifically lost (12/16 cases), while all other aspects of its expression remain normal (Fig. 4G,H). The retained **FGF8** expression marking the isthmus (midbrain-hindbrain boundary) at the rear of two simple vesicles, together with the homogeneous dorsal **Pax-6** expression throughout the more anterior of these vesicles (Fig. 4D, 11 cases), strongly suggests a general prosencephalic identity for the latter (Pera and Kessel, 1997). However, subregionalisation of this vesicle does not occur, since the
normal down-regulation of Pax-6 expression in the dorsoanterior midline (Fig. 4C) is not seen. Pax-6 expression is not expanded ventrally, consistent with the presence of normal inhibitory signals from ventral midline.

Following endoderm removal at stage 5+ to 6, where brain pattern is often complete but with eye vesicles extending anterolaterally due to the lack of foregut tunnel, brain-associated BF1 and GANF expressions can be normal in extent. The transitional result is a brain with very small optic vesicles protruding anteriorly from the lateral edges of the forebrain 'vesicle' (Fig. 3F). Such brains show small or partial expression patches of BF1 corresponding to partial restoration of dorsal telencephalic territory.

**Early patterning of the anterior neural plate after endoderm removal**

Otx2 is expressed throughout the forebrain and midbrain from very early stages (Bally-Cuif et al., 1995), and is essential for their development. In the experimental pattern-reduced brains at the 12-somite stage, the rostrocaudal extent of tissue expressing otx2 appears normal, although its architecture is simplified into two vesicles (Fig. 4A,B). Comparison of experimental FGF8 and Pax-6 expressions helps identify these two vesicles as prosencephalon and midbrain, and confirms the lack of subdivision in the former. Such specimens do not suggest a reduced initial allocation of ectoderm to neural plate anteriorly, or even a reduced allocation within the initial neural plate to forebrain territory. We nevertheless investigated more directly whether, following the endoderm removals at stage 4+ or 5, the overall extent of the neural plate might have been reduced because of failure of an anterior part of it to retain stable neural fate. Use of the pan-neural marker Sox3 at neural plate stages, 6-8 hours after operations, confirmed that in these experimental embryos the extent of the early neural rudiment remains normal (not shown, 4 cases). Neural plate otx2 expression at the same stages was also of normal relative extent (4 cases). In addition, early expression of GANF in the anterior neural plate, first seen at stages coinciding with those of effective endoderm removals, remains near-normal at headfold stages following such operations (9/11 cases normal, 2/11 reduced), even though it is later lost. Therefore a distinctive anterior neural territory is initiated as normal, but later subregionalisation within this territory is either not induced or not maintained.

Telencephalic expression of BF1 is not initiated until later, the 7- to 8-somite stage, in chick. In mouse, early FGF8 expression in the anterior neural ridge (ANR) has been implicated in induction and/or maintenance of BF1 (Shimamura and Rubenstein, 1997). We therefore examined FGF8 expression at the 5- to 6-somite stage, its first reliable ANR-associated appearance in normal chick, both in sham operated controls and following foregut endoderm removals expected to lead to anterior pattern loss. A striking deficit in initial activation of this gene was indeed associated with the removal of foregut endoderm (13/22 cases without expression, 3/22 cases with reduced expression), suggesting that the effects on neuraxial (and stomodeal) pattern might result, at least in part, from inadequate early expression of FGF8 signal in the ANR.

**Autonomous specification of stomodeal ectoderm**

Following endoderm removal too late to result in forebrain pattern reduction, a significant proportion of the axes display an unusual pipe-like tube of ectodermal-type epithelium stretching ahead of the brain. This tube extends in from the

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**Fig. 4.** Forebrain gene expressions at the 12-somite stage after definitive endoderm removal. Successive gene expressions are shown in whole-mount pairs from the dorsal aspect, with the control appearance on the left, and the pattern-reduced brain on the right (stage 4+ or 5 operation). For GANF, the normal dorsal, then ventral appearances are shown. (A,B) Otx2. In the experimental case, expression occurs in the anterior two simple brain vesicles. (C,D) Pax6. In experimental case, the expression occurs in the anteriormost simple brain vesicle, and in non-neural ectoderm that would in the normal case be applied to optic vesicles. (E,F) BF1. Experimental case lacks normal anterior neural expression. (G,H) FGF8. Experimental case lacks the normal forebrain-associated expression, but retains isthmic (mid-hindbrain junctional) expression. Comparison of FGF (H) with Otx2 (B) and Pax6 (D) helps diagnose the nature of the two simple brain vesicles as prosencephalon and midbrain, even though the former lacks subdivision. (I-K) GANF. In the experimental case, normal expression associated with forebrain folds and with more ventral facial (stomodeal) region is missing. Scale bar, 250 μm.
undersurface of ectoderm some way ahead of the forebrain, to end blindly at or near the infundibular region and its associated dispersed prechordal mesoderm (Figs 3H and 5). Such embryos sectioned at around the 8-somite stage reveal that this tube is already developed. GANF and Shh are both expressed in this tube, as they are in normal stomodeal ectoderm (compare Fig. 5A,E with B,F), while the ectodermal pattern of FGF8 expression near its base also strongly suggests its identity as stomodeum (Fig. 5C,D). It therefore appears that the stomodeum originates from its proper fate-map location in anterior ectoderm, but is unable to achieve its normal ventral position due to lack of foregut tunnel and proper face formation. This ectodermal region, once specified as stomodeum, apparently actively seeks cellular association with the other components of the infundibular brain region, necessary for subsequent development of its normal derivatives (Rathke’s pouch and anterior pituitary). In these embryos that lack ventral flexure and face formation, because anteriormost foregut endoderm was removed after having induced the stomodeal region, this association behaviour leads to the formation of the abnormal tube.

A further point of interest is that after later (stage 6) removals of prechordal and anterior chordamesoderm as well as the endoderm, embryos that lack dorsal forebrain regionalisation similar to that after stage 4+5 endoderm removals (see later subsection) may nevertheless display this anterior ‘pipe’ of stomodeal ectoderm leading to the infundibular region (Fig. 3M compared with H). Therefore, although after endoderm removal, stomodeum is only specified when forebrain regionalisation has also occurred due to the lateness of that removal, specification of stomodeum does not require intact anterior forebrain pattern itself.

Midline signalling gene expressions in axial mesoderm are unaffected by endoderm removal

Shh and chordin are intercellular signal genes, expressed throughout much of the axial mesoderm and the overlying neural midline in the chick head process. We examined both Shh and chordin expressions in axial head process mesoderm of appropriate endoderm-excised embryos, at both headfold stages 7-8 HH (2-4 somites; 9 cases each gene) and the 12 somites stage (Shh, 10 cases). There were no detectable deficits in expression of either gene (Fig. 6A-D). BMP7 expression in stage 7-8 chick prechordal mesendoderm has been implicated experimentally in conferring anterior character to ventral neural midline (Dale et al., 1997; Dale et al., 1999; Vesque et al., 2000). We therefore similarly examined this aspect of BMP7 expression in experimental embryos. Again, no appreciable deficit in prechordal mesodermal expression was apparent (Fig. 6E,F; 10/11 cases).

Significance of normal gene expressions in the removed endoderm region

The homeobox gene Hex has expression in pregastrulation mouse AVE, the extra-embryonic region that is necessary for complete anteriormost axial patterning (see Introduction). However, it has recently been demonstrated that the essential requirement for Hex in forebrain patterning resides in its later site of expression, the definitive embryonic endoderm (Martinez-Barbera et al., 2000). In chick, as well as expression in the extra-embryonic lower layer, there is also a prominent patch of Hex expression in anterior midline foregut endoderm, centrally within the removed region in our experiments (Fig. 6G and Yatskievych et al., 1999). We believe that normal chick Hex expression extends to cells of the prechordal mesoderm by later headfold stages, although this has been difficult to detect decisively on sagittal sections (see also Yatskievych et al., 1999). Conditions of lower layer removal that lead to anterior neural pattern reduction, by definition, result in absence of the prominent endodermal Hex expression underlying the prechordal mesoderm and anterior neural ridge around stage 7 (Fig. 6H; 10 cases). Furthermore, the complete absence of prechordal mesodermal expression in 8/10 of these whole-mount specimens suggests that any
normal middle layer Hex expression, whether imported by cell intercalation or induced due to presence of underlying endoderm, has also been prevented.

Chick Crescent encodes a putative secreted protein of the Frizbee group (Pfeffer et al., 1997), having the structure of the Frizzled receptor proteins for the Wnt signal pathway but lacking their intracellular signal transducing domain. Crescent is expressed in prestreak extra-embryonic lower layer, the hypoblast, and then strongly in the axial part of the early head process endoderm. We find a broad, intense expression domain in anterior definitive endoderm, centred on, but considerably more widespread than, the site of Hex expression. Seen in plan view, the area of Crescent expression at headfold stage corresponds rather closely with the lower layer area whose removal during stages 4+ or 5 distinctively reduces subsequent neural pattern (Fig. 6I). We find indeed that when removals are carried out at stage 4, normal endodermal Crescent expression is already recovered by headfold stages, as the gap in the endoderm layer is filled in and shrinks anteriorly. Following the stage 4+ and 5 operations where the window underlies the axial mesoderm, the massive lower layer expression is absent at this position as would be expected, and only the localised axial mesodermal expression remains (Fig. 6J; 8 cases).

**Foregut endoderm provides instructive signals and not just a maintenance environment, to head process**

We performed experiments to address the issue of positional distinctiveness in signals from anterior definitive endoderm. In one approach (Fig. 7A), embryos with this layer freshly removed in the stage 4+ or 5 operation were grafted with large heterotopic pieces of correctly orientated lower layer, either from the prenodal sector of stage 4 donors (i.e. with hypoblast and presumptive extra-embryonic endoderm that would be displaced ahead of head process endoderm; 8 cases), or from the perinodal and posterior head process level of stage 6 or 6+ donors (i.e. with more posterior presumptive foregut endoderm; 6 cases). Pieces were flattened to overlie an area centred on the exposed prechordal mesoderm of hosts. The difficulties of this operation meant that the exposed ‘window’ of the hosts was never entirely re-covered by lower layer, and many cases were rejected from analysis because the layer contact achieved did not sufficiently resemble that in normal development. Nevertheless, in no case did the graft appear to have rescued the anterior brain patterning deficiency as seen at the 12-somite stage. There was also little evidence for restoration of the ability of precardiac tissue to migrate across the re-covered anterior headfold area, or of foregut tunnel formation. In a second approach (Fig. 7B), endoderm from over and just ahead of the stage 5 prechordal plate (i.e. centrally positioned within the normal removed region), was excised and re-inserted heterotopically, between endoderm and presumptive mid- or hindbrain neural plate at one side of the regressing node of the same embryo. At headfold stages following a further 6-8 hours in culture, significant asymmetry of GANF expression was observed, biased to the grafted side (compare Fig. 7C with F; 8/15 cases). Four cases had separate areas of altered neural fold appearance with ectopic GANF expression in contact with the graft, well posterior to the gene’s normal expression site (Fig. 7D,E). Similar lower layer pieces from more lateral and peripheral positions caused no disturbance of either morphology or GANF expression (6 cases).
The relative roles of endoderm and of axial mesoderm

In our hands, extirpation during stage 5 of the entire emerged head process mesoderm, along with the definitive endoderm layer already described, results in embryos whose anterior CNS structure appears closely to resemble that resulting from lower layer removal alone. An indistinguishable result also follows later (stage 6) removals that include just this anterior chordamesoderm along with the endoderm (compare Fig. 3M,N with J-L). We have noted incidentally that these defects following mesoderm + endoderm removals, like those following the early removal of endoderm alone, differ morphologically from the defect resulting from Sonic hedgehog (Shh) gene disruption as such. Despite their loss of dorsal-anterior brain regionalisation, the embryos presented here retain a relatively expanded cross-section and pronounced pre-infundibular midline morphology (see earlier subsection). We thus see little to suggest a greater loss of early CNS midline specification or growth patterning, both believed to be due to Shh signal, when the mesoderm extirpation is added to that of endoderm (though see Pera and Kessel, 1997). By stage 6, as already described, complete selective removal of endoderm alone is not possible from over the prechordal region, and/or is ineffective in surrounding regions, since completely regionalised neural patterns develop after attempts at this operation (sagittal sections of 3E,F and anterior transverse of 3H).

DISCUSSION

We present evidence for a role of the chick definitive foregut endoderm in regionalisation of the forebrain. Following removal of this tissue as a complete epithelium-like layer at stages 4+ and 5, a simple-shaped but expanded prosencephalic vesicle forms, which lacks expression of telencephalic markers BF1, GANF and FGF8. This pattern deficit is not due to an overall reduction in the neurally induced area, which is believed to be already defined by stage 4 or 4+ in normal development (reviewed by Smith and Schoenwolf, 1998).

Indeed, anterior neural GANF expression is already detectable in normal embryos at the time of these operations, and is still seen at headfold stages following stage 4+ to 5 endoderm removal. The deficit is rather in the failure to maintain or refine subregionalisation within the prosencephalic territory, and this could be at least partly due to failure of early FGF8 expression in the ANR region. We have shown that the definitive endoderm emits specific signals, since it can induce ectopic GANF expression in presumptive hindbrain territory when transplanted. We also propose that the foregut endoderm is able directly to induce stomodeum in ectoderm at an anteriormost position, before the ventral flexure of head formation is due to bring the latter into contact with ventral diencephalon and the foregut tunnel.

Separation of dorsal-anterior and neural ventral midline patterning

At least following successful endoderm removal alone, the ‘information’ that is deleted in our experimental embryos is not that for neural ventral midline specification itself, via maintenance of Shh or chordin expressions. We observe considerable retention of early midline morphology, and Pax6 expression does not encroach abnormally upon the ventral midline, as occurs in midline-deficient embryos. Even the distinctive anteriorisation of midline character to give Rostral Diencephalic Ventral Midline (RDVM; Dale et al., 1997; Dale et al., 1999) may be unaffected, since prechordal mesodermal BMP7 expression at headfold stages appears unaffected by endoderm removal at stage 4+ to 5. Instead, there is a lack of one or more other signals that operate in combination with midline patterning, to achieve normal regionalisation of more dorsal forebrain territories, so that territories that would respond to midline-derived (Shh and other) signals by the distinctive growth programmes e.g. of optic vesicle formation,
have not been specified. More than Shh mutants, the present experimental embryos resemble other mouse mutants (Shawlot and Behringer, 1997; Andreazzoli et al., 1999; Dattani et al., 1998; Martinez-Barbera et al., 2000).

**Transfer of information between embryonic layers: the role of Hex**

Most recent work has assumed that neural-inducing signals present in vertebrates, as distinct from signals for regionalisation, come from the classical gastrular organiser, the node-equivalent or its precursors, principally by planar but perhaps by some later ‘vertical’ transfer. By head process stages, anterior neural regionalising signals in vivo would be expected to act only by locally modulating this already established state of general neural induction. While such signalling could in principle occur by further planar transfer along the early neural plate, underlying mesoendoderm is now believed to exert major influences. Correspondingly, ‘deep’ (thus mesendodermal) tissue, emerged from the newly regressing chick node, is reported to be a poor neural inducer, while being an effective neural anterioriser (Foley et al., 1997).

In chick, endoderm and axial mesoderm layers of the head process are clearly distinct both as tissue leaves the node and in the head process through stage 5. In mouse, axial mesoderm and definitive endoderm emerge from the node as a common head process population, only later separating, so that details of information transfer in the head process may differ. Complete anterior development in mouse has been shown to require a pregastrulation, extra-embryonic signalling component (Rhinn et al., 1998; reviewed by Beddington and Robertson, 1999). So far, the evidence is that this necessary component is not included in chick development (Knoetgen et al., 1999; S. Withington and J. Cooke, unpublished data). However, there appears also to be a subsequent necessary transfer of anteriorising information to the CNS from mouse definitive endoderm (Martinez-Barbera et al., 2000) and mesoderm (Ang et al., 1994; Shawlot et al., 1999) in the head process stage. We have shown here that the equivalent endoderm tissue in chick plays a similar anterior regionalising role. A further question concerns whether patterning information from endoderm acts directly on the ectoderm layer, or by modulating or stabilising the subsequent signalling properties of head process mesoderm (see also Vesque et al., 2000). By stage 6, there may have been incorporation of cells from the definitive endoderm into mesoderm at the prechordal region, where these components become indistinct (Seifert et al., 1993). Any crucial information transfer from lower layer into mesoderm could thus, in principle, have occurred through such cell intercalation as well as by influence of secreted molecules. During stage 6, dorsal-anterior forebrain patterning appears to have become stable in the presence of mesoderm alone, though not yet stable if anterior head process mesoderm is also removed.

Our results overall are consistent with either of two models. In a successive transfer model, ‘information’ influencing even the dorsal-lateral forebrain pattern that originates in definitive endoderm, would be first transmitted as an influence on (or even cellular incorporation into) head process mesoderm. The mesodermal inductive properties thus supported would then take some further time, to complete their own influence on ectoderm.

An alternative model is that the information our experiments reveal is transferred directly from endoderm to ectoderm, in parallel with any information from mesoderm that itself may proceed via midline patterning or independently of it. The presumptive anterior and lateral neural ridges at early stages, and the presumptive stomodeal ectodermal area ahead of them, lie outside the extent of head process mesoderm and are directly underlain by that region of foregut endoderm that displays, for instance, strong Crescent expression (see below). FGF8 expression in this ectodermal area is known to be important in organising its normal derivatives (Shimamura and Rubenstein, 1997; see also Martinez-Barbera et al., 2000). In this model, although there is a quite clear endodermal epithelial structure across the early head process midline, its removal there would be irrelevant to our results. Only in presumptive lateral-dorsal regions of neural plate would it be providing distinctive and direct information. We have been unable to make sufficiently controlled subregional removals to test this.

Of relevance to an ultimate discrimination between these two models, is the observation that the chick Hex expression is very circumscribed (Fig. 6G), underlying prechordal plate in midline endoderm and perhaps later invading it by an inductive process or by cell transfer. Our endoderm removals clearly ablate this Hex expression, and the dorsal anterior patterning deficits we see closely resemble the phenotype in mouse following ablation of this gene’s head process endodermal expression (Martinez-Barbera et al., 2000). Hex is thus likely to be in the pathway that supports the inter-layer signals, from endoderm to overlying structures, revealed by our experiments.

The developmental signals revealed in the present experiments would appear to be separate from those controlling CNS cross-sectional pattern in relation to the midline. We stress that our incidental morphological observations, that in the anterior region at least, the early signs of CNS midline patterning and growth appear immune to the removal of axial mesoderm, should be expanded in a separate study by use of suitable gene markers. Following our mesoderm extirpations, the rostral diencephalic midline or pre-infundibular region may already have been exposed to significant extrinsic midline Shh and chordin signals (see also Gunhaga et al., 2000). Moreover, intrinsic neural midline expression of these genes parallels or even precedes their mesodermal expressions in this region, and remains normal for hours following the mesoderm extirpation (S. Withington and J. Cooke, unpublished observations; see also Le Douarin and Halpern, 2000). However Pera and Kessel (1997), following presumed removals of both mesoderm and endoderm layers in prechordal region, observed synphalalia and loss of ventral neural gene expressions, and suggested that midline patterning remains immediately dependent upon axial mesodermal signals at these stages and positions, just as for later-developing more posterior ones (Placzek et al., 2000). If this is the case, then despite their similar appearance, our forebrain pattern losses following stage 6 mesoderm removals may not really be the equivalents of those following earlier loss of endoderm only. This would make harder the elucidation of any role for mesoderm in transfer of endodermal information for forebrain pattern (model 1 above), that is separate from its own midline patterning role.

**Positional specificity within the endoderm**

The expressions of several genes such as Shh, chordin and
anteriorly, *BMP7* are left intact in axial mesoderm for many hours following definitive endoderm removals, despite the abnormally exposed development. This suggests that anterior endoderm provides distinctive signals or conditions, and not just a more general, protective or maintenance environment for head process mesoderm. Positionally distinctive gene expressions in the definitive endoderm, such as *Hex* and *Crescent*, reinforce this view. We have also shown positional specificity within the endoderm; endoderm from just anterior to the prechordal mesoderm can induce ectopic GANF expression in presumptive hindbrain territory, while more peripheral and lateral endoderm cannot. The majority of operations removed the entire lower layer sector to the anterior limit of the area pellucida, but the similar effects of some more restricted removals from around and just ahead of prechordal mesoderm (see Fig. 1) again suggest that the patterning signals we are studying lie there rather than more peripherally. It has been difficult to find suitable other lower layer regions, from synchronous donor embryos, and to graft these successfully in place of anterior definitive endoderm in order to demonstrate the spatial specificity of its role. Most peripheral regions, known to have strong expression of ventralising BMP genes for instance, may superimpose their own actively distorting signals rather than revealing the effect of a ‘neutral’ epithelial covering for head process mesoderm. However, we have failed to observe any capacity of lower layer from ahead of the node at stage 4, or of stage 6 perinodal/posterior head process endoderm, to substitute for anterior foregut endoderm itself in rescuing either forebrain pattern or heart migration.

**Crescent expression in foregut endoderm**

*Crescent*, a chick homologue of the *Frizbee* group of genes, is strongly and distinctively expressed in the removed area of anterior foregut endoderm (Pfeffer et al., 1997). Frizbee proteins are candidates for secreted signals that may act by modulating or antagonising Wnt signal pathways, through competitive sequestration of the ligands. Anteriorly expressed genes of this group, which might locally antagonise Wnt signals in establishing the conditions for head determination, have been found in *Xenopus* as well as mouse, and have the appropriate properties on experimental ectopic expression in *Xenopus*. It is suggested that antagonism of a Wnt role, in spatial conjunction with antagonism of certain BMP and perhaps of Nodal signalling roles, provides the conditions for determination of anteriormost territories, and additional genes encoding proteins that exhibit more than one of these antagonist functions have indeed been characterised from various vertebrates (Leysn et al., 1997; Wang et al., 1997; Shawlot et al., 1998; Glinka et al., 1998; Piccolo et al., 1999; Pearce et al., 1999; Hoang et al., 1998). Genetic analysis is at an early stage (e.g. Simpson et al., 1999; Belo et al., 2000) but such genes of *Frizbee* and other families are quite numerous, and co-expressed, so may act redundantly in supporting this normal developmental step. After lower layer removals so timed as to result in later pattern truncation, the domain of *Crescent* expression is not restored, even though earlier removals followed by re-emigration of lower layer do restore it. Thus, while we do not claim that *Crescent* itself provides a unique endoderm-derived anteriorising signal that our embryological results reveal, we suggest that Crescent protein and others functionally parallel with it are candidates for such signals. A little-noted but relevant observation is that following early lithium treatment in *Xenopus* embryos, now thought to distort axial body pattern by global and sustained over-activation of the canonical Wnt pathway, a small but distinctive anteriormost sector of neural pattern is deleted as well as the larger and more widely recognised trunk-tail sector (Cook and Smith, 1988).

**Induction of stomodeal ectoderm by foregut endoderm**

It is of interest that a distinct region, lying at the conventionally defined anterior limit of neural induction, becomes predetermined as stomodeum. Acquisition of this stomodeal character is revealed by the relationships of *GANF, Shh* and *FGF8* expressions (Fig. 5), and by the capacity of this epithelium actively to seek interaction with its normally associated structures at the base of the brain. In the absence of face formation this produces a deep tube or ‘pipe’, extending back from beneath the anterior ectoderm to the ventrally exposed hypophyseal region (Fig. 3H,M). This stomodeal structure is specified if anterior endoderm has been in place long enough to support normal patterning of the forebrain itself, i.e. after lower layer removals delayed until stage 6. However, it is also produced after stage 6 axial mesoderm-plus-endoderm removals, which results in forebrains lacking telencephalic structure. This suggests that, rather than by planar ectodermal signalling, which depends on prior complete forebrain specification, stomodeum is specified by direct signals from an anteriormost position in definitive endoderm, where this layer directly contacts ectoderm.

We thank the following for gene probes; Anthony Graham for chordin, Ivor Mason and Anthony Graham for FGF8, Henk Roelink for Shh, Paul Thomas for cHex, Juan-Carlos Izpisua-Belmonte for Crescent, Philippa Francis-West for BMP7, Angela Nieto for BF1, Michael Kessel for GANF, Massimo Gulisano for Otx2, Fabienne Pituello for Pax6. We thank Juan Pedro Martinez-Barbera for instructive discussion and access to unpublished work, and Photo- Graphics section, NIMR, for invaluable help with figures.

**REFERENCES**


