Determination of the zebrafish forebrain: induction and patterning

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SUMMARY

We report an analysis of forebrain determination and patterning in the zebrafish Danio rerio. In order to study these events, we isolated zebrafish homologs of two neural markers, odd-paired-like (opl), which encodes a zinc finger protein, and fkh5, which encodes a forkhead domain protein. At mid-gastrula, expression of these genes defines a very early pattern in the presumptive neurectoderm, with opl later expressed in the telencephalon, and fkh5 in the diencephalon and more posterior neurectoderm. Using in vitro explant assays, we show that forebrain induction has occurred even earlier, by the onset of gastrulation (shield stage). Signaling from the early gastrula shield, previously shown to be an organizing center, is sufficient for activation of opl expression in vitro. In order to determine whether the organizer is required for opl regulation, we removed from late blastula stage embryos either the presumptive prechordal plate, marked by goosecoid (gsc) expression, or the entire organizer, marked by chordin (chd) expression. opl was correctly expressed after removal of the presumptive prechordal plate and consistently, opl was correctly expressed in one-eyed pinhead (oep) mutant embryos, where the prechordal plate fails to form. However, after removal of the entire organizer, no opl expression was observed, indicating that this region is crucial for forebrain induction. We further show that continued organizer function is required for forebrain induction, since beads of BMP4, which promotes ventral fates, also prevented opl expression when implanted during gastrulation. Our data show that forebrain specification begins early during gastrulation, and that a wide area of dorsal mesendoderm is required for its patterning.

Key words: Zebrafish, Forebrain, Neural induction, Organizer, odd-paired-like (opl), fkh-5

INTRODUCTION

Explant and transplant studies in amphibian, mouse and chick embryos indicate that neural determination has commenced by mid-gastrula stages (Ang et al., 1994; Holtfreter and Hamburger, 1955; Jones and Woodland, 1989; Roberts et al., 1991; Sive et al., 1989). Analysis using molecular markers is making it clear that, even before mid-gastrula, the presumptive neurectoderm has begun to be set aside (for example, see Kuo et al., 1998; Nakata et al., 1997). It is not clear, however, when the neurectoderm becomes patterned. During gastrulation, tissue initially specified as anterior neural (forebrain) includes at least some of the prospective posterior neurectoderm, suggesting that commitment to posterior neural fates may occur by converting anterior to more posterior tissue (Eyal-Giladi, 1954; Sive et al., 1989; reviewed in Kolm and Sive, 1997).

Despite the prevailing idea that forebrain is determined prior to more posterior neurectoderm, little information exists regarding the earliest patterning decisions within the presumptive forebrain. During neural plate stages, patterned gene expression divides the forebrain primordium into subregions (Bally-Cuif and Boncinelli, 1997; Simeone et al., 1993), while after neural tube closure, constrictions along the neural tube morphologically subdivide the future forebrain into prosomeres. Prosomeres can be grouped into two subregions: the diencephalon posteriorly, and more anteriorly, the secondary prosencephalon including the telencephalon (Puelles and Rubenstein, 1993; Rubenstein et al., 1994). However, it is not known when forebrain patterning begins, what genes divide the forebrain field initially, prior to neural plate formation, or what sources of patterning signals are responsible for this division.

Forebrain tissue can be induced by dorsal mesendoderm, also called the ‘organizer’, that has been found in all vertebrates. Organizing centers include the amphibian organizer (Spemann and Mangold, 1964), chick Hensen’s node (Storey et al., 1992; Waddington, 1932), the mouse node (Beddington, 1994) and the teleost shield (Oppenheimer, 1936; Sagerström et al., 1996; Shih and Fraser, 1996). The organizer can be separated into distinct domains which induce primarily anterior or primarily posterior neural tissue in Xenopus (Gerhart, 1996; Mangold and Spemann, 1927; Zoltewicz and Gerhart, 1997) and in teleosts other then zebrafish (Eakin, 1939; Oppenheimer, 1959). In Xenopus, anterior endoderm is a source of anterior ectodermal inducing signals (Bouwmeester et al., 1996; Bradley et al., 1996) and may be equivalent to the extraembryonic visceral endoderm in mouse that is required for forebrain formation (Tom and Beddington, 1996; Varlet et al., 1997). In zebrafish, a recent report indicates that the
anterior-most row of cells within the forming neural plate contains forebrain patterning signals (Houart et al., 1998).

Several molecules that are able to induce anterior neural tissue in Xenopus have been described (Kolm and Sive, 1997; Sasai and De Robertis, 1997). These include chordin, noggin and follistatin, which act by antagonizing the bone morphogenetic protein (BMP) signaling pathway through direct binding of BMPs (Piccolo et al., 1996; Zimmerman et al., 1996). Other inducers of anterior neural tissue are cerberus (Bouwmeester et al., 1996; Glinka et al., 1997), and dickkopf-1 (Glinka et al., 1998). Both are thought to antagonize the wnt signaling pathway, which is known to posteriorize neural tissue (McGrew et al., 1997). Corroborative data for the role of BMP-antiBMP signaling pathways have been obtained in zebrafish (Fisher et al., 1997; Hammerschmidt et al., 1996; Kishimoto et al., 1997; Schulte-Merker et al., 1997).

We have been studying neural commitment in the zebrafish, an excellent vertebrate paradigm (Driever et al., 1994; Kimmel, 1989; Rossant and Hopkins, 1992), where brain morphogenesis and patterning have been well described (Kimmel, 1993; Macdonald et al., 1994). Using explant assays we showed that zebrafish neurectoderm was not specified by late blastula; however, by early gastrula, the embryonic shield, which is a mixture of ectodermal and mesendodermal cell types, is committed to express both anterior and posterior neural markers (Sagerström et al., 1996). Since the shield is also a source of inducing signals (Sagerström et al., 1996; Shih and Fraser, 1996), it was not clear whether this reflected commitment of the early gastrula neurectoderm or induction of ectoderm during the culture period. Elegant transplant studies have shown that presumptive forebrain is not yet irreversibly committed by early gastrula (Woo and Fraser, 1997), but have not addressed whether initial forebrain induction has taken place at this stage.

In order to study determination and patterning of the zebrafish forebrain, we isolated zebrafish homologs of opl and fkh5, two genes defined as early neural-specific markers by subtractive cloning from Xenopus (Patel and Sive, 1996). We show here that zebrafish opl is expressed in the anterior neural plate which includes the presumptive telencephalon, and fkh5 in the presumptive diencephalon. Using in vitro explant assays (Grinblat et al., 1998; Sagerström et al., 1996) we analysed the timing of forebrain determination and the source of signals that pattern this region. We found that forebrain patterning begins early during gastrulation, and that the entire organizer, but not the prechordal plate, is necessary for forebrain determination in zebrafish.

**MATERIALS AND METHODS**

Isolation and characterization of opl and fkh5 cDNA clones

A cDNA library, prepared from early neurula (9-16 hpf) embryos by R. Rigglemann and D. Grunwald, was screened at low stringency (50°C; Church and Gilbert, 1984) with partial probes from the Xenopus opl and fkh5 cDNA. Complete nucleotide sequence was obtained for 3 zebrafish opl cDNAs (insert sizes 2.33, 2.33 and 1.86 kb) and 2 zebrafish fkh5 cDNAs (insert sizes 2.13 and 2.0 kb). Both contained the open reading frame predicted from other species, and were flanked by untranslated regions (for opl: 241 nt upstream and 693 nt downstream; for fkh5: 235 nt upstream and 1,010 nt downstream). Accession numbers are AF052435 for opl and AF052651 for fkh5.

**Microdissections and explant culture**

Explants were microdissected from embryos produced by natural crosses (Westerfield, 1995) and cultured at 29°C in 1× MBS supplemented with 50 μg/ml gentomycin and chemically defined lipids (0.01x, Gibco-BRL). Details of the dissection were as previously described (Grinblat et al., 1998; Sagerström et al., 1996). Late blastula (sphere stage) animal cap explants were cultured in groups of 10, early gastrula (shield stage) epiblast explants were cultured in groups of 5, and shield explants were cultured singly. Shield explants for conjugates were dissected from embryos lineage labeled with lysine fixable fluorescein dextran (FLDX, 10,000 M_. Molecular Probes). Each FLDX-labeled shield was conjugated to a group of 5 unlabeled animal caps within 30 minutes after dissection.

**Microdissections to remove dorsal margin**

The dorsal margin was identified by rotating embryos at 30-35% epiboly on their sides in the dissection dish until the blastoderm margin appeared clearly thinner on one side than on the opposite side (Schmitz and Campos-Ortega, 1994). With the embryo held in this orientation, a cut was made to mark the location. The embryo was then rotated and additional cuts were made to remove the regions shown in Fig. 7A. One of these cuts punctured the yolk cell and released some of the dorsally located yolk. Mock dissected control embryos were generated by making incisions on three sides, including the incision which punctured the yolk. Equivalent amounts of dorsal yolk were released in mock-dissected embryos. Operated embryos were cultured until unoperated controls reached 90% epiboly stage.

**Bead implantation**

Coated Sepharose beads (Biorad Affi-gel blue, 75-150 μm diameter) were prepared as follows; after rinsing in PBS, 1 μl of the beads were incubated with 5 μl of a solution of BMP4 (5 mg/ml in 0.1% trilfluoroacetic acid), or BSA (10 mg/ml in water) for 1 hour at room temperature. Just prior to implantation, the beads were transferred to a dissection plate filled with culture medium. An incision was made in the dorsal epiblast of a shield stage embryo, leaving the yolk cell intact, and a bead was pushed through the incision toward the animal pole, between the epiblast and the yolk cell. Incisions healed well and epiboly was not significantly affected.

**Relative quantitative RT-PCR analysis**

RNA was isolated and reverse transcribed as previously described by Sagerström et al. (1996). PCR amplification and analysis were carried out as described by Sagerström et al. (1996) for the number of cycles empirically defined as being within the linear range of amplification for individual primer pairs. After electrophoresis, a PhosphorImager gel reader and software (Fuji) was used to quantitate PCR products. PCR primers used to amplify gene-specific products are listed in Sagerström et al. (1996) and Table 1.

<table>
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*This paper; ‡G. Conway and W. Gilbert, personal communication; §Toyama et al., 1995; ¶Miller-Bertolvio et al., 1997.
Whole-mount in situ hybridization

In situ hybridization of whole embryos was carried out according to previously published protocol (Sagerström et al., 1996). The orange precipitate was formed when BCIP (Sigma) and 4-iodonitrotetrazolium violet (INT) (Molecular Probes) were added together at the developing step. The purple precipitate resulted from a combination of NBT (Research Organics) and BCIP.

RESULTS

Zebrafish opl is an early anterior neural marker

In a recent subtractive screening for early neural markers in Xenopus (Kuo et al., 1998; Patel and Sive, 1996), one of the earliest neural-specific genes identified was a gene encoding a zinc finger protein, opl, so called because it resembles Drosophila odd-paired (odd-paired-like). Since few ectoderm-restricted markers of early neural pattern were available in zebrafish, we isolated the zebrafish homolog of opl. Zebrafish opl encodes a 441 amino acid protein, which possesses a zinc finger domain which is highly similar (77%) to that of the Drosophila gene odd paired (opa) (Benedyk, 1994) (Fig. 1). Within vertebrates opl is highly conserved along the entire length of the protein. Zebrafish opl protein is 89% identical both to Xenopus opl (Kuo et al., 1998) and to its probable mouse homolog, Zic1 (Aruga et al., 1994).

Expression of zebrafish opl began at mid-gastrula, as seen both by northern analysis (data not shown) and by whole-mount in situ hybridization (Fig. 2A). Strong expression was restricted to an arc at the anterior of the future neural plate. Much weaker expression outlining the lateral edges of the future neural plate was detectable more posteriorly upon overstaining (not shown). Expression persisted in this domain until late gastrula (Fig. 2B), when the opl expression domain directly abutted but did not overlap ventral ectoderm, as seen in embryos double stained for opl (orange) and gta3 (purple), a marker of ventral ectoderm (Neave et al., 1995) (Fig. 2C).

After completion of gastrulation at tailbud stage, strong opl expression persisted in the presumptive forebrain (Fig. 2D). As somitogenesis progressed, opl expression persisted in this domain, with expression in the more posterior neural keel upregulated (arrowhead in Fig. 2E). By the end of somitogenesis, in prim-5 stage embryos, opl was strongly expressed throughout the brain and more weakly in the spinal cord.

![Fig. 1. Zebrafish opl is highly conserved. Alignment of the deduced amino acid sequences of the zebrafish opl protein and those of its putative homologs: Xenopus opl (Kuo et al., 1998), mouse Zic1 (Aruga et al., 1994) and Drosophila opa (Benedyk, 1994). Positions of identity are shaded. Amino acid positions are designated on the left hand side of the sequences. The five zinc finger domains found within the proteins are indicated with a line above.](attachment:image.png)
Fig. 2. Zebrafish opl is expressed in the anterior neural plate and dorsal neural tube. Staged embryos were stained for opl RNA (purple, except in C, where it is orange) and gta3 RNA (purple in C) using whole-mount in situ hybridization. (A) Mid-gastrula (75% epiboly) embryo, dorsal view, dotted line indicates blastoderm margin, dashed line indicates anterior neural boundary. (B) Late gastrula (90% epiboly), dorsal view; dotted line indicates blastoderm margin, arrow indicates plane of section shown in G. (C) Late gastrula (90% epiboly), dorsal view. (D) Early neurula (tailbud) embryo, dorsal view, dashed line outlines the neural plate. (E) Early somitogenesis (5 somites), side view, arrowhead indicates a weak posterior domain of neurula (tailbud) embryo, dorsal view. (F) Late somitogenesis (prim-5), side view, arrows indicate planes of section shown in H and I. (G) Transverse section through a late gastrula (90% epiboly) stage embryo as shown in D, showing that opl expression is confined to the epiblast (e) and is excluded from the hypoblast (h) (mesendoderm) layers (bracketed). Arrowheads mark dorsal and ventral limits of the blastoderm. (H,I) Transverse section through (H) the diencephalon, (I) the rhombencephalon; note restriction of expression to dorsal neural tube, arrowheads mark dorsal and ventral limits of the blastoderm. a, anterior; d, dorsal; v, ventral; p, posterior; t, telencephalon; d, diencephalon; m, mesencephalon; r, rhombencephalon; s, spinal cord.

The domains of opl and anterior fkh5 expression in the late gastrula embryo were remarkably similar in shape and position to the presumptive telencephalon and diencephalon, respectively, as defined by fate mapping (Woo and Fraser, 1995). Interestingly, a recent fine scale fate mapping of the late

cord (Fig. 2F). Sections of late gastrula (90% epiboly) stage embryos show that opl expression was restricted to the epiblast (ectoderm) (Fig. 2G). Sections of prim-5 stage embryos, which have formed the neural tube, show that opl expression was restricted to the dorsal neural tube in both diencephalon (Fig. 2H) and rhombencephalon (Fig. 2I).

These data identify opl as a very early neural marker, initially specific to the anterior neurectoderm, and later restricted to the dorsal region of the entire brain.

opl and fkh5 divide the future forebrain into anterior and posterior domains

We isolated the zebrafish homolog of another gene defined in the Xenopus subtraction study, the forkhead domain gene fkh5 (Gamse and Sive, unpublished data), which has also been isolated in mouse (Kaestner et al., 1996; Labosky et al., 1997; Wehr et al., 1997). We used in situ hybridization to compare the expression pattern of fkh5 RNA with that of opl. Zebrafish fkh5 (purple stain) was first expressed in mid-gastrula embryos in a wide stripe in the posterior dorsal ectoderm (Fig. 3A). At this stage, there was a slight gap between the posterior border of the opl (orange stain) expression domain and the anterior border of the fkh5 expression. By late gastrula (Fig. 3B) fkh5 was strongly expressed in an anterior domain which lay immediately posterior to, but did not overlap with, the opl-expressing territory, and was more weakly expressed in a separate posterior domain. Posteriorly, the strong anterior domain of fkh5 expression overlapped with the expression domain of pax2, a mesencephalic marker (Krauss et al., 1991) (data not shown). Both the anterior and posterior expression domains of fkh5 were maintained through neural keel formation which begins at tailbud stage (Fig. 3C). The anterior edge of the fkh5 domain remained directly juxtaposed with the posterior edge of the opl domain. A transverse section through the anterior expression domain at this stage (Fig. 3D) showed that fkh5 expression was restricted to the epiblast (ectoderm).

Fig. 3. opl and fkh5 are expressed in adjacent domains that mark presumptive telencephalon and diencephalon at gastrula. Staged embryos were stained for opl RNA (orange) and fkh5 RNA (purple) using whole-mount in situ hybridization. (A) Mid-gastrula (75% epiboly) embryo, dorsal view, dotted line marks blastoderm margin; dashed line outlines anterior neural plate. (B) Late gastrula (90% epiboly) embryo, dorsal view; ad, anterior domain of fkh5 expression; pd, posterior domain of fkh5 expression. (C) Early neurula (tailbud) embryo, dorsal view, arrow shows plane of section shown in D. (D) Transverse section through a tailbud stage embryo. Note that staining is strong in the epiblast (e) and excluded from hypoblast (h). (E) Early somitogenesis (5 somite) embryo, side view. (F) Late somitogenesis (prim-5) embryo, side view, asterisks indicate weak fkh5 staining. a, anterior; t, telencephalon; d, diencephalon; m, mesencephalon; p, posterior; r, rhombencephalon; s, spinal cord.
gastrula neural plate indicates that opl is expressed primarily in the future retina, which is initially adjacent to the presumptive telencephalon (Varga and Westerfield, personal communication). Later, opl is not expressed in the developing eye but is expressed in the telencephalon. The close proximity between the future retinal and telencephalic fields makes these domains indistinguishable in our assays, and we will therefore refer to opl as a telencephalic marker.

Strong fkh5 expression persisted through early somitogenesis, when it was restricted to the newly formed ventral diencephalon, mesencephalon, and spinal cord (Fig. 3E). By the end of somitogenesis, anterior fkh5 expression comprised two weak vertical stripes in the brain, one in the diencephalon and one in the posterior mesencephalon (Fig. 3F). The posterior domain was also downregulated, except for the posterior spinal cord and tailbud.

In summary, these data showed that opl and fkh5 expression divides the presumptive neurectoderm of the gastrula embryo into two distinct domains: the anterior domain, marked by opl, which includes the prospective telencephalon, and the posterior domain, marked by fkh5, which includes the prospective diencephalon, mesencephalon, and posterior hindbrain/spinal cord.

**Neural specification takes place by early gastrula (shield) stage**

Using explant assays and neural markers, which are expressed starting at late gastrula, we previously established that neural specification has not yet occurred at late blastula (sphere stage, 4 hpf) (Sagerström et al., 1996). We wished to repeat this analysis using the earlier, mid-gastrula onset, neural markers (4 hpf) (Sagerström et al., 1996). We also wanted to ask whether early gastrula (shield stage, 6 hpf) caps were specified as neural tissue. Explants of animal caps from sphere stage embryos or presumptive anterior neur ectoderm from shield stage embryos were cultured in saline, in groups of 5 to 10, until control embryos reached mid-somitogenesis (10 somite stage, 14 hpf). Explants were then analyzed by a reverse transcriptase-PCR (RT-PCR) assay for expression of a panel of ectodermal markers (see Materials and Methods).

The results shown in Fig. 4B are representative of 2-5 experiments for each marker. Late blastula animal caps (lane 1) were strongly specified to express the ventral ectodermal marker gta3 (Neave et al., 1995), and weakly specified to express early markers of anterior dorsal ectoderm opl, fkh5, and otx2 (Li et al., 1994). These caps were not specified to express the neural marker pax6 (Krauss et al., 1991) that is activated later during development, at late gastrula, in accord with our previous results (Sagerström et al., 1996). By early gastrula (shield stage) (lane 2), animal caps were much more weakly specified to express gta3 (by 3 to 35 fold, 5 fold on average, relative to sphere stage caps). In contrast, caps were much more strongly specified to express opl and otx2 at this stage than at earlier stages, with levels of opl upregulated by 3 to 30 fold (an average of 6 fold), and levels of otx2 by 5 to 8 fold (an average of 6 fold) relative to sphere stage caps. This strong specification occurred 2 hours prior to the normal onset of expression of these genes at mid-gastrula. Specification of the ability to express fkh5 did not increase significantly in shield stage caps relative to sphere stage caps. Specification of shield caps for expression of pax6, a neural marker with a later onset of expression than opl or fkh5, was observed for the first time at this stage.

In order to narrow the window of time during which neural specification occurred even further, we compared specification for opl expression in animal caps removed from sphere stage (4 hpf), from 30-35% epiboly (very late blastula, 4 hours 45 minutes postfertilization) and from shield stage (6 hpf)
embryos (Fig. 4A). As shown in Fig. 4C, op1 expression was specified at equivalently low levels in caps taken from sphere stage (lane 1) and from 30-35% epiboly (lane 2) embryos, and increased to high levels in shield stage embryos (lane 3). In summary, we conclude that neural specification takes place during a span of about 1 hour 15 minutes between the 30-35% epiboly and shield stages. This includes approximately 55 minutes before and 20 minutes after the onset of gastrulation.

Dorsal ectoderm is specified as anterior neural and patterned by early gastrula

Having established that the presumptive neuroectoderm of the shield stage embryo is specified as neural, we next asked whether any anteroposterior (A/P) pattern had been specified in this tissue. As diagrammed in Fig. 5A, four distinct regions of shield stage (6 hpf) ectoderm were explanted separately: ventral (V), anterior dorsal (A), posterior dorsal (P), and lateral (L), where V will give rise primarily to epidermis, A to telencephalon and retina with some diencephalon, P to diencephalon, retina and mesencephalon, and L to rhombencephalon. These assignments were made based on a fate map of Woo and Fraser (Woo and Fraser, 1995), constructed by dye-labeling cells at early gastrula (shield stage). The shield region (S), which contains a mixture of ectoderm, mesoderm and endoderm cells (Shih and Fraser, 1995), was also explanted. Explants were either harvested immediately, or cultured in groups of 5 until control embryos reached tailbud (10 hpf) or 10 somite (14 hpf) stage, when they were analysed for expression of marker genes using an RT-PCR assay (see Materials and Methods).

Since we wished to determine the specification state of pure ectoderm, we first asked whether explants were free of mesodermal contamination by harvesting explants immediately after dissection and analysing them for expression of mesodermal markers. A representative experiment (one of two with very similar results) is shown in Fig. 5B. The V explant (lane 1) was not specified to express the dorsal mesodermal markers gsc (Stachel et al., 1993; Thisse et al., 1994), lim1 (Toyama et al., 1995), or chd (Miller-Bertoglio et al., 1997), and was only weakly specified to express the pan mesodermal marker ntl (Schulte-Merker et al., 1994). The A explant did not express any of the 4 markers (lane 2), while very low (less then 1% of S expression) levels of gsc and chd were seen in the P explant (lane 3). Since both gsc and chd are expressed in the ectoderm soon after this stage, starting at mid to late gastrula (Miller-Bertoglio et al., 1997; Thisse et al., 1994), and since lim1 and ntl RNA was not detected in the P explant, the presence of small quantities of gsc and chd RNA in the P explant at early gastrula is likely to be due to low level ectodermal expression. In contrast, all four mesodermal markers were highly expressed in the S explant (lane 4), consistent with the fate map and our previous specification analysis (Sagerström et al., 1996).
In order to determine whether A/P polarity already exists in the dorsal ectoderm at shield stage, prior to the onset of *opl* or *fkh5* expression, we cultured explants until tailbud or 10 somite stage and analyzed them for marker gene expression (Fig. 5A). Results of a representative experiment (of 4 which gave similar results) are shown in Fig. 5C. V explants (lane 1) were strongly specified to express *gta3*, but only weakly specified to express the dorsal markers *opl* and *fkh5*. In contrast, the A explant (lane 2) was weakly specified to express *gta3* (with levels in the V explant 10 fold higher than those of the A explant) and strongly specified to express *opl*. The P explant (lane 3), like the A explant, was weakly specified to express *gta3* and strongly specified to express *opl*, but in contrast to the A explant, it was also strongly specified for *fkh5* expression (with levels of *fkh5* 15 fold higher in the cultured P than in the A explant). In addition, the P explant was less strongly specified to express *opl* than was the A explant (with levels approximately 1.5 fold lower in the P than in the A explant). The shield explant S (lane 4) was not specified to express *opl* or high levels of *gta3*, but was strongly specified to express *fkh5*. We also examined the specification state of the lateral ectoderm, L (lane 5) that is fated to form rhombencephalon. In order to assay expression of the rhombencephalic marker *krx20* (Octzy and Jowett, 1993), whose expression is activated at early neurula and reaches high levels by mid-somitogenesis, explants were cultured until control embryos reached the 10 somite stage. L explants were specified to express intermediate levels of *gta3* expression and strongly specified to express *opl* and *fkh5*, suggesting that this explant straddles the boundary between dorsal and ventral ectoderm. In contrast, *krx20* expression was not detected in this explant or in the V, A, and P explants. We have previously shown that isolated shields (S explant) go on to activate high levels of *krx20* (Sagerström et al., 1996).

In summary, this analysis showed that early during gastrulation the presumptive neurectoderm is not only specified, but contains a latent anteroposterior pattern. Specifically, telencephalic (marked by *opl*) and more posterior neural (marked by *fkh5*) regions are determined in discrete parts of the neurectoderm. Since a rhombencephalic marker (*krx20*) (Octzy and Jowett, 1993), whose expression is activated at early neurula and reaches high levels by mid-somitogenesis, was strongly induced in the portion of the animal cap juxtaposed to the most anterior region of the shield (arrowhead). In the remaining 16 conjugates (32%) no *opl* expression was observed. In both cases, the randomly distributed patches of weak *opl* expression observed in isolated caps were suppressed. We previously showed that shield could induce expression of the anterior neural markers otx2 and *pax6* in blastula caps with a frequency similar to the frequency of *opl* induction observed here (Sagerström et al., 1996). However, while *otx2* and *pax6* were expressed by isolated shield in culture (Sagerström et al., 1996), *opl* was not (Fig. 6Bc) (0 out of 40 explants analyzed). Since *otx2* and *pax6* are also expressed in more posterior domains than *opl*, this suggests that the shield is not capable of autonomously activating the most anterior neural fates.

In summary, these data demonstrated that although the early gastrula shield cannot autonomously activate expression of *opl*, it can efficiently induce *opl* expression in blastula stage ectoderm.

**The entire late blastula organizer, but not the future prechordal plate, is required for activation of *opl***

Having demonstrated that the early gastrula organizer (shield) can induce anterior neural tissue, we asked whether it is also required for induction of this tissue. Since we had shown that anterior neural tissue was specified by early gastrula stages, but had not yet been specified at very late blastula (30-35% epiboly, Fig. 4C), we asked whether removal of the presumptive shield from younger embryos prevented *opl* expression. The presumptive shield region of the late blastula organizer was identified morphologically and the entire region, or portions thereof, was removed. Operated embryos were cultured until the end of gastrulation (90% epiboly equivalent, 9 hpf) and assayed for *opl* expression by in situ hybridization (Fig. 7A).

The late blastula organizer was identified morphologically at the asymmetric onset of epiboly, which results in a thinner margin on the dorsal side then on the ventral side (Schmitz and Campos-Ortega, 1994). This difference can be most easily observed at late blastula, the 30-35% epiboly stage. In a pilot experiment using eight embryos, we confirmed the correlation by marking the thinner side with FLDX injected into the adjacent yolk. When observed at shield stage, all embryos had formed shields near the position marked by the injection (data not shown). Additionally, in order to determine how much tissue to remove, we mapped the late blastula organizer using in situ hybridization of two markers, *gsc* (purple) which marks the presumptive prechordal plate (Stachel et al., 1993) and *chd* embryos from which the shields were taken reached the 5 somite stage, and assayed for *opl* expression by in situ hybridization. In order to distinguish inducing and responding tissues after culture, embryos from which the shield was removed for conjugation were lineage labeled with FITC-conjugated dextran (FLDX) that was later visualized by immunohistochemistry (see Materials and Methods).

A representative set of explants is shown in Fig. 6B. Cultured late blastula animal caps expressed low levels of *opl*, distributed randomly (Fig. 6Ba), consistent with RT-PCR data shown in Fig 4. When animal caps were conjugated with a shield, two outcomes were observed (Fig. 6Bb). In 22 out of 38 conjugates (58%), generated in 7 experiments, *opl* was strongly induced in the portion of the animal cap juxtaposed to the most anterior region of the shield (arrowhead). In the remaining 16 conjugates (32%) no *opl* expression was observed. In both cases, the randomly distributed patches of weak *opl* expression observed in isolated caps were suppressed. We previously showed that shield could induce expression of the anterior neural markers *otx2* and *pax6* in blastula caps with a frequency similar to the frequency of *opl* induction observed here (Sagerström et al., 1996). However, while *otx2* and *pax6* were expressed by isolated shield in culture (Sagerström et al., 1996), *opl* was not (Fig. 6Bc) (0 out of 40 explants analyzed). Since *otx2* and *pax6* are also expressed in more posterior domains than *opl*, this suggests that the shield is not capable of autonomously activating the most anterior neural fates.

The early gastrula shield can induce and pattern anterior neural tissue

Having shown that the neur ectoderm near the animal pole is strongly specified as telencephalon by early gastrula, we asked what source of inducing signals might be responsible for this specification. By early gastrula the presumptive telencephalon is not yet underlain by involuting mesendoderm (Kimmel et al., 1990), suggesting either that signals arise from a source at a distance, particularly the dorsal mesendoderm (organizer), or that a proximal tissue other than the organizer is the source of inducing signals. We first asked whether the early gastrula shield, which contains organizer activity (Sagerström et al., 1996; Shih and Fraser, 1996), can induce *opl* during zebrafish embryogenesis. Animal caps were isolated from late blastula embryos and either cultured alone in groups of 10 or as conjugates with shields isolated from early gastrula (shield stage, 6 hpf) embryos (Fig. 6A). Shield explants cultured alone served as controls. Explants were cultured until control
removed an area which included the entire chd expression domain at 30% epiboly (entire stippled area in Fig. 7A, see Materials and Methods). This surgery did not remove cells at the animal pole, which contain forebrain precursors (Kimmel and Warga, 1987). In order to control for the effects of trauma due to surgery, embryos were generated in which a significant portion of the organizer domain (>80%, solid gray area in Fig. 7A) was removed, while the vegetal-most portion of it was left intact. Cultured embryos were processed by in situ hybridization to detect simultaneously expression of opl (orange) and chd (purple). Although epiboly was abnormal in control embryos, all of the control embryos expressed both chd and opl (5 out of 5 embryos generated in 1 experiment) with opl expressed anterior to the chd domain, as in normal embryos (Fig. 7Bc). In contrast, all of the embryos from which the late blastula organizer had been successfully removed (8 out of 23 operated embryos generated in 4 experiments) failed to express opl (Fig. 7Bd), although they appeared healthy, with no indication of excessive cell death as visualized by acridine orange staining at mid-gastrula (data not shown). The remaining 15 out of 23 operated embryos expressed both chd and opl, suggesting that the complete organizer had not been removed.

In order to test the requirement for the presumptive prechordal plate in opl activation, we removed a smaller portion of the late blastula organizer (hatched area in Fig. 7A). Control embryos were generated by mock dissection (see Materials and Methods). Embryos were cultured until late gastrula stage equivalent and stained for opl RNA (purple) by in situ hybridization. (B) Cultured explants, stained for the presence of opl RNA (purple) and lineage label (blue). (a) Two groups of 10 animal caps cultured in isolation. (b) Three conjugates representative of the typical outcomes of the experiment. Dots mark the anterior and posterior edges of the animal cap-derived tissues. (c) Shield explants cultured in isolation. A total of 38 conjugates were made in 7 independent experiments. Induction of opl was observed in 58% of them, and was always restricted to the animal cap-derived portion. No detectable opl expression has been observed in more than 40 shield explants generated in at least 5 experiments. Abbreviations: a, anterior; p, posterior.

(orange) that marks the entire dorsal mesendoderm (organizer region) (Miller-Bertoglio et al., 1997). At 30% epiboly, chd is expressed in a domain which extends from the blastoderm margin to about 30% of the distance between the margin and the animal pole, while gsc expression is restricted to the vegetal half of the chd domain (Fig. 7Ba).

Using the early asymmetry in epiboly as a landmark, we first
with the organizer. Kimmel and Warga (1987) showed that animal pole cells of a mid-blastula stage embryo remain at the animal pole until early gastrula and later contribute to forebrain structures. These cells can be induced to express opl in explants (Fig. 6), and therefore could presumably have been induced in the embryo, had appropriate inducing signals been present after surgery. Furthermore, when at least 80% of the mesendodermal region expressing chd was removed, leaving only a very thin strip of cells at the blastoderm margin as well as the animal pole cells, opl was efficiently induced. This indicates that failure to express opl after complete organizer removal was not a consequence of surgical trauma. Finally, using acridine orange staining (Furutani-Seiki et al., 1996), we have seen no increase in cell death between mock-dissected and organizer-ablated embryos.

In summary, these data demonstrated that the dorsal mesendodermal region marked by chd expression, the putative ablated embryos. Increase in cell death between mock-dissected and organizer-orange staining (Furutani-Seiki et al., 1996), we have seen no consequence of surgical trauma. Finally, using acridine orange staining (Furutani-Seiki et al., 1996), we have seen no increase in cell death between mock-dissected and organizer-ablated embryos.

When applied during gastrulation, BMP4 can ablate opl expression

Having shown that the late blastula organizer was required for opl activation, we next wanted to know whether organizer activity was also required during gastrulation for opl patterning and expression. This could not be done by removal of the early gastrula shield since this structure regenerates after removal at this stage (Y. G. and H. S., unpublished results). Bone Morphogenetic Proteins (BMPs), namely, BMP2, BMP4 and BMP7, promote ventral determination and must normally be antagonized by organizer-derived signaling to activate dorsal fates (Hawley et al., 1995; Sasai et al., 1995; Schmidt et al., 1995; Suzuki et al., 1997; Hammerschmidt et al., 1996; Kishimoto et al., 1997). We made use of BMP4 to ask whether signaling by the organizer during gastrulation is required for opl expression. Sepharose beads were coated with BMP4 protein (see Materials and Methods) and implanted into the dorsal side of early gastrula (shield, 6 hpf) stage embryos (Fig. 8A). Control beads were coated with bovine serum albumin (BSA). Embryos were cultured until late gastrula (90% epiboly, 9 hpf) and assayed for opl expression by in situ hybridization.

After embryo culture, control (BSA-coated) beads and BMP4-coated beads were found at similar positions with
Using explant and extirpation assays, we have shown that place by early gastrula, earlier than known previously. Third, we have found that induction of these regions takes more posterior region that includes the future diencephalon. Field into an anterior region, the future telencephalon, and a

Second, we have shown a very early molecular division of the forebrain

Three points. First, using two early neural markers, organizers-derived signaling in activating expression, except that beads needed to be located dorsally. Coated bead, however, we were not able to determine how close expressing cells were never found adjacent to a BMP4-bead, while in 3 out of 11 embryos it was ablated completely. Opl expression domain was located more caudally than in controls, except that beads needed to be to the blastoderm margin. Control beads had no effect on opl expression (7 out of 7 embryos) (arrowhead in Fig. 8Ba). In contrast, opl expression was partially or completely ablated in the vicinity of a BMP4-coated bead in all cases (11 out of 11 embryos) (arrowheads in Fig. 8Bb), and the opl expression domain was located more caudally than in controls, that is, toward the blastoderm margin. In 8 of the 11 embryos, opl expression was only partially ablated by the BMP4-coated bead, while in 3 out of 11 embryos it was ablated completely. opl-expressing cells were never found adjacent to a BMP4-coated bead, however, we were not able to determine how close the bead needed to be to the opl domain in order to inhibit opl expression, except that beads needed to be located dorsally.

In summary, these experiments indicate that BMP4 antagonizes opl expression, consistent with a continued role for organizer-derived signaling in activating opl during gastrula stages.

**DISCUSSION**

Our examination of zebrafish forebrain determination makes three points. First, using two early neural markers, opl and fkh5, we have shown a very early molecular division of the forebrain field into an anterior region, the future telencephalon, and a more posterior region that includes the future diencephalon. Second, we have found that induction of these regions takes place by early gastrula, earlier than known previously. Third, using explant and extirpation assays, we have shown that telencephalic induction can be effected by the embryonic shield, and that a broad region of the zebrafish organizer is required for activation of forebrain determination. These findings have implications for the timing of and tissue interactions required for commitment to forebrain fates in other vertebrates.

**Territories of the presumptive forebrain appear during gastrulation**

During gastrulation, the homeobox-containing gene otx2 demarcates the entire presumptive forebrain and midbrain in zebrafish (Li et al., 1994), Xenopus (Blitz and Cho, 1995; Panneese et al., 1995) and mouse (Simeone et al., 1993). After neural tube closure, multiple genes demarcate different transverse and longitudinal domains within the forebrain (Rubenstein et al., 1994). However, the issue of when the forebrain is first divided into territories has not been clarified. At late gastrula, the opl expression domain appears to correspond to the presumptive telencephalon while the anterior portion of the fkh5 expression domain appears to correspond to the presumptive diencephalon as defined by fate mapping (Woo and Fraser, 1995). Together, the expression patterns of opl and fkh5 define a very early molecular subdivision of the presumptive forebrain which precedes any morphological subdivision.

Starting at mid-gastrula, zebrafish opl and fkh5 are expressed in patterns which are very similar, both spatially and temporally, to those of their Xenopus homologs (Gamse and Sive, unpublished data). However, in Xenopus, opl and fkh5 are also strongly expressed by the onset of gastrulation, at an earlier stage than they are in zebrafish, suggesting that some levels of gene control are different between these organisms (Gamse and Sive, unpublished data). Mouse homologs of opl (Zic1) and fkh5 are expressed in analogous domains during early development: Zic1 in the neur ectoderm and then in the dorsal neural tube (Nagai et al., 1997) and fkh5 in the diencephalon, mesencephalon, rhombencephalon and spinal cord (Labosky et al., 1997; Wehr et al., 1997). These similarities may suggest that both the signaling pathways, which lead to the patterned expression of genes in the early neur ectoderm, and the functions of these genes are conserved among vertebrates.

**Timing of forebrain patterning**

We showed that neur ectoderm is specified by early gastrula (shield) stage, and that some neural pattern has also been specified by this stage. Three distinct domains of specification are present in the early gastrula ectoderm, shown as a ‘specification map’ in Fig. 9A. This map was drawn by summarizing the results of specification assays using explants made at different positions of the early gastrula ectoderm (Fig. 5). The overlap between ‘specification domains’ in this map reflects that none of the markers used was specified exclusively in one explant type. Instead, markers were specified primarily in one explant type, but also weakly in neighboring explants. This may be due to lack of sharp boundaries between specification domains or to our inability to explant precisely the same group of cells from different embryos.

Our data indicate the presence at early gastrula of a ventral domain, specified as ventral, primarily non-neural, ectoderm, an anterior dorsal domain, specified primarily as telencephalon, and a posterior dorsal domain, specified primarily as diencephalon. We compared our specification map of the early gastrula ectoderm to a fate map made by dye
marking cells of early gastrula embryos and scoring their fates after the brain had morphologically formed (Woo and Fraser, 1995). This comparison (Fig. 9A) shows a striking overlap between the domains we defined by specification assays and the domains defined by fate mapping. Since a fate map defines the regions of the embryo which will form given organs, but contains no information about when the specification to form such organs is first acquired, this overlap was not necessarily expected. Early gastrula is the earliest stage at which a fate map of the zebrafish embryo can be drawn, probably due to slowing down of cell mixing, and the coincidence of this with activation of neural specification and patterning suggests that inductive centers are in place prior to early gastrula.

The region of dorsal ectoderm which is fated to give rise to rhombencephalon is specified as anterior neural tissue at early gastrula, since after culture it expresses anterior markers opl and fkh5, but not the more posterior hindbrain marker krx20. This finding has recently been confirmed using several novel markers of posterior neurectoderm isolated in our laboratory (M. E. Lane, C. G. Sagerström, and H. S.). These data support the idea that in zebrafish the tissue fated to become rhombencephalon is first induced as anterior and later reprogrammed into more posterior neural tissue. Recent studies suggest that the zebrafish germ ring is a source of posteriorizing signals (Woo and Fraser, 1997; M. E. Lane and H. S., unpublished data) which may convert the neurectoderm, initially specified as anterior, into more posterior derivatives (Kolm and Sive, 1997).

**Tissues responsible for forebrain specification and patterning**

Previous studies using transplant (Shih and Fraser, 1996) and explant (Sagerström et al., 1996) analyses have shown that the zebrafish embryonic shield is a potent source of neural inducing signals. The shield encompasses the gsc expression domain, which has previously been shown to mark the prospective prechordal plate (Stachel et al., 1993). However, the zebrafish organizer is larger than the gsc domain and may correspond to the chd expression domain (Miller-Bertoglio et al., 1997). Since the organizer field has not been carefully mapped in fish, we relied on the evidence from Xenopus, where the functional organizer correlates well with the domain of chd expression at late blastula and early gastrula stages (Sasai et al., 1994; Stewart and Gerhart, 1990; Vodicka and Gerhart, 1995). By removing the entire chd expression domain we have shown that dorsal mesendoderm is required for anterior neural patterning marked by opl expression. This effect is not due simply to the removal of chd activity, since genetic deletion of the chd locus does not completely prevent the formation and patterning of neural tissue (Fisher et al., 1997; Hammerschmidt et al., 1996b; Schulte-Merker et al., 1997).

We used two methods to demonstrate that the presumptive prechordal plate is dispensable for opl activation and patterning but may contribute to the correct levels of gene expression: extirpation of the presumptive prechordal plate and analysis of oep mutant embryos, which fail to form the prechordal plate. Both methods allowed expression of opl in the correct position in the extreme anterior part of the neural plate. In oep mutant embryos opl expression levels were significantly reduced relative to wild type controls, but this was not the case in embryos from which the presumptive prechordal plate was removed microsurgically. One possible explanation for this difference is that the domain removed microsurgically also included posterodorsal mesendoderm. Postерodorsal
mesendoderm, including prospective notochord, may suppress extreme anterior neural determination and removal of this inhibitory region may compensate for the loss of positive signals coming from the anterior mesendoderm. This idea is supported by observations in *Xenopus*, where posterodorsal mesendoderm can inhibit anterior neural determination (Sive et al., 1990, 1989). Alternatively, since the gene identified by the *oep* mutation is highly expressed in the prospective forebrain itself by late gastrula (Zhang et al., 1998), it may play a direct role in maintaining high levels of anterior neural gene expression.

The early gastrula organizer is functionally heterogeneous (Oppenheimer, 1959; Zoltewicz and Gerhart, 1997). In amphibian and chick embryos, the presumptive prechordal plate (the *gsc*-expressing region) is correlated with head organizer activity (Foley et al., 1997; Holtfreter, 1938a,b; Pera and Kessel, 1997; Zoltewicz and Gerhart, 1997). However, consistent with our results, removal of this region from chick embryos does not prevent the correct patterning of the neural anteroposterior axis (Pera and Kessel, 1997) and organizers from which this region is deleted at early gastrula are fully functional in secondary axis induction (Psychoyos and Stern, 1996).

While the prechordal plate is not required for *opl* induction, the early gastrula shield, which includes the presumptive prechordal plate, is able to induce strong *opl* expression in blastula stage ectoderm. This was not necessarily expected, since isolated and cultured shield did not go on to express *opl*, and since transplanted shields do not activate head formation in secondary axes (Shih and Fraser, 1996). The ability of the shield to induce *opl* expression indicates that it contains molecules which activate determination of the telencephalon. The failure of isolated shield to express *opl*, together with the specification in intact embryos of *opl* at a distance from the presumptive shield, may suggest that telencephalon-inducing signals must act at a minimum distance from their source in the presumptive shield, perhaps after formation of a gradient. This idea is consistent with the observation that different doses of BMPs, or anti-BMP molecules such as noggin, lead to different readouts along the anteroposterior axis (Knecht and Harland, 1997; Wilson et al., 1997).

Although the blastula stage organizer (dorsal mesendoderm) is required for expression of *opl* by late gastrula, we do not know whether this is the tissue that initiates *opl* specification by early gastrula. Some inducing signals may be produced by the yolk cell, which contacts anterior ectoderm by the onset of gastrulation and which contains mesoderm-inducing and patterning capacity (Mizuno et al., 1996). It is possible, but not demonstrated, that this tissue is equivalent to the dorsoanterior endoderm in *Xenopus* and the extraembryonic endoderm of mice. However, since organizer removal prevents *opl* expression, the yolk cell is clearly not sufficient for *opl* activation. A recent interesting report indicates that during mid- to late gastrula stages in zebrafish a row of anterior ectodermal cells is required for forebrain patterning (Houart et al., 1998). The relationship between the forebrain patterning function of these cells and that of the late blastula organizer identified here is not clear since the two studies analyse events occurring at different times of gastrulation. In particular, it is not clear whether the neur ectodermal region (Houart et al., 1998) is active at earlier stages, or whether it is activated subsequent to the action of the late blastula organizer and initial neural patterning events we describe here.

**A model for induction and patterning of anterior neur ectoderm in zebrafish**

A model for early neural patterning in the zebrafish embryo which summarizes our findings is shown in Fig. 9B. In a late blastula embryo cells at the animal pole are specified primarily as ventral (non-neural) ectoderm, including prospective epidermis, with weak dorsal character. Signals that activate this specification may be cell autonomous, or may be derived from the yolk cell or from the blastoderm margin. Two hours later, at early gastrula, dorsal ectoderm is already specified as neural with an anteroposterior axis. The prospective telencephalon lies anteriorly and the prospective diencephalon posteriorly. We conclude that the initial induction and patterning of the forebrain in zebrafish takes place just prior to, or coincident with, the onset of gastrulation. Expression of the forebrain markers we have used does not begin until mid-gastrula, and it is not known whether a wave of patterned gene expression precedes that of *opl* and *fkh5*. Our data suggest that the signals responsible for forebrain patterning arise from the late blastula organizer (dorsal mesendoderm). Inducing signals may also originate from the yolk cell that underlies the future telencephalon, although these signals are not sufficient to activate expression of forebrain markers. A later source of inducing signals may also arise from the anterior neur ectoderm itself (Houart et al., 1998).

Future directions include analysis of the mechanisms of patterned gene expression within the forming forebrain, both with regard to the cis-acting promoter elements and the trans-acting factors responsible for generating this pattern.

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