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Overexpression of the forebrain-specific homeobox gene *six3* induces rostral forebrain enlargement in zebrafish

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SUMMARY

The *Drosophila* homeobox gene *sine oculis* is expressed in the rostral region of the embryo in early development and is essential for eye and brain formation. Its murine homolog, *Six3*, is expressed in the anterior neural plate and eye anlage, and may have crucial functions in eye and brain development. In this study, we describe the cloning and expression of zebrafish *six3*, the apparent ortholog of the mouse *Six3* gene. Zebrafish *six3* transcripts are first seen in hypoblast cells in early gastrula embryos and are found in the anterior axial mesendoderm through gastrulation. *six3* expression in the head ectoderm begins at late gastrula. Throughout the segmentation period, *six3* is expressed in the rostral region of the prospective forebrain. Overexpression of *six3* in zebrafish embryos induced enlargement of the rostral forebrain, enhanced expression of *pax2* in the optic stalk and led to a general disorganization of the brain. Disruption of either the Six domain or the homeodomain abolish these effects, implying that these domains are essential for *six3* gene function. Our results suggest that the vertebrate *Six3* genes are involved in the formation of the rostral forebrain.

Key words: *sine oculis*, *six3*, Homeobox, Rostral forebrain, Zebrafish, Optic stalk, Eye, Brain

INTRODUCTION

Understanding the organization of the vertebrate embryonic forebrain is one of the most important issues in biology, especially since, unlike in the case of more posterior regions of the central nervous system (CNS), little is known about the molecular mechanisms underlying forebrain organization. Recent progress towards elucidating the control of embryonic forebrain development has been made through the identification of regionally specific genes whose combinatorial expression may direct the development of distinct regions in the forebrain. Rubenstein and his colleagues proposed that the forebrain is subdivided into six transverse domains named prosomeres (reviewed in Puelles and Rubenstein, 1993; Rubenstein et al., 1994). The prosomeres can be grouped into the diencephalon (p1 to p3) and the secondary prosencephalon (p4 to p6). P6, the most rostral subregion of the forebrain, includes some distinct tissues such as optic stalk, olfactory bulb, commissural plate and chiasm. Recent studies have shown that homeobox genes, such as members of the *Emx* and *Otx* family, have a central role in the patterning of more caudal regions of the vertebrate forebrain and other homeobox genes may participate in the regionalization of its most rostral sections. The newly identified homeobox gene *Six3*, which is expressed in the most rostral aspect of the forebrain in the mouse and chicken, is a candidate for such a function (Oliver et al., 1995a; Kawakami et al., 1996b; Bovolenta et al., 1998).

Mammalian *Six* family genes were identified by homology to the *Drosophila sine oculis* (*so*) gene (Oliver et al., 1995a,b) and by the binding of a Six protein to the promoter of the Na,K-ATPase α1 subunit gene (Kawakami et al., 1996a,b). The *so* gene encodes a homeodomain protein and is essential for *Drosophila* eye formation (Cheyette et al., 1994; Serikaku and O'Tousa, 1994). Five members of the mouse *Six* gene family have been characterized. Of these, *Six1* and *Six2* are expressed in head and body mesenchyme, limb muscles and tendons (Oliver et al., 1995b); *Six3* is expressed in the anterior forebrain and in the eyes (Oliver et al., 1995a; Kawakami et al., 1996b); *Six4/AREC3* is expressed in neural tissues and encodes the transcription factor regulating the Na,K-ATPase α1 subunit gene (Kawakami et al., 1996a; Ohto et al., 1998), and *Six5/mDMAHP* is expressed in a wide variety of mouse tissues (Boucher et al., 1995; Kawakami et al., 1996b; Heath et al., 1997). The human *SIX5/DMAHP* gene maps immediately 3′ to the CTG repeat which is known to associate with myotonic dystrophy (Boucher et al., 1995; Klesert et al., 1997; Thornton et al., 1997). It has been reported that mutations in *Drosophila so* genes lead to defects of the adult and/or larval visual system (Cheyette et al., 1994; Serikaku and O'Tousa, 1994) and that ectopic expression of mouse *Six3* in medaka embryos promotes ectopic lens formation in the area of the otic vesicle (Oliver et

al., 1996). A recent report by Pignoni et al. (1997) has demonstrated that ectopic co-expression of *so* with *eyes absent* (*eya*) leads to ectopic eye tissues in the *Drosophila* antennal, wing and leg discs. These findings suggest a conserved function for vertebrate *Six3* and *Drosophila so* in visual system development. In *Drosophila*, the structure of the brain is also affected by the *so* mutation (Serikaku and O'Tousa, 1994), implying that *Six3* may have a function in brain formation.

Organization and subdivision in the vertebrate forebrain remain more controversial than those in the posterior brain. Molecular studies of the embryonic forebrain in lower vertebrates such as the zebrafish, *Danio rerio*, may reveal evidence of such subdivision. In this article, we report the cloning of the zebrafish *six3* gene and a study of its expression pattern during embryonic development, demonstrating conservation of *Six3* sequence and expression pattern among the zebrafish, mouse and chicken. Overexpression of *six3* in zebrafish embryos induced an enlargement of the rostral region of the forebrain including the optic stalk, providing evidence for a role of *six3* in the development of the rostral forebrain.

MATERIALS AND METHODS

Isolation of *six3* cDNA

Degenerate oligonucleotide primers against the conserved regions among mouse *Six1*, *Six2*, *Six3* and *Six4/AREC3* (Oliver et al., 1995a,b; Kawakami et al., 1996a) were synthesized, and RT-PCR was performed using these primers and total RNA from zebrafish embryos. The following oligonucleotide primers were used (see also Fig. 1A,C): CCGGATCCAT(A/C/T)TGGGA(C/T)GG(A/C/G/T)GA-(A/G)GA(A/G)AC (sense strand corresponding to IWDGEET plus *Eco*RI linker), CCGGATCCGT(A/C/G/T)TA(C/T)TG(C/T)TT(C/T)-AA(A/G)GA(A/G)AA (sense strand corresponding to VYCFKEK plus *Eco*RI linker), and CCGTCGACAACCA(A/G)TT(A/C/G/T)-(A/T)(G/C)(A/C/G/T)AC(C/T)TG(A/C/G/T)GT (antisense strand corresponding to TQVSNWF plus *Sal*I linker). Conditions of PCR were the same as described previously (Taira et al., 1992). PCR product was purified from an agarose gel and was subcloned into pBluescript II KS+. The zebrafish *six3* cDNA was isolated from a λZap shield stage cDNA library (Rebagliati et al., 1998) using these PCR-derived clones as probes.

Embryo isolation and RNA injection

Adult fish were kept at 28.5˚C on a 14-hour light/10-hour dark cycle, and embryos were obtained by natural matings (Westerfield, 1995). Some embryos were raised in 0.2 μM phenylthiocarbamide (Sigma) to inhibit pigment formation, as described previously (Hyatt et al., 1992). For sections, embryos were embedded in Technovit 7100 (Heraeus Kluzer GmbH, Germany) after fixation with 4% paraformaldehyde/PBS. Transverse sections were prepared at 10 μm, and were stained with hematoxylin. RNA injection was performed as described previously (Toyama et al., 1995b).

Plasmid construction and in vitro transcription

The *six3* injection construct was made by inserting the coding region of zebrafish *six3* into the vector pCS2+ (Rupp et al., 1994) to make pCS2six3. For the deletion constructs, *six3* cDNA in pBluescript II KS+ was digested with *Msc*I-*Hin*dIII, *Hin*dIII-*Kpn*I, or *Kpn*I-*Not*I, followed by being digested both protruding ends with Mung Bean nuclease or filled recessed 3′ ends with Klenow fragment, and was religated with T4 DNA ligase. The coding regions of these deleted *six3* were inserted into the pCS2+ to generate pCS2six3d1, pCS2six3d2 and pCS2six3d3, respectively. All constructs were verified by sequencing. Synthetic capped RNA was transcribed from these plasmids or pSP64-Xβm (Krieg and Melton, 1984) using the SP6 Megascript in vitro transcription kit (Ambion) with $m^7G(5')ppp(5')G$ (Boehringer Mannheim).

Whole-mount in situ hybridization and immunostaining

Embryos were analyzed by whole-mount in situ hybridization as described by Toyama et al. (1995b). The antisense *six3* probe contained the entire region of *six3* cDNA. Digoxigenin-labeled RNA probes were prepared according to the instruction of the manufacturer (Boehringer-Mannheim). We used RNA probes prepared from cDNAs of the zebrafish *emx2* (Morita et al., 1995), *dlx3* (Akimenko et al., 1994), *gsc* (Stachel et al., 1993), *Islet-1* (Inoue et al., 1994), *lim3* (Glasgow et al., 1997), *lim5* (Toyama et al., 1995a), *ntl* (Schulte-Merker et al., 1992), *otx2* (Mori et al., 1994), *pax2* (Krauss et al., 1991) and *shh* (Ekker et al., 1995). Frozen sections of whole-mount-stained embryos were prepared at 10 μm. Whole-mount immunostaining using antibody against acetylated α-tubulin (Sigma, 1:500 dilution) and the Vectastain Elite ABC kit (Vector Laboratories) was performed by the protocol of Miyagawa et al. (1996).

RESULTS

Isolation of zebrafish *six3* cDNA

To search for zebrafish *Six* genes, we designed two sets of degenerate primers based on conserved sequences in the Six domain and the homeodomain (Kawakami et al., 1996a; Oliver et al., 1995a,b). RT-PCR analyses using these primers were performed using total RNA prepared from zebrafish embryos of various stages as templates and the resulting PCR products were subcloned. The nucleotide sequences of these clones revealed the existence of at least seven zebrafish *Six* genes (data not shown). RT-PCR analyses with specific primer pairs based on these sequences showed that four different *Six* genes, including a *Six3* homolog, are expressed at the shield stage. A shield stage cDNA library was screened with a mixture of these four *Six* probes at low stringency, resulting in 83 positives among 2×10^6 phage, three of which were independent isolates of the zebrafish *six3* homolog; the other zebrafish *Six* genes will be discussed elsewhere. An in-frame termination codon exists 105 bp upstream of the putative translation initiation codon in the zebrafish *six3* cDNA sequence (data not shown), making it likely that this methionine is the actual N terminus of the protein. The amino acid sequence of zebrafish six3 protein has striking similarity with that of Six3β, deduced from one of the three known alternatively spliced *Six3* mRNAs in the mouse (Fig. 1A; Kawakami et al., 1996b). The overall identity between mouse Six3β and zebrafish six3 is 76% at the nucleotide level and 85% at the amino acid level. The only prominent difference between these two proteins is that zebrafish six3 does not have the glycine tracts in the N-terminal region present in mouse Six3β (Fig. 1A). Intermediate numbers of glycine residues in the chicken homolog cSix3 suggests that this glycine tract may expand during evolution for an unknown reason (Fig. 1A; Bovolenta et al., 1998). While all Six family proteins, as well as the entire bicoid class (Bürglin, 1994), share a lysine at position 50 of the homeodomain, sequence similarity between zebrafish six3 and other family members is much lower than with mouse Six3β and chicken cSix3 (Fig. 1B,C). It is notable that the nematode clone W05E10.3 encodes a Six-related protein with a homeodomain highly homologous

to vertebrate Six3 (Fig. 1B,C), while clone C10G8.7 encodes a protein more similar with mouse Six1 (data not shown). Thus it appears that divergence within the Six family is ancient in metazoans, and that Six3 homeodomain sequences have been conserved during a long period of evolution.

six3 expression in anterior axial mesendoderm at gastrula and anterior CNS during segmentation

The expression patterns of zebrafish *six3* during embryogenesis were examined by whole-mount in situ hybridization. The *six3* transcript was undetectable until the shield stage (data not shown). *six3* is expressed in hypoblast cells at the shield stage (Fig. 2A), and at the anterior edge of involuting axial mesendoderm during gastrulation (Fig. 2B). As gastrulation proceeds, *six3* mRNA-expressing cells become increasingly limited to the anterior, the presumptive prechordal plate, not overlapping with *ntl* mRNA-expressing cells which form the presumptive notochord (Fig. 2C,G; Schulte-Merker et al., 1992). The expression pattern in the axial mesendoderm is similar to that of *gsc* (Stachel et al., 1993; Schulte-Merker et al., 1994; Thisse et al., 1994), although *six3* is more limited to the most anterior edge (Fig. 2D-F). *Six3* expression in the mesendoderm has been also observed in the chicken (Bovolenta et al., 1998).

six3 starts being expressed at the anterior edge of the presumptive neuroectoderm at the 80-85% epiboly stage (data not shown). At the beginning of the segmentation period, *six3* is expressed in the rostral end of both mesendodermal and ectodermal cells (Figs 2D, 3A,D), with the former fading during somitogenesis, leaving *six3* mRNA-expressing cells confined to the rostral region of the prospective forebrain (Figs 2H,I, 3B). These cells will develop into the telencephalon, retina and part of diencephalon according to the fate map of Woo and Fraser (1995). A gap between *six3* and *pax2* mRNA-expressing cells implies that most of the prospective midbrain is *six3* negative (Fig. 2H). In the prospective forebrain, *six3* is expressed in superficial cells in the neural rod and in the overlying ectoderm at the 6-somite stage (Fig. 3B,E) and, by the 16-somite stage, becomes confined to the lateral surfaces of the retina and lens (Fig. 3C,F).

In the 24 hour embryo, *six3* is expressed in rostral cells of the telencephalon and ventral diencephalon (Fig. 4A). These regions correspond to p6, the most rostral subregion of the forebrain, as defined by Rubenstein and colleagues in the mouse (Puelles and Rubenstein, 1993; Rubenstein et al.,

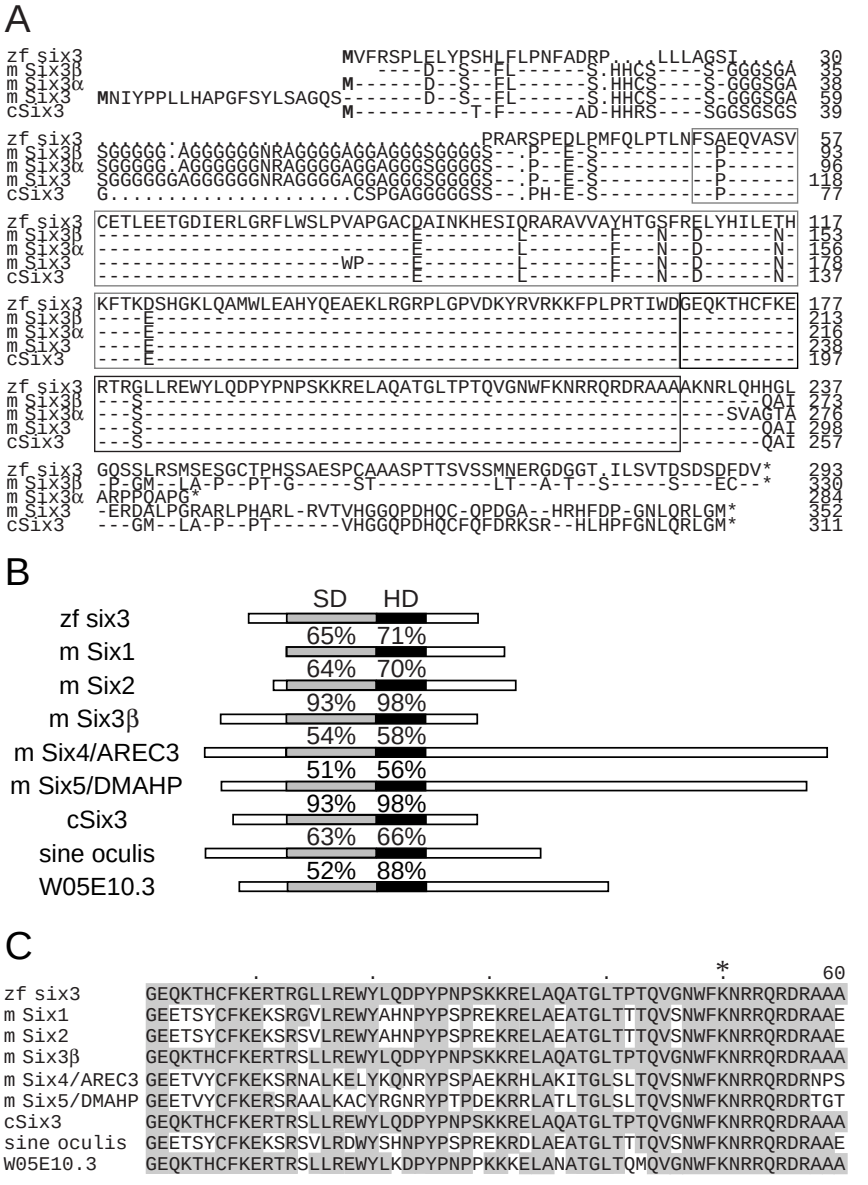


Fig. 1. Sequence comparison of zebrafish six3 protein and other Six proteins predicted from cDNA sequences. (A) Sequence alignment of zebrafish (zf) six3, mouse (m) Six3 and chicken cSix3. The Six domain and the homeodomain are boxed with dotted and solid lines, respectively. Dash indicates identity, dot indicates gap inserted for better alignment, asterisk denotes the termination codon, bold M denotes putative initiation methionine. The nucleotide sequence data of zebrafish *six3* has been deposited in the DDBJ/EMBL/GenBank databases with the accession number AB004881.
(B) Comparison of Six proteins. The gray and black boxes indicate the Six domain (SD) and the homeodomain (HD), respectively. Percentage of sequence identity between zebrafish six3 and other Six proteins are indicated. (C) Sequence alignment of the homeodomains. Identities with zebrafish six3 proteins are in gray background. Asterisk indicates the conserved lysine at position 50 in the homeodomain. Sources: mouse Six1 (Oliver et al., 1995b), mouse Six2 (Kawakami et al., 1996b), mouse Six3β (Kawakami et al., 1996b), mouse Six3α (Kawakami et al., 1996b), mouse Six3 (Oliver et al., 1995a), mouse Six4/AREC3 (Kawakami et al., 1996a), mouse Six5/DMAHP (Kawakami 1996b), chicken cSix3 (Bovolenta et al., 1998), *Drosophila* sine oculis (Cheyette et al., 1994), *Caenorhabditis elegans* W05E10.3 (accession number, Z77670).

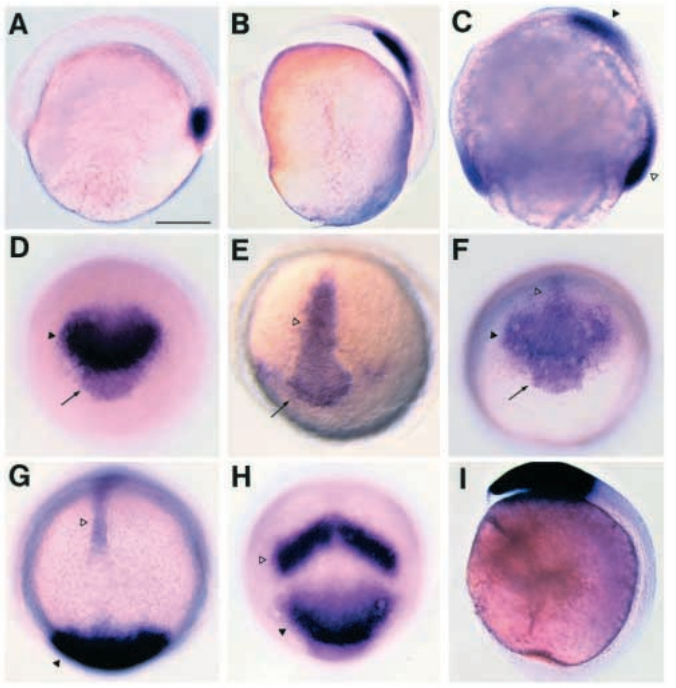


Fig. 2. Expression of *six3* in early embryogenesis. (A-C,I) Lateral views with dorsal to the right; (D-H), dorsoanterior views with posterior to the top. (A) 55% epiboly; *six3* starts being expressed in the hypoblast of the embryonic shield. (B) 75% epiboly; *six3*signal is localized in the anterior axial mesendoderm. (C) 80% epiboly; embryo was hybridized with *six3* and *ntl* probes together using the same color; *six3* is expressed only in the anterior (black triangle), whereas *ntl* is expressed posteriorly (open triangle). (D-H) Tail bud stage; embryos were hybridized with *six3* (D), *gsc* (E), *six3+gsc* (F), *six3+ntl* (G), *six3+pax2* (H), using the same color. (D-F) *six3* is expressed in ectodermal cells of the prospective forebrain (black triangle). *six3* expression in the anterior axial mesendoderm is delimited to the polster (arrow), while *gsc* expression is posteriorly expanded (open triangle). (G) *six3* is not expressed in the presumptive notochord; there is a gap between *six3* (black triangle) and *ntl* (open triangle) mRNA-expressing cells. (H) *six3* is expressed in the prospective forebrain, indicated by a gap between *six3* (black triangle) and *pax2* (open triangle) mRNA-expressing cells. (I) Two-somite stage; *six3* expression is sharply delimited to the rostral brain. Scale bar, 200 μm.

1994). Ross et al. (1992) proposed that the rostral surface of the brain bends downward at the transverse ventral flexure, making a dorsal surface to be the most anterior portion of the embryo; this rostral surface is essentially identical to the *six3* mRNA-expressing region. This broad rostral expression pattern is transient and, by 36 hours, *six3* mRNA are confined to specific areas within the rostral surface. To identify *six3*-positive cells at this stage, we double-stained embryos with *six3* probe and with antibody against acetylated α-tubulin to visualize the axonal tracts (Chitnis and Kuwada, 1990). *six3* is expressed in medially located cells of the telencephalon, the ventral tip of the olfactory bulb and the optic stalk (Fig. 4B,C), and also in the pituitary anlage visualized as *lim3*-positive cells (Fig. 4D,E; Glasgow et al., 1997). The cells around the postoptic commissure (POC) and those around the intersection of the supraoptic tract (SOT) and the anterior commissure (AC) are also *six3* positive (Fig. 4B,C). These tissues were shown to have a common origin from the anterior neural ridge (ANR) in *Xenopus* (Eagleson et al., 1995). *six3* starts being expressed in the ventral midbrain tegmentum at 24 hours and the expression becomes stronger at later stage (Fig. 4A,B,D). *six3* is expressed in the entire eyes at 24 hours, with the expression fading later except in the lens and its neighbors (Fig. 4C; data not shown).

In summary, *six3* is expressed at the anterior end of the mesendoderm and in the prospective forebrain in early development, and in the rostral surface of the brain and its derivatives in later development.

Overexpression of *six3* leads to enlarged anterior head structures

With the goal of studying *six3* function during development, we injected synthetic *six3* mRNA into zebrafish embryos at the 2-cell stage, resulting in mosaic but fairly even distribution of the injected mRNA by early somitogenesis. Injected embryos exhibited specific morphological defects of the brain area (Fig. 5A,B). In control embryos, three ventricles were apparent in the brain, the diencephalic, mesencephalic and rhombencephalic ventricles. These ventricles were not well formed in *six3* mRNA-injected embryos, despite the obvious presence of a lumen within the neural tube. In addition, the constriction normally present at the midbrain-hindbrain boundary was absent. Most *six3* mRNA-injected embryos showed defects in the head region while tissues in the trunk such as notochord, somites and spinal cord appeared normal (Fig. 7B; data not shown).

six3 mRNA-injected embryos showed an enlargement of the telencephalon between AC and epiphysis as seen by *Islet-1* staining (Fig. 5C,D; Inoue et al., 1994). In contrast, the region between epiphysis and otic vesicles, including the midbrain and anterior hindbrain, was shortened and broadened. Immunostaining with antibody against acetylated α-tubulin (Fig. 5E-H) revealed that the axonal tracts of AC, POC and SOT are elongated in *six3* mRNA-injected embryos, and the preoptic area which is surrounded by these axonal tracts is enlarged (Fig. 5G,H); in contrast, axons of the posterior commissure (PC) were reduced. Telencephalic neuronal clusters were enlarged and disorganized (e.g., in Fig. 5F).

Transverse sections at the level of the prospective forebrain of *six3* mRNA-injected embryos at early somitogenesis stages show that cell number in the dorsal but not ventral neural tube was increased (Fig. 6A,B) while, at the level of the midbrain, the structure was disorganized, but cell number appeared largely unchanged (Fig. 6C,D). These observations suggest that one reason for the disorganization of the head may be an excessive accumulation of cells in the anterior/dorsal neural tube, enlarging the forebrain and compressing the midbrain and anterior hindbrain into a short and broadened shape.

Six domain and homeodomain are essential for *six3* gene function

Both the Six domain and the homeodomain are conserved among vertebrate and invertebrate Six proteins (Fig. 1). The C-terminal half of the Six domain and the entire homeodomain of mouse Six4/AREC3 have been shown to be essential for sequence-specific DNA binding (Kawakami et al., 1996a). In addition, the Six domain of *Drosophila* so

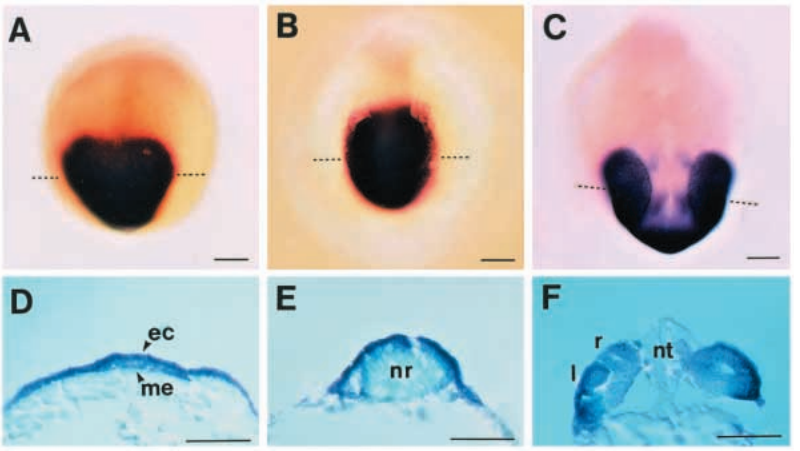


Fig. 3. *six3* expression in the head ectoderm. Dorsoanterior views (A-C, posterior to the top) and corresponding transverse sections (D-F). Dotted lines in A-C indicate approximate levels of the sections. (A,D) Two-somite stage; *six3* is expressed in both ectodermal and mesendodermal cells. (B,E) Six-somite stage; *six3* mRNA is present primarily in ectodermal cells. (C,F) 16-somite stage; *six3* expression is restricted to the eye and the rostral regions of the forebrain. The section shows that *six3* expression in both retina and lens is dominant in surface cells, while the neural tube is largely *six3* negative. Abbreviations: ec, ectoderm; l, lens; me, mesendoderm; nr, neural rod; nt, neural tube; r, retina. Scale bars, 100 μm.

binds to eya protein, a presumptive transcriptional co-activator, and may mediate their synergistic function (Pignoni et al., 1997). To examine whether these domains are required for *six3* function, we overexpressed *six3* derivatives with deletions in the Six domain and/or the homeodomain in zebrafish embryos (Fig. 7A). Northern blots revealed that the stability of injected six3d1, six3d2 and six3d3 mRNAs was similar to that of wild-type mRNA at 24 hours (data not shown). About 70% of wild-type *six3* mRNA-injected embryos showed head-specific defects, while no embryos displayed such a phenotype after mRNA injection of either *six3* derivatives, even when the amount of injected mRNA was increased five-fold (Fig. 7B). General defects seen in some six3d1 mRNA-injected embryos may due to nonspecific effects of the homeodomain. These results not only confirm that the phenotype in *six3* mRNA-injected embryos is due to overexpression of *six3* gene product but also demonstrate that both the Six domain and the homeodomain are essential for *six3* gene function.

six3 overexpression modulates marker genes expression

While no changes were observed in *gsc* expression in the anterior axial mesendoderm at the 75% epiboly or tail bud stages (data not shown), *six3*-overexpressing embryos exhibited modified expression of subregion-specific marker genes in the CNS during later embryogenesis. During somitogenesis, *lim5* mRNA forms a tightly delimited stripe in the diencephalon in normal embryos (Toyama et al., 1995a), which moved posteriorly in *six3* mRNA-injected embryos especially in its dorsal aspect (Fig. 8A,B), indicating an expansion of the rostral forebrain. Such expansion was also indicated by the pattern of *otx2*, which is expressed in the dorsal diencephalon and optic tectum in normal 24-hour embryos (Li et al., 1994; Mori et al., 1994); overexpression of *six3* led to an enlarged rostral *otx2*-negative region and a shortened *otx2*-positive region (Fig. 8C,D). The domain of *shh* expression in ventral regions of the brain (Krauss et al., 1993; Ekker et al., 1995) showed only slight expansion after *six3* mRNA injection (Fig. 8E,F). In the case of *emx2*, which is expressed both dorsally in the telencephalon and ventrally in the hypothalamus (Morita et al., 1995), overexpression of *six3* caused an expansion of the expression domain in the dorsal but not ventral forebrain, even though both regions appear enlarged (Fig. 8G,H). Genes expression in areas posterior to the otic vesicles was unaffected, such as *Islet-1* expression in the spinal chord and *shh* expression in the floor plate and notochord (data

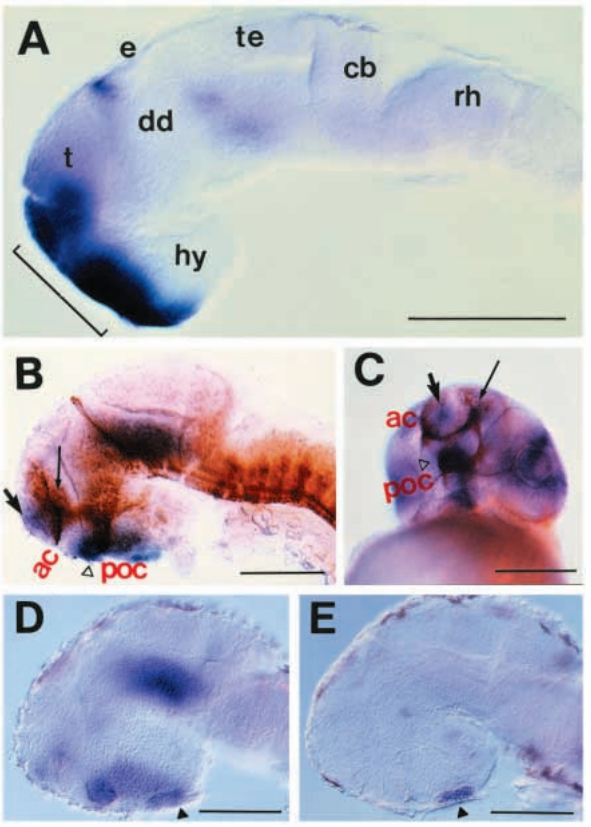


Fig. 4. *six3* expression in later development. (A) Lateral view of 24-hour embryo showing *six3* expression at the rostral surface of the forebrain (bracket); the eyes were removed. (B,C) Lateral (B) or frontal (C) view of *six3* expression at 36 hours. The *six3* signal (blue) is localized in the medial telencephalon (arrow), optic stalk (open triangle) and near the olfactory nerve (thin arrow). Axons were stained with antibody against acetylated α-tubulin (brown). (D,E) Lateral views of *six3* (D) or *lim3* (E) expression at 48 hours. *six3* is expressed in the pituitary anlage (black triangle). Additional abbreviations: ac, anterior commissure; cb, cerebellum; dd, dorsal diencephalon; e, epiphysis; hy, hypothalamus; poc, postoptic commissure; rh, rhombomeres; t, telencephalon; te, tectum. Scale bars, 200 μm.

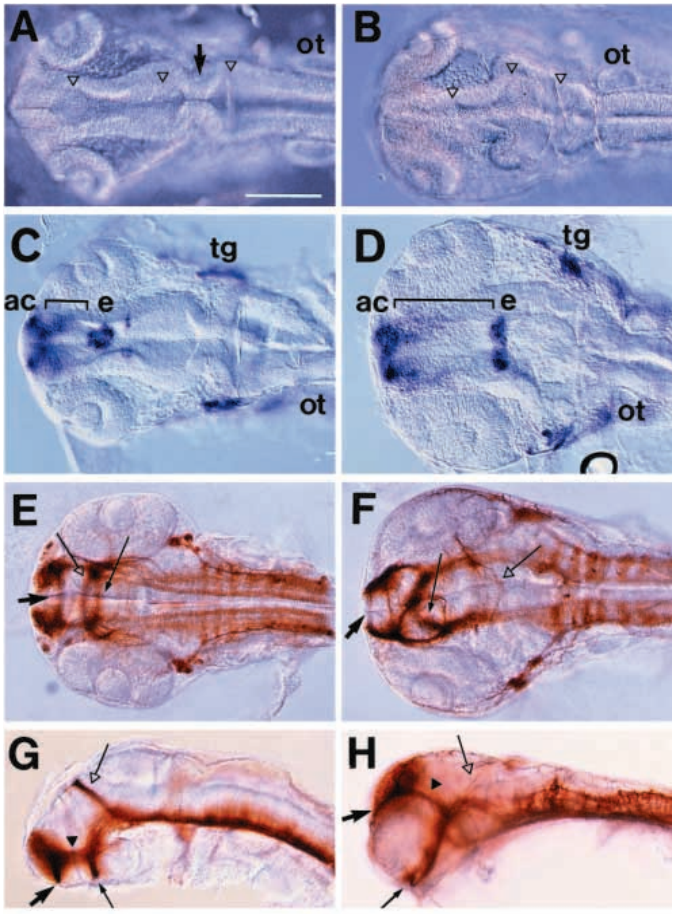


Fig. 5. Disorganized head formation in *six3* mRNA-injected zebrafish embryos. 20 pg of globin (A,C,E,G) or *six3* mRNA (B,D,F,H) were injected into 2-cell-stage zebrafish embryos; see Fig. 7 for quantitative results. (A,B) Dorsal views at 24 hours. Three ventricles (open triangles) in the brain are filled with masses of cells and the midbrain-hindbrain boundary (arrow) is disorganized in *six3* mRNA-injected embryos. (C,D) Dorsal views of *Islet-1* expression at 24 hours. The region between AC and epiphysis (bracket) is enlarged in *six3* mRNA-injected embryos. Likewise, the distance between left and right trigeminal ganglia is expanded. (E-H) Dorsal (E,F) or lateral views (G,H) of 36 hour embryos labeled with antibody against acetylated α-tubulin. AC (arrow), POC (thin arrow), SOT (black triangle) and region surrounded by them are enlarged, while axons in PC (open arrow) are reduced in *six3* mRNA-injected embryos. Additional abbreviations: ot, otic vesicle; tg, trigeminal ganglion. Scale bar, 200 μm.

not shown). Furthermore, no obvious changes were observed in *lim3* or *dlx3* expression in the pituitary anlage and olfactory placodes, respectively (data not shown).

Thus, the expression of marker genes indicates an expansion of the rostral forebrain in *six3* mRNA-injected embryos especially in the dorsal domain (Fig. 8), but gross morphological appearance (Fig. 8) and the modified arrangement of axonal pathways (Fig. 5G,H) suggests that ventral regions are enlarged as well.

six3 overexpression induces enlargement of the optic stalk

Because evolutionary considerations implicate the *six3* gene in visual system development, we tested *pax2* expression in *six3* mRNA-injected embryos. During segmentation, *pax2* is expressed primarily in the optic stalk, midbrain-hindbrain boundary and otic vesicles (Krauss et al., 1991). Of these major expression sites, *pax2* staining in the prospective optic stalk at the 10-somite stage was enlarged in *six3* mRNA-injected embryos, while the signal at the midbrain-hindbrain boundary and in the otic vesicles appeared unaffected (Fig. 9A,B). In normal 24-hour embryos, the optic stalk as visualized by *pax2* staining, was located ventrally because of the bending of the rostral forebrain (Fig. 9C). In contrast, the optic stalk in *six3* mRNA-injected embryos was displaced to a frontal location (Fig. 9D), indicating that the flexure of the forebrain had failed to occur. The base of the optic stalk in the vicinity of the choroid fissure at the anterior retina was dramatically enlarged in *six3* mRNA-injected embryos (Fig. 9E-H), indicating that *six3* is involved in optic stalk formation. Though the region of *pax2*-negative cells in the retina remained essentially unchanged, the extension of *pax2*-positive regions caused an enlargement of the retina.

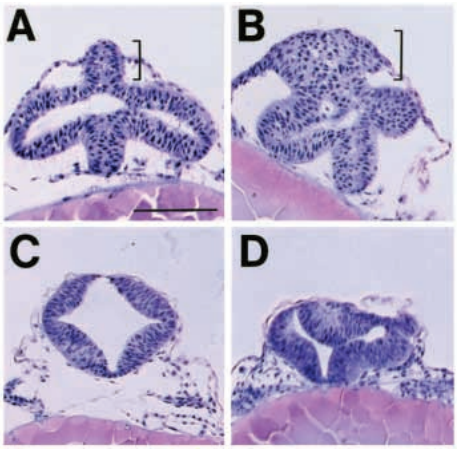


Fig. 6. The dorsal neural tube expands in *six3* mRNA-injected embryos. Transverse sections of embryos injected with globin (A,C) or *six3* mRNA (B,D). (A,B) Six-somite stage; sections through the prospective forebrain and optical vesicles. Dorsal (bracket) but not ventral region contains extra cells in *six3* mRNA-injected embryos. The size of cells is basically unchanged. (C,D) 24 hours; Sections through the midbrain. Structure of dorsal portion of the neural tube is disorganized, but cell number is not changed substantially. Scale bar, 100 μm.

DISCUSSION

Six3 is highly conserved among zebrafish, mouse and chicken

The amino acid sequence of the zebrafish six3 protein is highly similar to mouse Six3β and chicken cSix3 (Fig. 1). We suggest that zebrafish *six3* is the ortholog of mouse *Six3* and chicken *cSix3* by the following criteria. (1) The entire amino acid sequence of these proteins is highly conserved and is distinct as compared to other Six proteins. (2) The expression pattern is similar for zebrafish *six3*, mouse *Six3* and chicken *cSix3* (Oliver et al., 1995a; Bovolenta et al., 1998); these genes are

first expressed at early gastrula and later are limited to the rostral forebrain.

The amino acid sequence of *Drosophila* sine oculis (so) is more similar to those of mouse Six1 and Six2 than of Six3. Although the predicted amino acid sequence of so is not closely similar to vertebrate Six3, there are some similarities in the expression pattern and suggested function of these genes. Thus, no clear orthology relationships can be proposed between so and vertebrate Six proteins. It is therefore somewhat surprising that the nematode, *Caenorhabditis elegans*, has both a *Six1*-related and a *Six3*-related gene in its genome. In this context, it is possible that there is another *Six3*-related *so* gene in *Drosophila*.

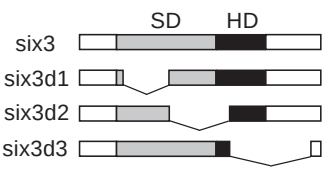
six3 may function in anterior forebrain formation

Zebrafish *six3* is expressed in the most rostral regions of the forebrain throughout early development. On the basis of explant culture experiments in mouse embryos, Shimamura and Rubenstein (1997) have proposed that an anterior domain, the ANR, is a local organizing center for the patterning of the rostral forebrain. Similarly, a recent report by Houart et al. (1998) has implicated a signal from a small group of anterior neuroectodermal cells in zebrafish embryos at the 75% epiboly stage in the induction of the anterior forebrain-specific genes and patterning of the rostral brain. Since *six3* expression in the neuroectoderm begins at the 80-85% epiboly stage, and overexpression of *six3* induces the expansion of rostral forebrain, *six3* might be a downstream target and possible mediator of this putative rostral brain organizing signal.

While it is not clear whether *six3* or any other member of the *Six* family is an ortholog of *Drosophila so*, there are important functional similarities between *so* and *six3*. Serikaku and O'Tousa (1994) have shown that the most prominent defect in the so^{mda} allele is the absence of an optic stalk, which prevents a physical connection between the developing eye and the brain; thus, *so* appears to be essential for optic stalk formation. This conclusion is consistent with our results based on overexpression experiments, implicating *six3* in the formation and patterning of the rostral forebrain, especially of the optic stalk.

Oliver et al. (1996) reported that ectopic expression of mouse *Six3* from DNA expression constructs in medaka, a fish distantly related to zebrafish, induced ectopic lens formation within the otic vesicles in 2.5% of injected embryos. In our experiments, the effect of mRNA injection was more drastic, generating effects in the retina/optic stalk and the forebrain at

A



B

Injected mRNA		Total embryos	Survived at 24 hours	Normal	Head defect /normal trunk	General defects
six3	20 pg	613	467	41 (9%)	329 (70%)	97 (21%)
six3d1	20 pg	89	89	88 (99%)	0 (0%)	1 (1%)
	100 pg	37	36	25 (69%)	0 (0%)	11 (31%)
six3d2	20 pg	83	80	80 (100%)	0 (0%)	0 (0%)
	100 pg	43	43	43 (100%)	0 (0%)	0 (0%)
six3d3	20 pg	82	81	80 (99%)	0 (0%)	1 (1%)
	100 pg	44	42	42 (100%)	0 (0%)	0 (0%)
globin	20 pg	381	336	333 (99%)	0 (0%)	3 (1%)
	100 pg	38	33	33 (100%)	0 (0%)	0 (0%)

Fig. 7. Effects of *six3* overexpression in zebrafish embryos. (A) Schematic view of the products from *six3* and its derivative mRNAs used in the experiments. The gray and black boxes indicate the Six domain (SD) and the homeodomain (HD), respectively. The six3d1, six3d2 and six3d3 constructs contain residues 1-54/108-293, 1-105/189-293 and 1-186/262-293, respectively. (B) Overexpression of *six3* results in head-specific defects (scored as missing midbrain ventricle; see Fig. 5), and both the Six domain and the homeodomain are essential for this phenotype. Numbers in parenthesis give the percentage showing the phenotype among surviving embryos. Phenotypes listed under general defects include kinked tail, no eyes, split somites and defects in both head and trunk. About 15% of *six3* mRNA-injected embryos died at gastrula.

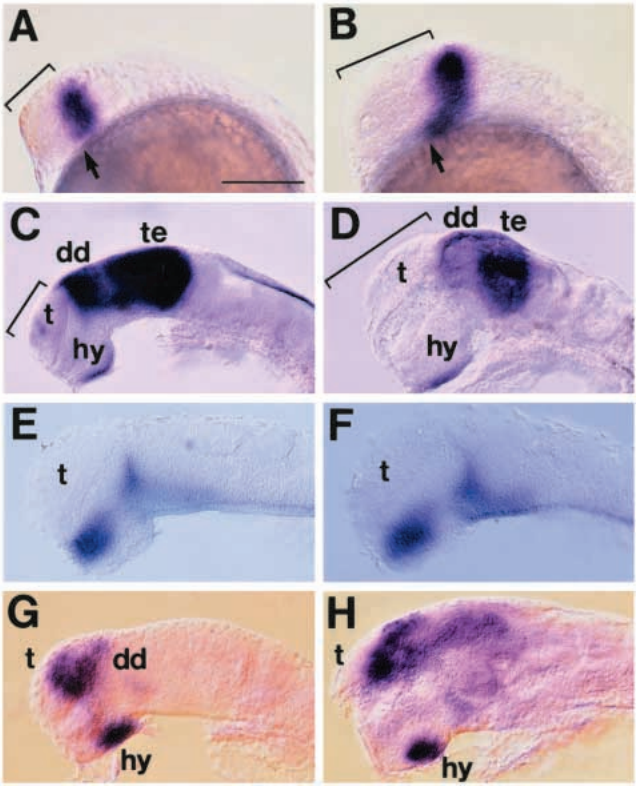


Fig. 8. Effects of *six3* overexpression on expression patterns of brain subregion-specific genes. Globin (A,C,E,G) or *six3* mRNA-injected (B,D,F,H) embryos. (A,B) Lateral views of *lim5* expression at the 16-somite stage. The rostral *lim5*-negative region (bracket) expands in *six3* mRNA-injected embryos. The apparent posterior displacement of ventral *lim5*-positive cells (arrows) is smaller than that of dorsal cells. (C,D) Lateral views of *otx2* expression at 29 hours. The rostral *otx2*-negative region (bracket) is enlarged in *six3* mRNA-injected embryos, while the *otx2* mRNA-expressing region moves posteriorly and becomes shorter. (E,F) Lateral views of *shh* expression at 24 hours; *shh* expression is not changed substantially. (G,H) Lateral views of *emx2* expression at 24 hours. The *emx2*-positive region in the telencephalon, but not in the hypothalamus, is enlarged. Scale bar, 200 μm.

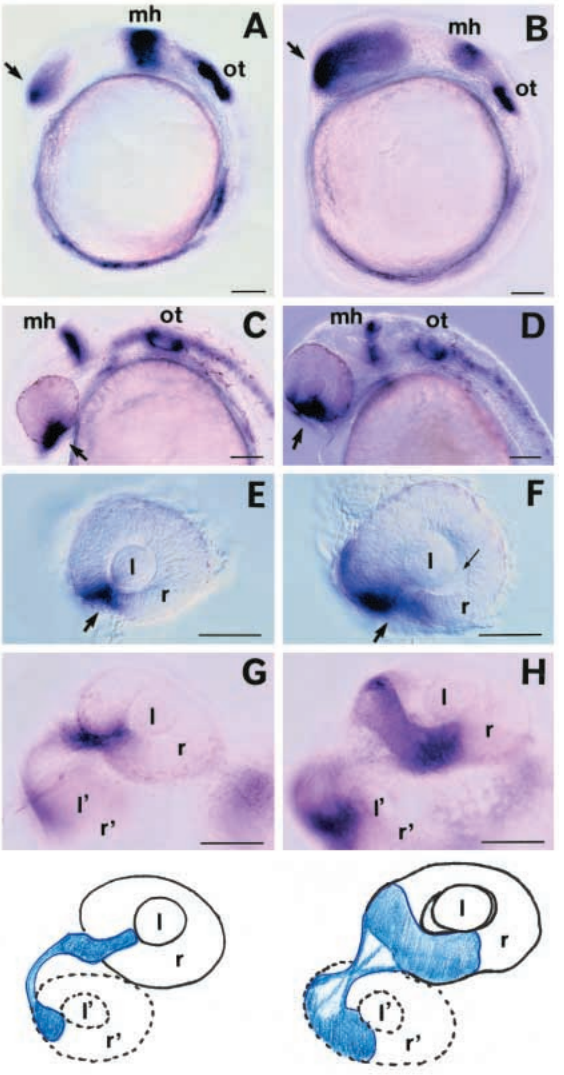


Fig. 9. Accumulation of *pax2*-positive cells in *six3* mRNA-injected embryos. *pax2* expression in globin (A,C,E,G) or *six3* (B,D,F,H) mRNA-injected embryos was analyzed at the 10-somite stage (A,B) or at 24 hours (C-H). (A,B) *pax2* mRNA-expressing cells in the anlage of the optic vesicles are increased in *six3* mRNA-injected embryos, while expression at the prospective midbrain-hindbrain boundary and at the otic placode is unchanged. (C,D) In control embryos *pax2*-positive optic stalk cells (arrow) are located in the ventral diencephalon, while these cells are located more dorsally in *six3* mRNA-injected embryos. (E,F) In the eye, the *pax2*-positive region at the choroid fissure, i.e., the base of the optic stalk (arrow), is enlarged in *six3* mRNA-injected embryos. Since the lens is unaffected, a gap arose between lens and retina (thin arrow). (G,H) Oblique ventral-lateral views of the optic stalk and eyes show that the base of optic stalk is substantially enlarged in *six3* mRNA-injected embryos. Schematic views show *six3* expression as blue areas. Circles drawn in solid and dotted lines indicate the proximal and distal eyes, respectively. Scale bars, 100 μm. Additional abbreviations: l', lens in the distal eye; mh, midbrain-hindbrain boundary; r', retina in the distal eye.

high penetrance (Fig. 7). While no embryos with an ectopic lens in the otic vesicles or in any other part of the body were observed among more than one thousand *six3* mRNA-injected embryos, it is not excluded that we failed to observe a low-level or delayed effect on the lens in the face of the high-frequency effects reported here. Instability of injected mRNA may also be involved, although we found that the level of injected *six3* mRNA in 24-hour embryos was three times higher than that of endogenous mRNA.

Genes involved in the *six3*-dependent regulation

Overexpression of *six3* leads to an increase in *pax2* expression in the optic stalk; therefore, the question arises whether the *six3* effect is mediated by *pax2*, and whether *six3* directly regulates *pax2* expression. An analysis of *pax2*-defective mutants in zebrafish and in the mouse has shown that optic stalk cells were intact but failed to intercalate across the midline (Torres et al., 1996; Macdonald et al., 1997), suggesting that *six3* may carry out functions in optic stalk formation beyond the induction of *pax2*. Furthermore, overexpression studies have suggested that *shh* can regulate optic stalk formation (Ekker et al., 1995; Macdonald et al., 1995), yet *shh* expression is not substantially affected in *six3* mRNA-injected embryos (Fig. 8) and *six3* expression is normal in *cyclops* mutant (unpublished results) even though *shh* expression is defective (Krauss et al., 1993; Ekker et al., 1995), suggesting that *six3* and *shh* influence optic stalk formation separately.

Six3 may be involved not only in optic stalk formation but also in the formation of other tissues in the anterior forebrain. In experiments using explant culture from mouse embryo brain, Shimamura and Rubenstein (1997) showed that the expression of *BF1*, a winged helix transcription factor that is essential for telencephalon and eye development (Xuan et al., 1995), depends on a signal from the ANR. *Fgf8*, known to be expressed in the ANR (Heikinheimo et al., 1994; Ohuchi et al., 1994; Fürthauer et al., 1997), is capable of mediating this signal (Shimamura and Rubenstein, 1997). Since *Six3* is expressed in the anterior forebrain at an earlier stage than *Fgf8* (Crossley and Martin, 1995; Oliver et al., 1995a; Fürthauer et al., 1997), it is possible that *Six3* induces *Fgf8* expression, thereby regulating patterning during the formation of the forebrain.

Cooperating factors may be required for *six3* function

Injection of *six3* mRNA generated defects only in the anterior CNS, mostly the forebrain, and, even in this region, no duplications, formation of ectopic optic stalk or ectopic domains of *pax2* expression were seen. These results suggest that the competence to respond to *six3* overexpression is limited to those domains in which *six3* functions normally. Such a restriction of competence could be due to a requirement for cooperating factors for *six3* function that are limited to the rostral forebrain. Studies on *so* function in *Drosophila* are consistent with this suggestion, in that no dominant gain-of-function phenotypes associated with ectopic expression of *so* were observed without co-expressing *eya* (Serikaku and O'Tousa, 1994; Pignoni et al., 1997). The result that the Six domain, demonstrated to be required for eya binding in *Drosophila*, is also essential for achieving a head-specific phenotype in zebrafish (Fig. 7) suggests that a cooperating factor for *six3* could be a vertebrate *eya* homolog. Three *eya* homologs have been isolated in the mouse, but their expression is not limited to the anterior forebrain (Xu et al., 1997; Zimmerman et al., 1997). It will be interesting to isolate

zebrafish *eya* homologs and to test for overexpression phenotypes together with *six3* in zebrafish embryos.

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REFERENCES

Akimenko, M.-A., Ekker, M., Wegner, J., Lin, W. and Westerfield, M. (1994). Combinatorial expression of three zebrafish genes related to *distal-less*: part of a homeobox gene code for the head. *J. Neurosci.* **14,** 3475-3486.

Boucher, C. A., King, S. K., Carey, N., Krahe, R., Winchester, C. L., Rahman, S., Creavin, T., Meghji, P., Bailey, M. E. S., Chartier, F. L., Brown, S. D., Siciliano, M. J. and Johnson, K. J. (1995). A novel homeodomain-encoding gene is associated with a large CpG island interrupted by the myotonic dystrophy unstable (CTG)n repeat. *Hum. Mol. Genet.* **4,** 1919-1925.

Bovolenta, P., Mallamaci, A., Puelles, L. and Boncinelli, E. (1998). Expression pattern of *cSix3*, a member of the Six/sine oculis family of transcription factors. *Mech. Dev.* **70,** 201-203.

Bürglin, T. R. (1994). A comprehensive classification of homeobox genes. In *Guidebook to the Homeobox Genes* (ed. D. Duboule), pp. 27-71. Oxford: Oxford University Press.

Cheyette, B. N. R., Green, P. J., Martin, K., Garren, H., Hartenstein, V. and Zipursky, S. L. (1994). The Drosophila *sine oculis* locus encodes a homeodomain-containing protein required for the development of the entire visual system. *Neuron* **12,** 977-996.

Chitnis, A. B. and Kuwada, J. Y. (1990). Axonogenesis in the brain of zebrafish embryos. *J. Neurosci.* **10,** 1892-1905.

Crossley, P. H. and Martin, G. R. (1995). The mouse *Fgf8* gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* **121,** 439-451.

Eagleson, G., Ferreiro, B. and Harris, W. A. (1995). Fate of the anterior neural ridge and the morphogenesis of the *Xenopus* forebrain. *J. Neurobiol.* **28,** 146-158.

Ekker, S. C., Ungar, A. R., Greenstein, P., von Kessler, D. P., Porter, J. A., Moon, R. T. and Beachy, P. A. (1995). Patterning activities of vertebrate *hedgehog* proteins in the developing eye and brain. *Curr. Biol.* **5,** 944-955.

Fürthauer, M., Thisse, C. and Thisse, B. (1997). A role for FGF-8 in the dorsoventral patterning of the zebrafish gastrula. *Development* **124,** 4253-4264.

Glasgow, E., Karavanov, A. A. and Dawid, I. B. (1997). Neuronal and neuroendocrine expression of *lim3*, a LIM class homeobox gene, is altered in mutant zebrafish with axial signaling defects. *Dev. Biol.* **192,** 405-419.

Heath, S. K., Carne, S., Hoyle, C., Johnson, K. J. and Wells, D. J. (1997). Characterisation of expression of *mDMAHP*, a homeodomain-encoding gene at the murine DM locus. *Human Mol. Genet.* **6,** 651-657.

Heikinheimo, M., A., L., Shackleford, G. M., Wilson, D. B. and MacArthur, C. A. (1994). *Fgf-8* expression in the post-gastrulation mouse suggests roles in the development of the face, limbs and central nervous system. *Mech. Dev.* **48,** 129-138.

Houart, C., Westerfield, M. and Wilson, S. W. (1998). A small population of anterior cells patterns the forebrain during zebrafish gastrulation. *Nature* **391,** 788-792.

Hyatt, G. A., Schmitt, E. A., Marsh-Armstrong, N. R. and Dowling, J. E. (1992). Retinoic acid-induced duplication of the zebrafish retina. *Proc. Natl. Acad. Sci. USA* **89,** 8293-8297.

Inoue, A., Takahashi, M., Hatta, K., Hotta, Y. and Okamoto, H. (1994). Developmental regulation of Islet-1 mRNA expression during neuronal differentiation in embryonic zebrafish. *Dev. Dyn.* **199,** 1-11.

Kawakami, K., Ohto, H., Ikeda, K. and Roeder, R. G. (1996a). Structure, function and expression of a murine homeobox protein AREC3, a homologue of *Drosophila sine oculis* gene product, and implication in development. *Nucleic Acids Res.* **24,** 303-310.

Kawakami, K., Ohto, H., Takizawa, T. and Saito, T. (1996b). Identification and expression of *six* family genes in mouse retina. *FEBS Lett.* **393,** 259-263.

Klesert, T. R., Otten, A. D., Bird, T. D. and Tapscott, S. J. (1997). Trinucleotide repeat expansion at the myotonic dystrophy locus reduces expression of *DMAHP*. *Nature Genet.* **16,** 402-406.

Krauss, S., Johansen, T., Korzh, V. and Fjose, A. (1991). Expression of the zebrafish paired box gene *pax[zf-b]* during early neurogenesis. *Development* **113,** 1193-1206.

Krauss, S., Concordet, J.-P. and Ingham, P. W. (1993). A functionally conserved homolog of the Drosophila segment polarity gene *hh* is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75,** 1431-1444.

Krieg, P. A. and Melton, D. A. (1984). Functional messenger RNAs are produced by SP6 *in vitro* transcription of cloned cDNAs. *Nucleic Acids Res.* **12,** 7057-7070.

Li, Y., Allende, M. L., Finkelstein, R. and Weinberg, E. S. (1994). Expression of two zebrafish *orthodenticle*-related genes in the embryonic brain. *Mech. Dev.* **48,** 229-244.

Macdonald, R., Barth, K. A., Xu, Q., Holder, N., Mikkola, I. and Wilson, S. W. (1995). Midline signalling is required for Pax gene regulation and patterning of the eyes. *Development* **121,** 3267-3278.

Macdonald, R., Scholes, J., Strähle, U., Brennan, C., Holder, N., Brand, M. and Wilson, S. W. (1997). The Pax protein Noi is required for commissural axon pathway formation in the rostral forebrain. *Development* **124,** 2397-2408.

Miyagawa, T., Amanuma, H., Kuroiwa, A. and Takeda, H. (1996). Specification of posterior midbrain region in zebrafish neuroepithelium. *Genes Cells* **1,** 369-377.

Mori, H., Miyazaki, Y., Morita, T., Nitta, H. and Mishina, M. (1994). Different spatio-temporal expressions of three *otx* homeoprotein transcripts during zebrafish embryogenesis. *Mol. Brain Res.* **27,** 221-231.

Morita, T., Nitta, H., Kiyama, Y., Mori, H. and Mishina, M. (1995). Differential expression of two zebrafish *emx* homeoprotein mRNAs in the developing brain. *Neurosci. Lett.* **198,** 131-134.

Ohto, H., Takizawa, T., Saito, T., Kobayashi, M., Ikeda, K. and Kawakami, K. (1998). Tissue and developmental distribution of *Six* family gene products. *Int. J. Dev. Biol.* **42,** 141-148.

Ohuchi, H., Yoshioka, H., Tanaka, A., Kawakami, Y., Nohno, T. and Noji, S. (1994). Involvement of androgen-induced growth factor (FGF-8) gene in mouse embryogenesis and morphogenesis. *Biochem. Biophys. Res. Commun.* **204,** 882-888.

Oliver, G., Mailhos, A., Wehr, R., Copeland, N. G., Jenkins, N. A. and Gruss, P. (1995a). *Six3*, a murine homologue of the *sine oculis* gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development. *Development* **121,** 4045-4055.

Oliver, G., Wehr, R., Jenkins, N. A., Copeland, N. G., Cheyette, B. N. R., Hartenstein, V., Zipursky, S. L. and Gruss, P. (1995b). Homeobox genes and connective tissue patterning. *Development* **121,** 693-705.

Oliver, G., Loosli, F., Köster, R., Wittbrodt, J. and Gruss, P. (1996). Ectopic lens induction in fish in response to the murine homeobox gene *Six3*. *Mech. Dev.* **60,** 233-239.

Pignoni, F., Hu, B., Zavitz, K. H., Xiao, J., Garrity, P. A. and Zipursky, S. L. (1997). The eye-specification proteins so and eya form a complex and regulate multiple steps in Drosophila eye development. *Cell* **91,** 881-891.

Puelles, L. and Rubenstein, J. L. R. (1993). Expression patterns of homeobox and other putative regulatory genes in the embryonic mouse forebrain suggest a neuromeric organization. *Trends Neurosci.* **16,** 472-479.

Rebagliati, M. R., Toyama, R., Fricke, C., Haffter, P. and Dawid, I. B. (1998). Zebrafish nodal-related genes are implicated in axial patterning and establishing left-right asymmetry. *Dev. Biol.* (in press).

Ross, L. S., Parrett, T. and Easter, S. S., Jr. (1992). Axonogenesis and morphogenesis in the embryonic zebrafish brain. *J. Neurosci.* **12,** 467-482.

Rubenstein, J. L. R., Martinez, S., Shimamura, K. and Puelles, L. (1994). The embryonic vertebrate forebrain: the prosomeric model. *Science* **266,** 578-580.

Rupp, R. A. W., Snider, L. and Weintraub, H. (1994). *Xenopus* embryos regulate the nuclear localization of XMyoD. *Genes Dev.* **8,** 1311-1323.

Schulte-Merker, S., Ho, R. K., Herrmann, B. G. and Nüsslein-Volhard, C. (1992). The protein product of the zebrafish homologue of the mouse *T* gene is expressed in nuclei of the germ ring and the notochord of the early embryo. *Development* **116,** 1021-1032.

Schulte-Merker, S., Hammerschmidt, M., Beuchle, D., Cho, K. W., De Robertis, E. M. and Nüsslein-Volhard, C. (1994). Expression of zebrafish

goosecoid and *no tail* gene products in wild-type and mutant *no tail* embryos. *Development* **120,** 843-852.

Serikaku, M. A. and O'Tousa, J. E. (1994). *sine oculis* is a homeobox gene required for Drosophila visual system development. *Genetics* **138,** 1137-1150.

Shimamura, K. and Rubenstein, J. L. R. (1997). Inductive interactions direct early regionalization of the mouse forebrain. *Development* **124,** 2709-2718.

Stachel, S. E., Grunwald, D. J. and Myers, P. Z. (1993). Lithium perturbation and *goosecoid* expression identify a dorsal specification pathway in the pregastrula zebrafish. *Development* **117,** 1261-1274.

Taira, M., Jamrich, M., Good, P. J. and Dawid, I. B. (1992). The LIM domain-containing homeo box gene *Xlim-1* is expressed specifically in the organizer region of *Xenopus* gastrula embryos. *Genes Dev.* **6,** 356-366.

Thisse, C., Thisse, B., Halpern, M. E. and Postlethwait, J. H. (1994). *goosecoid* expression in neurectoderm and mesendoderm is disrupted in zebrafish *cyclops* gastrulas. *Dev. Biol.* **164,** 420-429.

Thornton, C. A., Wymer, J. P., Simmons, Z., McClain, C. and Moxley III, R. T. (1997). Expansion of the myotonic dystrophy CTG repeat reduces expression of the flanking *DMAHP* gene. *Nature Genet.* **16,** 407-409.

Torres, M., Gómez-Pardo, E. and Gruss, P. (1996). *Pax2* contributes to inner ear patterning and optic nerve trajectory. *Development* **122,** 3381-3391.

Toyama, R., Curtiss, P. E., Otani, H., Kimura, M., Dawid, I. B. and Taira, M. (1995a). The LIM class homeobox gene *lim5*: implied role in CNS patterning in *Xenopus* and zebrafish. *Dev. Biol.* **170,** 583-593.

Toyama, R., O'Connell, M. L., Wright, C. V., Kuehn, M. R. and Dawid, I. B. (1995b). nodal induces ectopic *goosecoid* and *lim1* expression and axis duplication in zebrafish. *Development* **121,** 383-391.

Westerfield, M. (1995). *The Zebrafish Book*. 3rd ed. Eugene: University of Oregon Press.

Woo, K. and Fraser, S. E. (1995). Order and coherence in the fate map of the zebrafish nervous system. *Development* **121,** 2595-2609.

Xu, P.-X., Woo, I., Her, H., Beier, D. R. and Maas, R. L. (1997). Mouse *Eya* homologues of the *Drosophila eyes absent* gene require *Pax6* for expression in lens and nasal placode. *Development* **124,** 219-231.

Xuan, S., Baptista, C. A., Balas, G., Tao, W., Soares, V. C. and Lai, E. (1995). Winged helix transcription factor BF-1 is essential for the development of the cerebral hemispheres. *Neuron* **14,** 1141-1152.

Zimmerman, J. E., Bui, Q. T., Steingrímsson, E., Nagle, D. L., Fu, W., Genin, A., Spinner, N. B., Copeland, N. G., Jenkins, N. A., Bucan, M. and Bonini, N. M. (1997). Cloning and characterization of two vertebrate homologs of the *Drosophila eyes absent* gene. *Genome Res.* **7,** 128-141.