

## Genes involved in forebrain development in the zebrafish, *Danio rerio*

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### SUMMARY

We identified four zebrafish mutants with defects in forebrain induction and patterning during embryogenesis. The four mutants define three genes: *masterblind* (*mbl*), *silberblick* (*slb*), and *knollnase* (*kas*). In *mbl* embryos, the anterior forebrain acquires posterior forebrain characteristics: anterior structures such as the eyes, olfactory placodes and the telencephalon are missing, whereas the epiphysis located in the posterior forebrain is expanded. In *slb* embryos, the extension of the embryonic axis is initially delayed and eventually followed by a partial fusion of the eyes. Finally, in *kas* embryos, separation of the telencephalic

primordia is incomplete and dorsal midline cells fail to form a differentiated roof plate. Analysis of the mutant phenotypes indicates that we have identified genes essential for the specification of the anterior forebrain (*mbl*), positioning of the eyes (*slb*) and differentiation of the roof plate (*kas*).

In an appendix to this study we list mutants showing alterations in the size of the eyes and abnormal differentiation of the lenses.

Key words: neurogenesis, neural development, forebrain, eye, lens, zebrafish, *Danio rerio*

### INTRODUCTION

The process of neural induction in vertebrates has been postulated to be subdivided into two steps (for a review see Saxen, 1989): first there is an induction of anterior fates within the whole neural plate, called 'activation'. This is followed by a 'transformation' of the neural plate to give posterior character; a gradient emanating from the posterior of the embryo regionalizes the neural axis into brain and spinal cord. This morphogenetic gradient is established by the mesendoderm; early involuting mesendoderm induces anterior neural fates and late involuting mesendoderm induces posterior ones (Eyal-Giladi, 1954).

Several candidate molecules involved in neural induction and subsequent patterning of the neural plate have been identified. Noggin, a secreted polypeptide (Smith and Harland, 1992; Lamb et al., 1993), follistatin, a secreted antagonist of activin (Hemmati-Brivanlou et al., 1994) and chordin, the vertebrate homologue of the *Drosophila* gene *short gastrulation* (*sog*) (Sasai et al., 1994, 1995; Francois and Bier, 1995) can induce neuroectoderm of anterior character, thereby mimicking the 'activation' step during neural induction. Retinoic acid as well as basic fibroblast growth factor may be involved in generating the morphogenetic gradient leading to the 'transformation' of the neural plate, since both have been

shown to induce the development of neural tissue with posterior character (Durstont et al., 1989; Kengaku and Okamoto, 1995). However, the role of these factors in vivo and the mechanisms by which these signals are transmitted to the ectoderm remain unclear, as results of a complete 'knock-out' of the genes have not been published (except for follistatin, where neural induction appears to be unaffected; Matzuk et al., 1995).

A number of experiments in amphibians suggest that signals needed for induction and patterning of the neural plate can be transmitted in different ways (Spemann, 1938; Kintner and Melton, 1987). There is evidence for both vertical transmission of signals from the underlying mesendoderm to the ectoderm and horizontal signaling within the plane of the ectoderm: exogastrulated embryos and explants of dorsal tissue, which mimic exogastrulae ('Keller sandwiches'), have been employed to analyze neural-specific gene expression within the ectoderm in the absence of vertical signals (Keller and Danilchik, 1988; Ruiz i Altaba, 1990). Exogastrulated embryos form mesendoderm, which does not involute, therefore neural inducing signals from the mesendoderm must be exclusively transmitted horizontally through the plane of the ectoderm (Holfreter, 1933; Hamburger, 1988). In exogastrulae and 'Keller sandwiches', the expression of neural-specific genes along the anterior-posterior axis of the ectoderm is induced, suggesting

that vertical signals from the underlying axial mesendoderm are not required (Ruiz i Altaba, 1992; Doniach et al., 1992; Papalopulu and Kintner, 1993). However, differentiation of specific cell types characteristic of the forebrain and ventral neural tube is not observed. These cell types are therefore likely to depend on both vertical and planar signals (Ruiz i Altaba, 1992, 1994; Papalopulu and Kintner, 1993).

The underlying axial head mesendoderm, the prechordal plate, is known to be essential for induction and patterning of the forebrain. Whereas the expression of genes specific for the forebrain anlage has been shown to be independent of vertical signals from the prechordal plate, the differentiation of forebrain derivatives like eyes, as well as the capability of the presumptive forebrain to induce midbrain (Nieuwkoop and Albers, 1990), depends on both vertical and planar signals (Ruiz i Altaba, 1992, 1994; Papalopulu and Kintner, 1993). Moreover analysis of the zebrafish *cyclops* (*cyc*) and *one-eyed-pinhead* (*oep*) mutants shows that a reduction of the prechordal plate is associated to a deletion of ventral forebrain tissue, suggesting that vertical signaling is involved in the formation of the dorsal-ventral forebrain axis (Hatta et al., 1994; Thisse et al., 1994; Hammerschmidt et al., 1996; Warga et al., unpublished data).

Subdivision of the forebrain along its major axes, similar to that shown for the hindbrain and spinal cord (Keynes and Stern, 1984), is still a matter of debate (for review see Fraser, 1993; Puelles and Rubenstein, 1993). Boundaries of expression domains of various markers found in the forebrain have been shown in many cases to coincide with morphological boundaries seen at different stages of forebrain development (Puelles et al., 1987; Figdor and Stern, 1993; Puelles and Rubenstein, 1993; Macdonald et al., 1994; Barth and Wilson, 1995). The diencephalon for example has been shown to be composed of four neuromeres along its anterior-posterior axis which are anatomically, molecularly and functionally definable (Figdor and Stern, 1993). However, many of the other proposed neuromere subdivisions, in particular within the telencephalon, must still be examined in more detail with respect to functional properties such as lineage restriction.

In this study we describe the isolation and initial characterization of zebrafish mutants exhibiting specific defects in the formation of the forebrain: patterning of the forebrain along the anterior-posterior axis is affected in *mbl*, positioning of the eyes is aberrant in *slb*, and differentiation of the roof plate separating the telencephalic primordia is defective in *kas*. The analysis of these mutant phenotypes may be helpful in elucidating important steps in forebrain patterning and differentiation.

## MATERIALS AND METHODS

### Screening, maintenance and breeding

Maintenance and breeding of fish has been described by Mullins et al. (1994). Identification and isolation of mutants together with the complementation analysis is described in an accompanying paper by Haffter et al. (1996).

### Whole-mount in situ hybridization and immunohistochemistry

Antibody and in situ stainings were performed as described previously (Hammerschmidt and Nüsslein-Volhard, 1993). For antibody stainings, anti-islet (Korzh et al., 1993; 1:500), anti-pax6 (also called

pax[zf-a]) (Macdonald et al., 1994; 1:400), anti-pax2 (also called pax[zf-b]) (Mikkola et al., 1992; Püschel et al., 1992b; 1:100), anti-ntl (Schulte-Merker et al., 1992; 1:1000), and anti-fkd2 (Warga et al., unpublished data; 1:2000) polyclonal antibodies, as well as anti-acetylated tubulin (Sigma; 1:1000) and anti-fret43 (Larison and Bremiller, 1990) monoclonal antibodies were used. For in situ hybridization digoxigenin-labeled RNA probes were synthesized from the full-length *shh* (Krauss et al., 1993), *zotx-2* (Li et al., 1994), *zash-1b* (Allende and Weinberg, 1994), *myoD* (Weinberg et al., 1996), *pax2* (Krauss et al., 1991a,b), and *fkd3* (Odenthal et al., unpublished data) cDNA clones. For double stainings (in situ and antibody labeling), embryos first underwent complete in situ hybridization, followed by complete antibody staining. Sections of embryos were prepared after whole-mount stainings. Embryos were dehydrated (methanol, 10 minutes), permeabilized (acetone, 10 minutes), incubated in araldite/acetone (1:1; 12 hours) and embedded in araldite. The araldite-blocks were polymerized (12 hours, 80°C) and sectioned on a vibrotome in 10 µm intervals. Sections were mounted in araldite. Photographs were taken using an Axiophot photomicroscope (Zeiss).

### Mosaic analysis

Transplantations were done as described by Ho and Kane (1990). Cells from wild-type donor embryos labeled with rhodamine-dextran at the one-cell stage were transplanted into the animal pole blastoderm of 4-hour host embryos obtained by crossing *mbl* heterozygotes. The results were analyzed at 30 hours. A quarter of the hosts were expected to be mutant. Only partial rescue of the eye and forebrain phenotype could be identified since a complete rescue was expected to result in a phenotype indistinguishable from the wild-type siblings.

## RESULTS

Screening for various classes of mutants was done by visual inspection of live embryos on the second, third and sixth day of development. 131 mutants were isolated and kept on the basis of morphologically recognizable defects of the nervous system. 18 of these mutants show regionally restricted defects within the nervous system (Brand et al., 1996a,b; Jiang et al., 1996; and this paper) whereas the others exhibit more general defects of neuronal tissue (Jiang et al., 1996; Furutani-Seiki, 1996). We also isolated mutants that showed defects of the axial mesendoderm accompanied by a general reduction of the ventral central nervous system (CNS) such as alleles of the *cyclops* (*cyc*) mutation (Hatta et al., 1991; Thisse et al., 1994; Warga et al., unpublished data). These mutants will be described elsewhere (Brand et al., 1996b; Hammerschmidt et al., 1996; Odenthal et al., 1996).

The zebrafish embryonic forebrain (prosencephalon) forms at the anterior end of the neural keel and is subdivided into a dorsal-anterior telencephalon and a ventral-posterior diencephalon (Ross et al., 1992). Structures formed or induced by the forebrain such as the retinae, olfactory placodes and the epiphysis are clearly visible in 24-hour embryos (Kimmel et al., 1995) and were used as morphological landmarks to discriminate forebrain mutants.

Four mutants defining three genes which exhibit a clear neuroectodermal defect restricted to the forebrain will be described in this paper: one allele of *masterblind* (*mbl*), two alleles of *silberblick* (*slb*) and one allele of *knollnase* (*kas*) (Table 1).

### *mbl* affects anterior-posterior patterning of the forebrain

The single *mbl* allele found (*tm13*) exhibits a recessive lethal

phenotype which does not resemble any other phenotype seen in the screen. The strength of the phenotype is slightly variable within a single egg-lay and depends on the genetic background.

The phenotype of live *mbl* embryos is characterized by the absence of optic vesicles seen already at 14 hours (Fig. 1A,D). Eventually the eyes (Fig. 1B,E) and olfactory placodes do not develop (Fig. 1C,F). The degree of variation ranges between the complete lack of eyes in severe cases to the formation of very small eyes (about 1/10 of wild type size) in milder cases. Phenotypic alterations are restricted to the anterior region of the CNS in *mbl* mutants; no other specific defects could be detected in *mbl* embryos as compared to wild type siblings. General necrosis of the mutant embryo is observed by 120 hours.

Primary neurons and their axons form a simple and well-described arrangement within the zebrafish embryonic CNS (Wilson et al., 1990; Ross et al., 1992). To determine which structures are altered in the CNS of *mbl* embryos, stainings using antibodies that recognize these elements were performed. Labeling of 24-hour mutant embryos for acetylated tubulin (Chitnis and Kuwada, 1990) revealed that the telencephalic clusters of primary neurons and olfactory placodes are missing (Fig. 2A,B,D,E). Evaluation of the axonal scaffold at 24 hours showed that the anterior commissure connecting the bilateral telencephalic neuronal clusters and the postoptic commissure connecting the bilateral nuclei of the tract of the postoptic commissure (nTPOC) do not form in the mutant (Fig. 2B,E). To test if the absence of a postoptic commissure is due to *mbl*

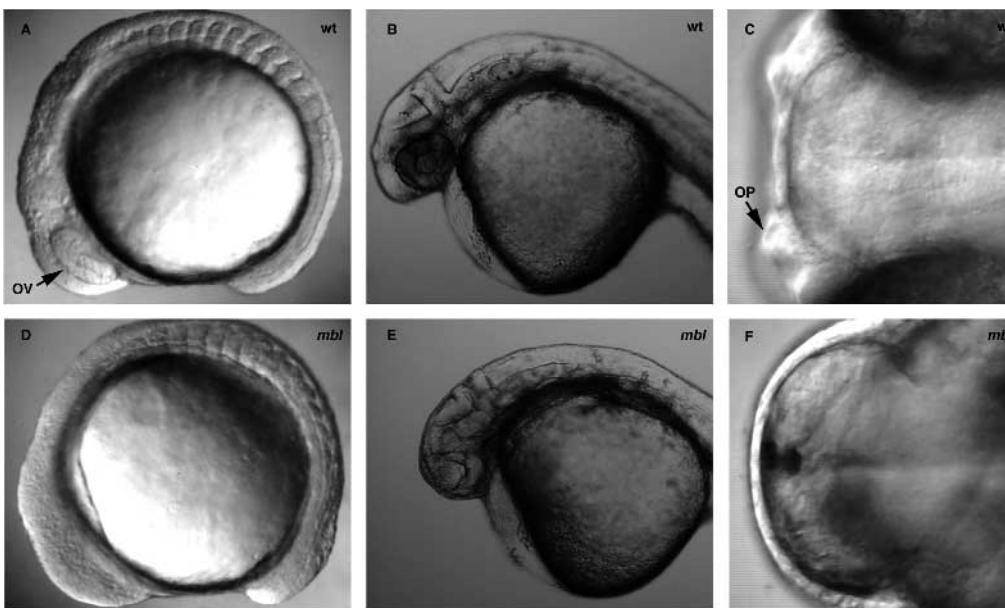
embryos lacking the neurons which normally pioneer this commissure, we labeled embryos for acetylated tubulin and islet proteins (using an anti-pan-islet antiserum recognizing members of the LIM homeodomain protein family; Korzh et al., 1993). Anti-tubulin labeling showed that many differentiated neurons are present in the nTPOC of 24-hour *mbl* embryos (Fig. 2A,D). However, anti-islet labeling revealed major differences in the neurons between wild-type and mutant embryos in that none of the nTPOC cells express islet proteins in *mbl* embryos at 24 hours (Fig. 2C,F). This result is consistent with the possibility that an islet-proteins-expressing subpopulation of the nTPOC cells, normally responsible for pioneering the postoptic commissure, is absent in *mbl*. The alterations in the organization of the CNS in *mbl* embryos are schematically illustrated in Fig. 2G.

Structures associated with the ventral diencephalon, such as the anterior pituitary, appear to be reduced in size, as seen by a reduction in the number of pax6-positive cells within the anterior pituitary of *mbl* embryos compared to wild type at 30 hours (Fig. 3A,D) (Krauss et al., 1991b,c; Püschel et al., 1992a; Macdonald et al., 1994). In contrast, the epiphysis, a dorsal diencephalic structure, is strongly expanded in mutant embryos, as seen by staining neurons within the epiphysis for islet proteins (Fig. 3B,E) and for *fret43* (Larison and Bremiller, 1990) at 28 hours (Fig. 3C,F).

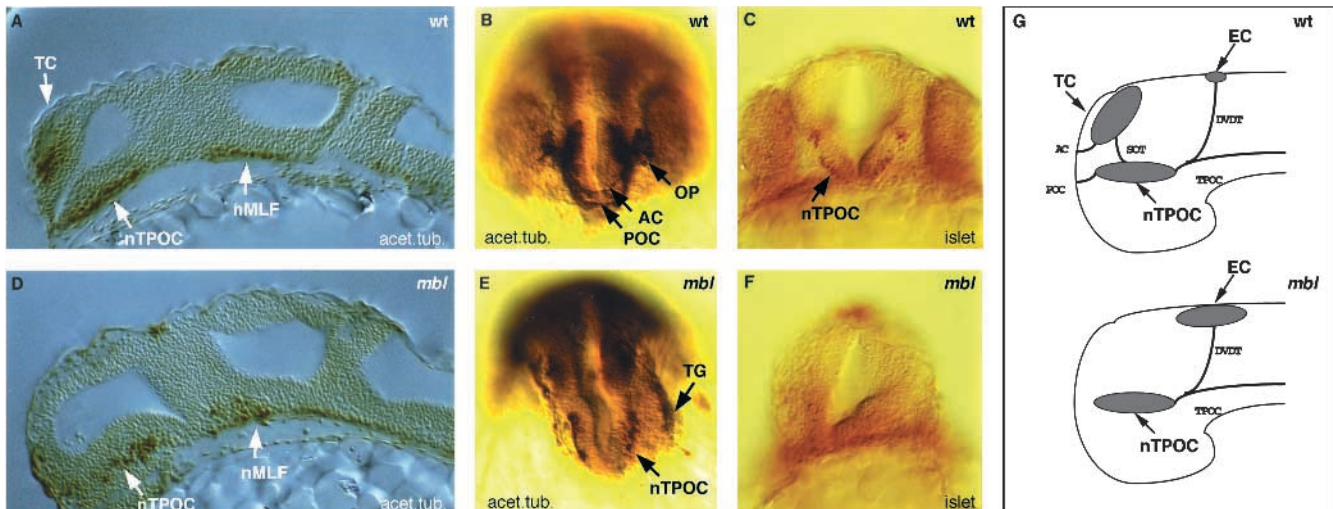
We examined whether the alterations in the architecture of the forebrain in *mbl* embryos are preceded by changes in the anlage of these structures at earlier stages of development. Therefore we analyzed the expression of genes in specific subdomains of the embryonic forebrain by in situ hybridization. The diencephalic expression domain of *zotx-2* (a zebrafish relative of *Drosophila orthodenticle*, Li et al., 1994) is expanded anteriorly in mutant embryos at 14 hours (Fig. 4A,D). Correspondingly the telencephalic expression domain of *zash-1b* (a zebrafish relative of *Drosophila* genes of the *achaete-scute* complex, Allende and Weinberg, 1994) is absent as shown at 12 hours (Fig. 4B,E). In wild-type embryos, *sonic hedgehog* (*shh*) (a zebrafish homologue of *Drosophila hedgehog*, Krauss et al., 1993) is expressed within the

**Table 1. Synopsis of mutants with specific defects in the forebrain summarizing the phenotypes, gene names, allele designations and the papers where the phenotype is described**

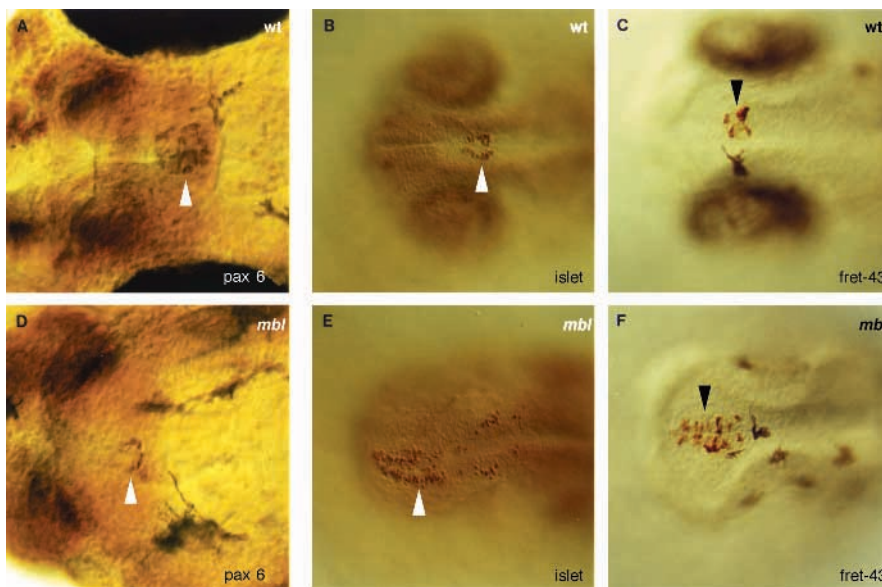
Affected structures	Gene	Alleles
Telencephalon, eyes, nose	<i>masterblind (mbl)</i>	<i>tm13</i>
Eyes (cyclopia)	<i>silberblick (slb)</i>	<i>tx226, tz216</i>
Roof plate	<i>knollnase (kas)</i>	<i>ty122</i>
Unresolved		<i>tp71e</i>



**Fig. 1.** Phenotypes of live wild type (A-C) and *mbl* (D-F) embryos. (A,D) Lateral view of 14-hour embryos showing the absence of optic vesicles in *mbl*. (B,E) Lateral view of 24-hour embryos showing the absence of eyes in *mbl*. (C,F) Dorsal view of 96-hour embryos showing the absence of olfactory placodes in *mbl*. OV, optic vesicles; OP, olfactory placodes. Anterior to the left.



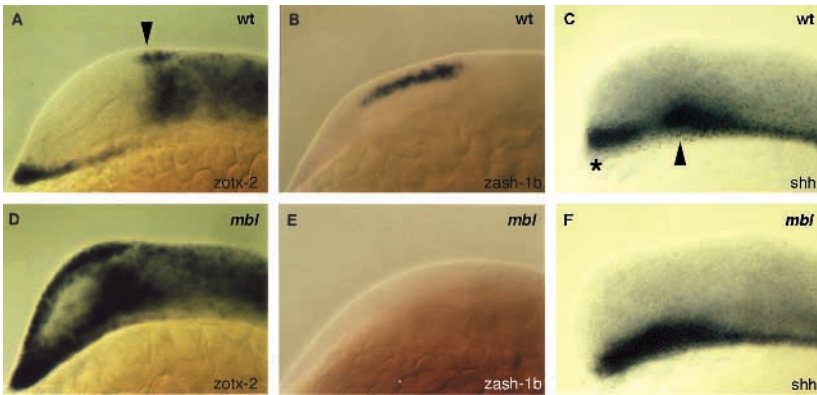
**Fig. 2.** Whole-mount antibody stainings of 24-hour embryos for acetylated tubulin and islet proteins (anti-pan-islet antibody) visualizing primary neurons and their axons in wild-type (A-C) and *mb1* (D-F) embryos. (A,D) Sagittal sections through the head region stained for acetylated tubulin showing the absence of the telencephalic neuronal clusters in *mb1* embryos (anterior to the left). (B,E) Frontal view of the head of embryos stained for acetylated tubulin showing the absence of olfactory placodes, and anterior postoptic commissures and the expansion of the trigeminal ganglia in *mb1* embryos. (C,F) Frontal views of the head (optical section) of embryos stained for islet proteins showing the absence of islet-positive cells within the nTPOC in *mb1*. (G) Schematic drawings of lateral views of the head summarizing the results from A-F (anterior to the left). TC, telencephalic cluster; nTPOC, nucleus of the tract of the postoptic commissure; nMLF, nucleus of the medial longitudinal fasciculus; EC, epiphysial cluster; AC, anterior commissure; POC, postoptic commissure; SOT, supraoptic tract; TPOC, tract of the postoptic commissure; TG, trigeminal ganglion; OP, olfactory placodes; DVDT, dorsal ventral diencephalic tract.



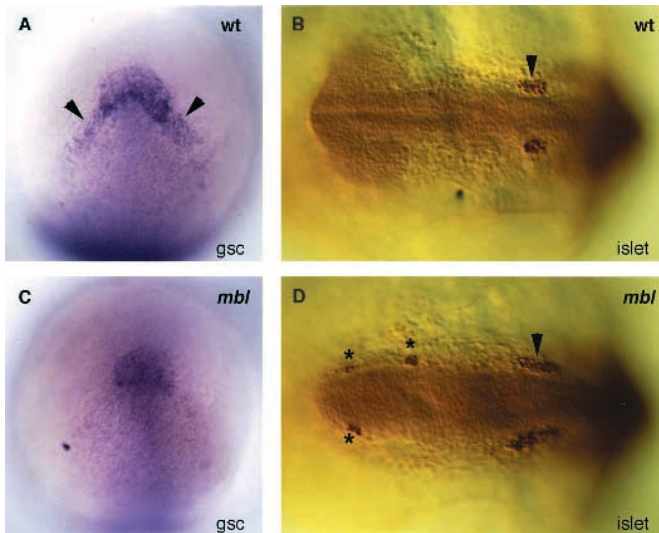
**Fig. 3.** Whole-mount antibody stainings of embryos for pax6 (30 hour), islet proteins (anti-pan-islet antibody; 24 hour), and fret43 (24 hour) visualizing the anterior pituitary and epiphysis in wild-type (A-C) and *mb1* (D-F) embryos. (A,D) Ventral view of the head of embryos stained for pax6 showing a reduction in the number of pax6-positive neurons within the anterior pituitary (arrowhead) of *mb1* embryos. (B,C,E,F) Dorsal view of the head of embryos stained for islet proteins (B,E) and fret43 (C,F) showing an increase in the number of islet/fret43-positive cells within the epiphysis (arrowhead) of *mb1*. Anterior to the left.

forebrain in the posterior diencephalon and in the presumptive anlage of the hypothalamus at 14 hours (Krauss et al., 1993; Barth and Wilson, 1995). In *mb1* mutants it forms a continuous band of strong expression extending to the anterior end of the neural keel (Fig. 4C,F). This is consistent either with an anterior extension of the posterior diencephalic expression domain or a posterior extension of the anterior expression field. In summary, these results suggest that the anlage of the telencephalon is already strongly reduced in mutant embryos at early neural keel stages whereas the anlage of the posterior diencephalon is extended anteriorly.

Since the trigeminal ganglion in *mb1* embryos appears to be abnormally shaped at 24 hours (Fig. 2B,E), we examined the anlage of the trigeminal placodes at earlier stages. By 12 hours, cells within the trigeminal placode are islet-positive in the wild type (Korzhan et al., 1993). In *mb1* embryos there are ectopic clusters of islet-positive cells anterior to the trigeminal placodes. Moreover, the trigeminal placodes that are positioned normally appear slightly enlarged in mutant embryos (Fig. 5B,D). Thus the trigeminal placodes are expanded anteriorly at the expense of the lens and olfactory placodes which are not formed in *mb1* (Fig. 1B,E and data not shown).

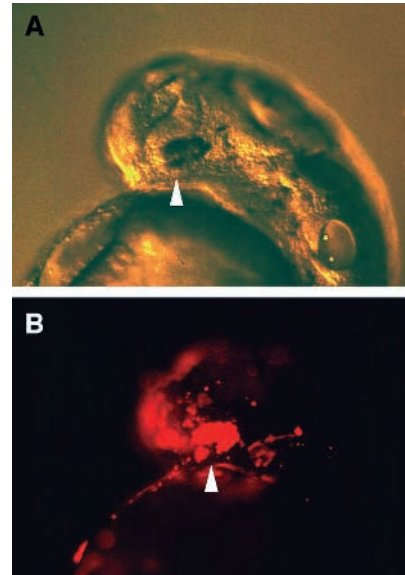


**Fig. 4.** Whole-mount in situ labeling of embryos visualizing the forebrain expression domains of *zotx-2* (14 hour), *zash-1b* (12 hour), and *shh* (14 hour) of wild-type (A-C) and *mbl* (D-F) embryos. (A,D) Lateral view of the head showing an expansion of the *zotx-2* expression domain anteriorly in *mbl* (arrowhead points to the epiphysis anlage in wild type). (B,E) Lateral view of the head showing the absence of the telencephalic expression domain of *zash-1b* in *mbl*. (C,F) Lateral view of the head showing an expansion of the hypothalamic (asterisk) and/or diencephalic expression domain (arrowhead) of *shh* in *mbl*. Anterior to the left.



**Fig. 5.** Whole-mount antibody and in situ stainings of embryos for islet proteins (anti-pan-islet-antibody) (12 hour) and *gsc* (10 hour) visualizing the trigeminal placodes (islet) and prechordal plate (*gsc*) of wild type (A,B) and *mbl* (C,D) embryos. (A,C) Dorsal view of the head showing a reduction of *gsc* expression overlying the anterior-lateral edge of the prechordal plate (arrowheads) in *mbl* (anterior up). (B,D) Dorsal view of the head showing ectopic clusters of islet-positive cells (stars) anterior to the trigeminal placodes (arrowhead) in *mbl* (anterior to the left).

The forebrain defects in *mbl* embryos may be caused by the lack of signals from the prechordal plate to the neuroectoderm. We therefore examined the expression of zebrafish *gooseoid* (*gsc*) (Stachel et al., 1993; Schulte-Merker et al., 1994a) and *fkf2* (a zebrafish forkhead domain transcription factor, Odenthal et al., unpublished data; Warga et al., unpublished data) which predominantly stain mesendodermal cells within the prechordal plate during gastrulation. Expression of *gsc* and *fkf2* within the prechordal plate before the tailbud stage (10 hours) appears normal (data not shown). At 10 hours and during the early somite stages, *gsc* expression overlying the anterior-lateral edge of the prechordal plate is clearly reduced in *mbl* mutants (Fig. 5A,C). However, this reduction of *gsc* expression may reflect a neuroectodermal rather than a mesendodermal defect since *gsc* has been reported to be primarily expressed in neuroectodermal cells in this region



**Fig. 6.** Transplantation of rhodamine-dextran labeled wild-type cells into *mbl* mutants. (A) Phenotype of a live, rescued, 30-hour *mbl* embryo showing small eyes (arrowhead). (B) UV light image of the same embryo showing that the rescued eye is exclusively formed of labeled (transplanted wild-type) cells (arrowhead). Anterior to the left.

(Thisse et al., 1994). We therefore have no clear evidence for the prechordal plate being affected in *mbl*.

#### Cell-autonomous requirement of *mbl* function within the neuroectoderm

To determine if *mbl* acts in a cell-autonomous manner, cell transplantation experiments were performed. We transplanted 10-20 rhodamine-dextran-labeled wild-type cells into mutant embryos at late cleavage stages prior to the onset of gastrulation and asked if these cells could bring about the formation of eyes in *mbl* embryos. Only egg-lays in which none of the untransplanted mutant embryos showed any sign of eyes were included in this study. In 7 out of 33 cases, eye-like structures (exhibiting characteristics of eyes such as production of melanin, expression of *pax6*; data not shown) composed of 10-30 labeled cells were found in mutant embryos (Table 2 and Fig. 6). Mutant cells were not seen to be recruited into these structures, indicating that *mbl* acts in a cell-autonomous

manner within the neuroectoderm. In all transplantations leading to the formation of eye-like structures in *mbl*, other labeled wild-type cells were observed in various regions of the forebrain (Table 2). Therefore it cannot be excluded that *mbl* is also needed in a non-cell-autonomous way within other regions of the forebrain to promote the formation of eyes.

Although two cases of transplantation of wild-type cells into the head mesenchyme of *mbl* embryos were observed (Table 2) these experiments were not performed in sufficient detail to gain insight into whether *mbl* function is also needed outside the CNS in a cell-non-autonomous manner to induce the formation of eyes.

### The zebrafish homologue to the murine *pax6* gene is not linked to *mbl*

*Pax6*, a member of the murine paired-box-containing gene family (Walther and Gruss, 1991), and its homologues have been shown to be essential for the development of eyes in such divergent species as mice and flies (Hill et al., 1991; Quiring et al., 1994). Since *mbl* is the only mutant in our screen which lacks eyes, a phenotype reminiscent of mice homozygous for mutations in the *pax6* gene (Hill et al., 1991), we tested whether *mbl* is linked to the zebrafish *pax6* homologue. A recombination analysis using a restriction fragment length

polymorphism (RFLP; as previously described by Schulte-Merker et al., 1994b) showed that *mbl* is not linked to *pax6* (data not shown).

### *silberblick (slb)* displays both a gastrulation and neuroectodermal phenotype

*slb* is a zygotic mutation with two alleles (*tx226*, *tz216*) of approximately the same phenotypic strength. *slb* displays a recessive phenotype which is variable within a single egg-lay and depends on the genetic background. The phenotype is fully penetrant at early stages, but only partially penetrant at later stages of development. Adult viability was not tested.

During the early somite stages, *slb* embryos are shorter than wild type. The polster, a structure of the anterior prechordal plate thought to contribute to the hatching gland (Ballard, 1973; Kimmel et al., 1990), appears smaller (Fig. 7A,D). At 24 hours, in about half of the mutant embryos, the retinae are not properly separated anteriorly (Fig. 7B,E) whereas other parts of the embryo appear normal. Mutants with a strong phenotype show a nearly complete fusion of the retinae, mutants exhibiting a weak phenotype have only slightly closer retinae. At 120 hours the jaw is deformed (Fig. 7C,F).

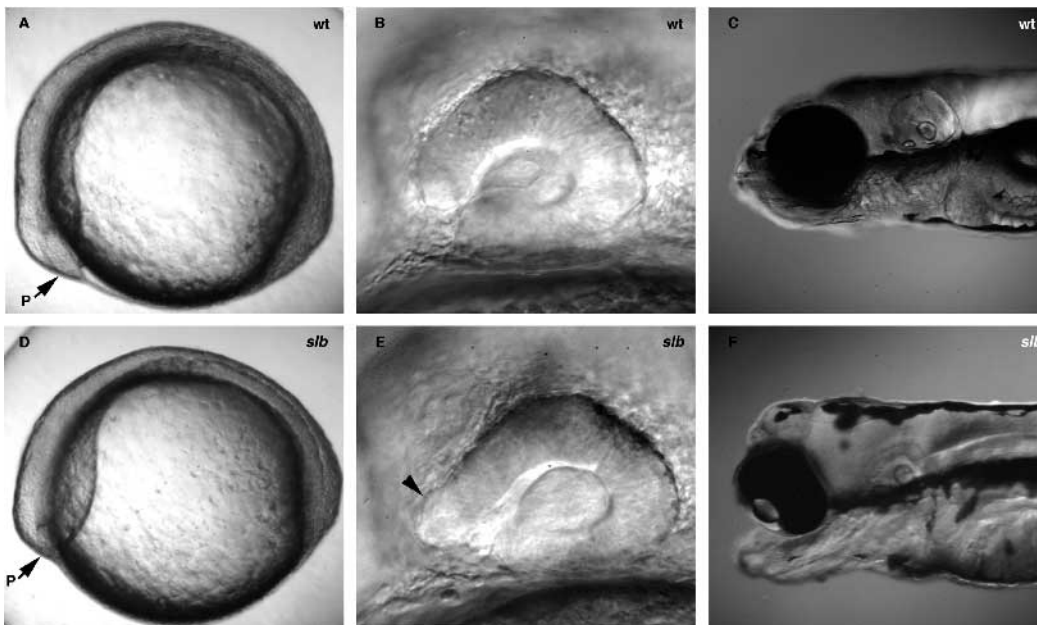
In order to elucidate the eye-phenotype of *slb* embryos, we stained embryos for *pax2* (Krauss et al., 1991a,b), which labels the presumptive optic stalk region, and *pax6* (Macdonald et al., 1994), which labels the developing retinae. At 20 hours the *pax2* expression domain is broader across the midline, suggesting that the optic stalks may be thicker (Fig. 8A,C), whereas the retinae are still separated from each other (Fig. 8B,D).

A gastrulation phenotype in *slb* mutants is transiently visible between 10 and 12 hours: the anlage of the notochord in the posterior axial mesendoderm is broadened and shortened, as visualized by the expression of no tail (*ntl*) in notochord precursor cells at 10 hours (Fig. 9) (Schulte-Merker et al., 1992). In contrast, formation of the paraxial mesoderm appears to be unaffected in *slb* mutants, as assayed by the expression of *myoD*, a marker for paraxial/presomitic mesoderm

**Table 2. Summary of the results obtained by the transplantation experiments**

Cluster position	Rescue (eye)	No rescue (no eye)	Total
Forebrain	2	6	8
Other brain	0	3	3
Others	0	2	2
Forebrain/other brain	5	12	17
Other brain/others	0	3	3
Total	7	26	33

Cluster position is the location where labeled (wild-type) cells were found in 30-hour *mbl* embryos. Other brain, mid- and hindbrain; others, head mesenchyme.



**Fig. 7.** Phenotypes of live wild-type (A-C) and *slb* embryos (D-F). (A,D) Lateral views of 11-hour embryos showing a reduced body length and a smaller polster in *slb*. (B,E) Lateral views of the head of 24-hour embryos showing a fusion of the eyes in *slb* (arrowhead points to the region of the optic stalks in *slb*). (C,F) Lateral views of the head of 120-hour embryos showing a fusion of the eyes and deformation of the jaw in *slb* embryos. P, polster. Anterior to the left.

(Weinberg et al., 1996) which is unperturbed in mutants at 10 hours (Fig. 9B,C). The prechordal plate in the anterior axial mesendoderm appears to be abnormally shaped, as shown by staining mesendodermal cells within the prechordal plate for *fkd2*. Clustering of *fkd2*-positive cells at the anterior edge of the prechordal plate, where the polster forms (Warga et al., unpublished data), is not seen in mutant embryos at 10 hours (Fig. 10A,C). In parallel to the situation in the hypoblast, the presumptive neural plate in *slb* embryos appears to be broader during gastrulation, particularly in the head region as shown by the expression of *fkd3* (a zebrafish forkhead domain transcription factor, Odenthal et al., unpublished data) and *pax2* (Mikkola et al., 1992; Püschel et al., 1992b) in the diencephalon anlage at 10 hours (Fig. 10A-D). The expression domain of *shh* in the anterior-most part of the neural keel overlying the prechordal plate is shortened in *slb* mutants (Fig. 10B,D). At later stages of development (16 hours), however, *slb* embryos show normal expression of *shh* (data not shown).

### ***knollnase (kas)* affects the formation of the telencephalic ventricle**

*kas* is a zygotic lethal mutation. A single allele (*ty122*) was found with a recessive phenotype which is distinct from all other phenotypes seen in the screen. In *kas* embryos formation of the telencephalic ventricle which underlies the dorsal midline roof plate tissue and separates the telencephalic primordia is defective, as shown in a 24-hour embryo in Fig. 11A,D. Horizontal sections through the telencephalon of 24-hour embryos stained for acetylated tubulin show that the arrangement of cells at the presumptive midline is severely disorganized in *kas* mutants (Fig. 11B,E). The malformation of the roof plate is accompanied by a defasciculation of the anterior commissure connecting the telencephalic clusters as shown by staining axons for acetylated tubulin at 24 hours (Fig. 11C,F). In contrast, commissures crossing the ventral midline such as the postoptic commissure appear normal in *kas* (data not shown). Thus in *kas* mutants the dorsal midline within the anterior forebrain does not differentiate properly.

## **DISCUSSION**

### **Screening for forebrain mutants**

The mutants analyzed in this study were isolated in the course of a large scale mutagenesis screen for mutants affecting embryonic development in zebrafish (Haffter et al., 1996). Only mutants with easily visible morphological changes in the architecture of the forebrain could have been detected. Since the forebrain in teleosts is a rather inconspicuous structure in comparison to other parts of the embryo (for instance the notochord) we may have missed a significant portion of forebrain mutants exhibiting more subtle defects. Furthermore, there may be mutants with still undiscovered defects in the forebrain, which were identified and subsequently classified on the basis of different phenotypic criteria like motility (Granato et al., 1996) and changes in the retinotectal projection (Trowe et al., 1996; Karlstrom et al., 1996).

Both *mbl* and *kas* were identified by single alleles and are therefore clearly underrepresented in comparison to other genes in this screen (Haffter et al., 1996). In the case of *kas*,

the phenotype appears so subtle that weaker alleles might have been missed. In the case of *mbl*, a mutant with an identical phenotype was found and subsequently lost, which might have been allelic to *mbl* considering the singularity of this phenotype.

### **Reduction of telencephalic and expansion of diencephalic brain structures in *mbl***

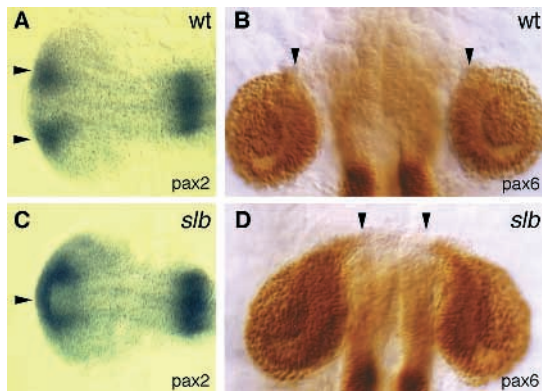
In *mbl* mutants the telencephalon, the anterior pituitary and the optic vesicles are absent or reduced in size. The epiphysis, a dorsal diencephalic structure, is expanded anteriorly. We propose that in *mbl* embryos the part of the forebrain which is reduced or absent is actually transformed into the enlarged forebrain structures. Both the expression pattern of various markers within the forebrain at early neural keel stages and the morphological alterations seen in later stages of development are consistent with this hypothesis.

The situation in the forebrain of *mbl* mutants is reminiscent of homeotic transformations of body segments described in *Drosophila* and subsequently in vertebrates (for review see Lawrence and Morata, 1994; Krumlauf, 1994): combinatorial expression of homeobox genes is known to specify segmental identities. Altering this combinatorial code by either shifting or turning off the expression of these genes leads to a transformation of segmental identities, so that a whole segment takes on a new identity. In recent studies the expression patterns of various homeobox genes within the vertebrate forebrain have been mapped and correlated to the proposed neuromere subdivisions (Simeone et al., 1992). Since the *mbl* phenotype presumably represents a transformation within the forebrain, these homeobox genes are likely candidates for involvement in the generation of the *mbl* phenotype.

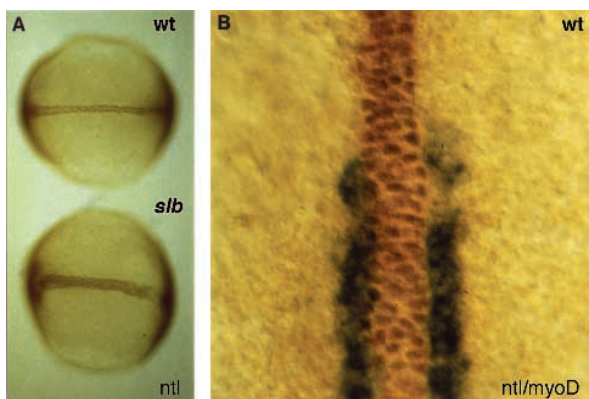
### **Induction and patterning of the forebrain in *mbl***

The nearly complete loss of a restricted subset of forebrain structures such as the telencephalon and the eyes in *mbl* mutants indicates that there may be a common pathway for the determination of these structures. This may indicate that they share segmental identity. How could such a 'segment' fit into the proposed neuromere subdivisions of the forebrain? The forebrain is thought to be subdivided into neuromeres, which are defined by morphological landmarks and the expression domains of various genes respecting the proposed neuromere boundaries (Figdor and Stern, 1993; Puelles and Rubenstein, 1993; Macdonald et al., 1994). In *mbl* mutants the reduced structures are located anteriorly to the epiphysis, whereas the epiphysis itself is enlarged. Just anterior to the epiphysis lies the middiencephalic boundary (MDB), presumably equivalent to one of the proposed neuromere boundaries separating the ventral from the dorsal thalamus, the zona limitans interthalamica described in other species (Figdor and Stern, 1993; Puelles and Rubenstein, 1993; Barth and Wilson, 1995). The MDB has been reported to overlap with the anterior diencephalic expression boundary of both *shh* and *zotx-2* (Krauss et al., 1993; Li et al., 1994; Barth and Wilson, 1995), which is shifted to the anterior end of the neural keel in *mbl* mutants. This observation, together with the finding that the morphological alterations in *mbl* embryos are restricted to an area within the forebrain anterior to the MDB, supports the view that the MDB defines a segmental border between neuromeres.

The outcome of the transplantation experiments indicates

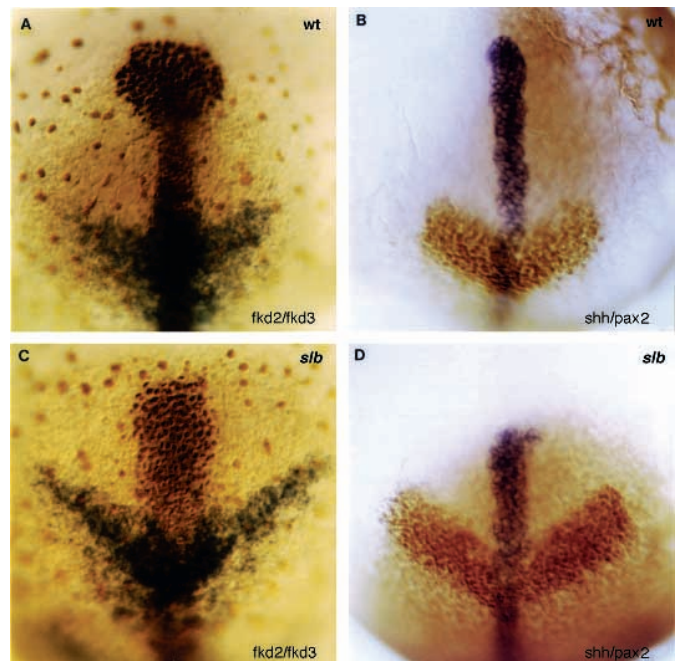


**Fig. 8.** Whole-mount antibody stainings for *pax6* and in situ stainings for *pax2* of 20-hour embryos visualizing the optic stalks and retinæ of wild-type (A,B) and *slb* (C,D) embryos. (A,C) Dorsal views of the head stained for *pax2* showing a fusion of the optic stalk region in *slb* (anterior to the left). (B,D) Dorsal views of the head stained for *pax6* showing separated retinæ in *slb*. Arrowheads indicate the region of the optic stalks (anterior up).



**Fig. 9.** Whole-mount antibody and in situ stainings of 10-hour embryos visualizing the expression of *ntl* and *myoD* in wild-type (A,B) and *slb* (A,C) embryos. (A) Dorsal view showing that the notochord anlage stained for *ntl* is broadened in *slb* embryos (anterior to the right). (B,C) Dorsal view of double labeled embryos (antibody and in situ) showing that the notochord anlage (*ntl*, brown colour) is broadened in *slb*. At the same time *myoD* expression (blue colour) in the presomitic mesoderm of *slb* embryos looks wild type (anterior up).

that *mb1* acts in a cell-autonomous manner within the neuroectoderm. It therefore appears likely that *mb1* is needed by a population of cells at the anterior end of the neural keel to differentiate into the structures missing in the mutant. What



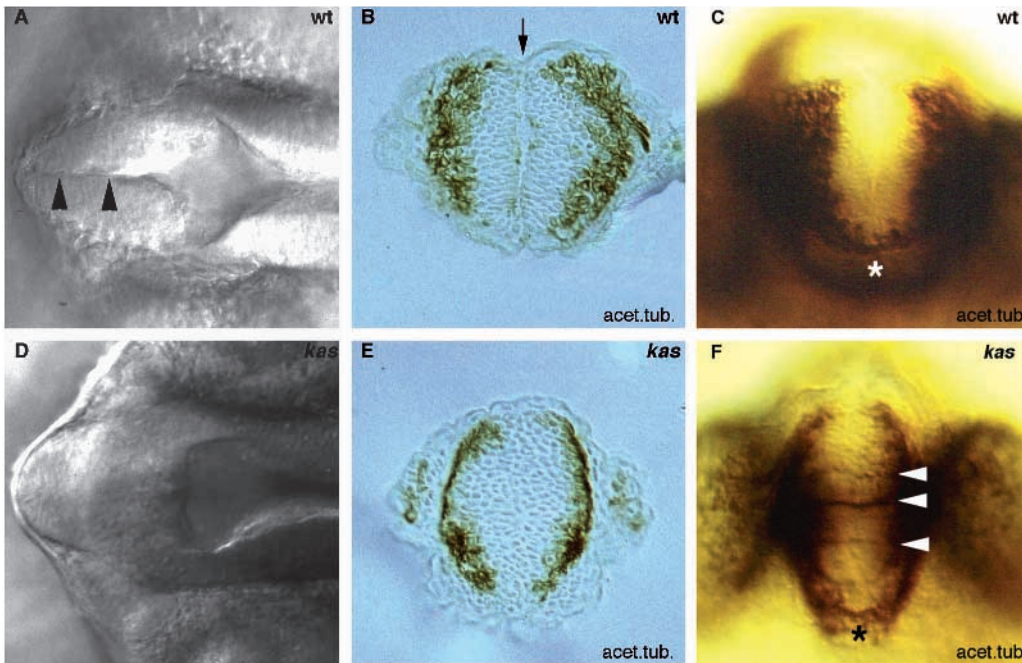
**Fig. 10.** Whole-mount antibody and in situ stainings of 10-hour embryos visualizing the expression domains of *fkd2*, *fkd3*, *pax2* and *shh* in wild-type (A,B) and *slb* (C,D) embryos. (A,C) Dorsal views of the head of double labeled (antibody and in situ) embryos showing an altered shape of the prechordal plate stained for *fkd2* (brown colour) and a broadened neuroectodermal expression domain of *fkd3* at the level of the diencephalon (blue colour) in *slb*. (B,D) Dorsal views of the head of double labeled (antibody and in situ) embryos showing a shortened expression domain of *shh* in the anterior-ventral neuroectoderm (blue colour) and broadened neuroectodermal expression domain of *pax2* (brown colour) at the level of the mid-hindbrain-boundary anlage in *slb*. Anterior up.

makes this population of cells distinct from other cells located in more posterior parts of the neural keel? It could be either the presence of signals originating from the underlying axial mesendoderm, the prechordal plate, or planar signaling within the neural plate. The observation that a nearly complete loss of the prechordal plate in the zebrafish *one-eyed-pinhead* (*oep*) mutant does not substantially affect anterior-posterior patterning of the forebrain (Hammerschmidt et al., 1996) indicates that the prechordal plate is not the source of signals specifically patterning the anterior-posterior forebrain axis. Planar signaling within the neural plate is therefore likely to be involved in determining the identity of the cells affected in the *mb1* mutant. If they lose their ability to respond to these signals, as may be the case in *mb1*, they adopt more posterior fates, resulting in a transformation of anterior into more posterior forebrain structures.

### Partitioning of the eye field in *slb*

*slb* mutants display a very restricted defect within the CNS: an anterior fusion of the eyes. The zebrafish *cyclops* (*cyc*) mutant, which has been isolated and analyzed previously (Hatta et al., 1991), shows a similar but stronger eye phenotype. The ventral midline tissue within the neural keel in *cyc* embryos is reduced (Hatta et al., 1991, 1994; Patel et al., 1994; Macdonald et al., 1994), accompanied by a reduction in the underlying axial





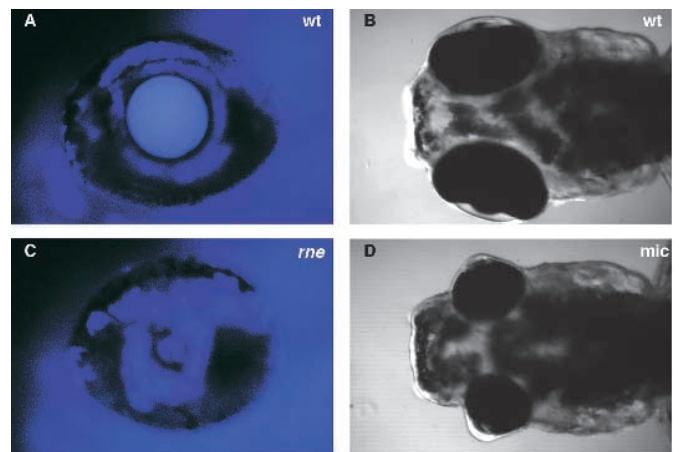
**Fig. 11.** Phenotype of live embryos (A,D) and stainings for acetylated tubulin (B,C,E,F) in 24-hour wild type (A-C) and *kas* (D-F) embryos. (A,D) Dorsal views of the head showing the absence of the dorsal midline in the anterior forebrain (black arrowheads in wild type) in *kas* (anterior to the left). (B,E) Frontal sections of the head at the level of the anterior telencephalon of embryos stained for acetylated tubulin showing a disorganization of cells at the presumptive midline (arrow in wild type) between these clusters in *kas*. (C,F) Frontal views of the head of embryos stained for acetylated tubulin showing that the anterior commissure (white arrowheads) is defasciculated in *kas*. Star indicates the position of the anterior commissure in wild type.

mesendoderm (Thisse et al., 1994; Warga et al., unpublished data). In contrast, there is no general reduction of midline tissue detectable in *slb* mutants (data not shown). Also, the type of eye fusion in *slb* differs from the *cyc* phenotype, in that the eye primordia contain *pax2*-expressing cells demarcating the area of the forming optic stalk. *Pax2*-positive cells are reduced or absent and so little or no optic stalk tissue is detectable in *cyc* mutants (Hatta et al., 1991, 1994; Ekker et al., 1995; Macdonald et al., 1995). The *slb* eye phenotype therefore seems to be due to an aberrant differentiation of midline tissue restricted to the anterior forebrain rather than the result of a massive deletion of the ventral CNS tissue as may be the case in the *cyc* mutant.

The partial fusion of the eyes in *slb* embryos may be due to the shortened expression domain of *shh* along the anterior-posterior axis during early somite stages: it has been shown that overexpression of *shh* induces *pax2* and represses *pax6* expression which is followed by a hypertrophy of optic stalk-like structures and a reduction of the retinae (Macdonald et al., 1995; Ekker et al., 1995). Anteriorly shortened *shh* expression, as seen in *slb*, may therefore lead to an ectopic induction of *pax2* and/or ectopic repression of *pax6* expression resulting in deformed optic stalks and retinae being placed closer together.

### Extension of the embryonic axis and positioning of the eyes

From the tailbud stage until the early somite stages (10-14 hours) the elongation of the body axis in *slb* mutants is delayed, as indicated by a short and broad notochord and an abnormally shaped prechordal plate. However, at the 10 somite stage (14 hours) the differentiation of the notochord and prechordal plate appears normal. The primary defect in *slb* may be a reduction in medial-lateral intercalation of cells in the axial mesoderm. Medial-lateral intercalation is thought to mediate convergence and extension movements at the dorsal side of the gastrula (Keller and Tibbets, 1989; Warga and Kimmel, 1990). Alternatively, migration of the prechordal plate towards the



**Fig. 12.** Phenotypes of live 120-hour wild type (A) and *rne* (C) embryos. (A,C) Lateral views of the eye (UV images) showing degenerating lenses in *rne* embryos. Phenotypes of live 120-hour wild type (B) and *mic* (D) embryos. (B,D) Dorsal view of the head showing smaller eyes in *mic* embryos. Anterior to the left.

animal pole may be impaired, which in turn may slow medial-lateral intercalation and thereby extension of the axis.

How may this gastrulation phenotype be linked to the neuroectodermal phenotype? It has been speculated for a long time that the initially singular eye field within the neural plate is split into two bilateral fields by means of vertical signals originating from the axial head mesendoderm, the prechordal plate, to the neuroectoderm (Adelmann, 1930; Brun, 1981). Thus the partial eye fusion in *slb* may be causally related to the deformation of the prechordal plate. We propose that the delay in the differentiation of the anterior prechordal plate into the polster (Ballard, 1973; Kimmel et al., 1990) causes the neuroectodermal phenotype in *slb*. If this is true, the polster should be involved in the separation of the eye fields within the neuroectoderm.

The analysis of the zebrafish *cyc* mutant has shown that a reduction of the prechordal plate and chordal mesendoderm is associated with both a severe reduction of the ventral CNS and a fusion of the eyes (Thisse et al., 1994; Warga et al., unpublished data). In contrast, in *slb* mutants the ventral CNS appears relatively unaffected and the mesoendodermal defect is most prominent in the polster region. It is therefore tempting to speculate that posterior prechordal plate and chordal mesendoderm are needed for general induction of ventral CNS whereas the polster is specifically required for positioning of the eye fields within the neural plate. This hypothesis is supported by the initial analysis of the *schmalspur* (*sur*) mutant where the posterior prechordal plate but not the polster is reduced, accompanied by a reduction of ventral CNS tissue but not a fusion of the eyes (Brand et al., 1996b; Warga et al., unpublished data).

An alternative scenario which may explain the eye fusion in *slb* mutants is suggested by a recent publication presenting a fate map of the zebrafish nervous system (Woo and Fraser, 1995). It has been proposed that the initial singular eye field is physically separated into two bilateral fields by diencephalic progenitor cells moving towards to the animal pole during gastrulation. An impaired extension of the neural plate in *slb* as reflected by the shortened *shh* and broadened *pax2* and *fdk3* expression fields may slow down the migration of diencephalic progenitors, leading secondarily to an incomplete separation of the eye field.

### ***kas* affects the formation of the dorsal midline in the anterior forebrain**

The analysis of the *kas* mutant phenotype shows that the dorsal midline (roof plate) separating the bilateral telencephalic clusters in the anterior forebrain is malformed in the mutant. As a consequence, axons connecting the telencephalic clusters are not bundled into one anterior commissure anymore, instead they cross the midline between the telencephalic clusters at multiple locations. It seems that *kas* specifically affects the formation of the telencephalic ventricle, which forms the roof plate in the anterior-most part of the neural keel. It will be interesting to see if anterior-lateral parts of the neural plate, which are thought to give rise to anterior-dorsal structures of the neural keel (Kimmel et al., 1994; Papan and Campos-Ortega, 1994) are already affected in this mutant.

### **Conclusion**

We isolated a class of mutants with specific defects in the development of the forebrain. The analysis of these phenotypes provides new insight into the mechanisms by which the forebrain is patterned along its anterior-posterior and dorsal-ventral axis: specification of the anterior forebrain in *mbl*, positioning of the eyes in *slb* and formation of the roof plate in *kas*. Taking advantage of the zebrafish as an experimental system, further analysis of these mutants may help in understanding the fundamental processes underlying induction and differentiation of the vertebrate forebrain.

## **APPENDIX**

### **Mutants with alterations in the size of the eyes**

We isolated several mutants which show smaller eyes at

various stages of embryonic development. A preliminary characterization of the mutant phenotypes did not reveal specific defects in the process of eye differentiation. We divided these mutants into subgroups based on the association of the eye phenotype with other phenotypes like brain necrosis and pigmentation (Table 3A). We did not find mutants with smaller eyes in which the rest of the embryo develops normally. This preliminary synopsis of mutants showing alterations in the size of the eyes will be followed by a more detailed analysis of the various eye phenotypes, including mutants which were isolated on the basis of other phenotypic criteria like retinotectal projection and motility (Trowe et al., 1996; Karlstrom et al., 1996; Granato et al., 1996). An example of a mutant with a strongly reduced eye size associated with a reduction in the total length

**Table 3A. Eye-mutants (small eyes)**

Additional phenotypes	Complementation groups	Reference
Reduced body length	<i>microps(tm329)</i>	a
Pigmentation	<i>fading vision</i> <i>sahme</i> <i>fade out</i> <i>cookie</i> <i>choco</i> <i>sunbleached</i> <i>blurred</i>	b
Pigmentation and brain degeneration	<i>vanille</i> <i>sallow</i> <i>ivory</i> <i>delayed fade</i> <i>bleached</i>	b
Pigmentation and small head	<i>milky</i>	b
Brain degeneration	<i>pistachio</i> <i>flotte lotte</i> <i>superglue</i> and multiple unnamed mutants	c
Brain degeneration and jaw	<i>flathead</i> <i>baby face</i> <i>facelift</i> <i>screamer</i> <i>duckbill</i>	e f
Motility and pigmentation	<i>touch down</i> <i>blanched</i>	b
Unresolved	<i>tc234d,tj266c,tk254b,tq262a,tu235b,tu274b,tz284,tm15</i>	a

**Table 3B. Lens mutants**

Phenotype	Complementation group	Description
Degenerating lens	<i>bumper(to20,tm127d,tg413b)</i> <i>rosine(tm70h)</i> <i>korinthe(tm292b)</i>	a
Protruding lens	<i>sunrise(tq253a)</i>	a
Colour of lens	<i>helderziend(tq291)</i>	a
Degenerating lens and small ear	<i>dreumes</i> <i>leprechaun</i> <i>earache</i> <i>ukkie</i>	g,h
Unresolved	<i>tf201,tl243</i>	a

The mutants have been subdivided on the basis of their primary and secondary (additional) phenotypes. For each subgroup the gene names and the paper where the main description of the phenotypes can be found are added.

References: a, this paper; b, Kelsh et al., 1996; c, Chen et al., 1996; d, Furutani-Seiki et al., 1996; e, Piotrowski et al., 1996; f, Schilling et al., 1996; g, Whitfield et al., 1996; h, van Eeden et al., 1996.

of the body (*microps*) at the sixth day of development is shown in Fig. 12B,D.

### Mutants with abnormal lenses

Mutants that show abnormal lens differentiation were subdivided into seven complementation groups (Table 3B): in *bumper* (3), *rosine* (1) and *korinthe* (1) the lenses begin to degenerate at the fourth day of development, in *sunrise* (1) the lenses protrude from the eyes and the cornea is closely apposed to the lens and in *helderziend* (1) the colour of the lenses appears clear instead of greenish at the sixth day of development. The remaining complementation groups, *dreumes* (1), *leprechaun* (2), *earache* (1) and *ukkie* (1) display changes in the size of the pupil combined with an abnormal differentiation of the ears and fins (Whitfield et al., 1996; van Eeden et al., 1996). We have no information as yet about which processes of lens differentiation are affected in these mutants. An example of a mutant with degenerating lenses at the sixth day of development is shown in Fig. 12A,G.

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