Overexpression of the forebrain-specific homeobox gene \textit{six3} induces rostral forebrain enlargement in zebrafish

Makoto Kobayashi$^{1,*}$, Reiko Toyama$^2$, Hiroyuki Takeda$^3$, Igor B. Dawid$^2$ and Kiyoshi Kawakami$^1$

$^1$Department of Biology, Jichi Medical School, Minamikawachi, Tochigi 329-0498, Japan
$^2$Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA
$^3$Division of Biological Science, Nagoya University Graduate School of Science, Nagoya 464-0814, Japan

$^*$Present address: Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan

1 Department of Biology, Jichi Medical School, Minamikawachi, Tochigi 329-0498, Japan
2 Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA
3 Division of Biological Science, Nagoya University Graduate School of Science, Nagoya 464-0814, Japan

Accepted 22 May; published on WWW 9 July 1998

SUMMARY

The \textit{Drosophila} homeobox gene \textit{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, \textit{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 \textit{six3}, the apparent ortholog of the mouse \textit{Six3} gene. Zebrafish \textit{six3} transcripts are first seen in hypoblast cells in early gastrula embryos and are found in the anterior axial mesendoderm through gastrulation. \textit{six3} expression in the head ectoderm begins at late gastrula. Throughout the segmentation period, \textit{six3} is expressed in the rostral region of the prospective forebrain. Overexpression of \textit{six3} in zebrafish embryos induced enlargement of the rostral forebrain, enhanced expression of \textit{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 \textit{six3} gene function. Our results suggest that the vertebrate \textit{Six3} genes are involved in the formation of the rostral forebrain.

Key words: \textit{sine oculis}, \textit{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 Puuelles 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 \textit{Enx} and \textit{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 \textit{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 \textit{Drosophila} \textit{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 \textit{\alpha}1 subunit gene (Kawakami et al., 1996a,b). The \textit{so} gene encodes a homeodomain protein and is essential for \textit{Drosophila} eye formation (Cheyette et al., 1994; Serikaku and O’Tousa, 1994). Five members of the mouse \textit{Six} gene family have been characterized. Of these, \textit{Six1} and \textit{Six2} are expressed in head and body mesenchyme, limb muscles and tendons (Oliver et al., 1995b); \textit{Six3} is expressed in the anterior forebrain and in the eyes (Oliver et al., 1995a; Kawakami et al., 1996b); \textit{Six4/AREC3} is expressed in neural tissues and encodes the transcription factor regulating the Na,K-ATPase \textit{\alpha}1 subunit gene (Kawakami et al., 1996a, Ohto et al., 1998), and \textit{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 \textit{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 \textit{Drosophila} \textit{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 \textit{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): CCGGATCCCAT(A/C)/TGGGA(A/G)/CTGGGA(A/G)/ACG(T/G)/GAG(A/G)/A(antisense strand corresponding to IWDGET plus EcoRI linker), CCGGATCCGT(A/C/G)T/A(C/G)/TG(C/T)/TTCG(A/G)/A(antisense strand corresponding to TVSNWF plus SalI linker). Conditions of PCR were the same as described previously (Taira et al., 1992). PCR product was purified from agarose gel and was subcloned into pBluescript II KS+. The zebrafish six3 cDNA was isolated from a 13Zap 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 collected 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 MscI-HindIII, HindIII-KpnI, or KpnI-Ncol, 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-XpM (Krieg and Melton, 1984) using the SP6 Megascript in vitro transcription kit (Ambion) with m7G(5′)ppp(5′)G (Boehringer Mannheim).

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 2×105 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 mRNA in the mouse (Fig. 1A; Kawakami et al., 1996a,b). 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 (Stachel et al., 1993; 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,1, 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 pb, the most rostral subregion of the forebrain, as defined by Rubenstein and colleagues in the mouse (Puelles and Rubenstein, 1993; Rubenstein et al., 1993).

### 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 *Six3* (Kawakami et al., 1996b), mouse *Six1/AREC3* (Kawakami et al., 1996a), mouse *Six5/DMAP* (Kawakami et al., 1996b), chicken cSix3 (Bovolenta et al., 1998). *Drosophila* sine oculis (Chayette et al., 1994), *Caenorhabditis elegans* W05E10.3 (accession number, Z77670).
positive cells at this stage, we double-stained embryos with probes to specific areas within the rostral surface. To identify the rostral surface 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.

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.

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...
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 \textit{six3} function, we overexpressed \textit{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 \textit{six3d1}, \textit{six3d2} and \textit{six3d3} mRNAs was similar to that of wild-type mRNA at 24 hours (data not shown). About 70% of wild-type \textit{six3} mRNA-injected embryos showed head-specific defects, while no embryos displayed such a phenotype after mRNA injection of either \textit{six3} derivatives, even when the amount of injected mRNA was increased five-fold (Fig. 7B). General defects seen in some \textit{six3d1} mRNA-injected embryos may due to nonspecific effects of the homeodomain. These results not only confirm that the phenotype in \textit{six3} mRNA-injected embryos is due to overexpression of \textit{six3} gene product but also demonstrate that both the Six domain and the homeodomain are essential for \textit{six3} gene function.

\textbf{\textit{six3} overexpression modulates marker genes expression}

While no changes were observed in \textit{gsc} expression in the anterior axial mesendoderm at the 75% epiboly or tail bud stages (data not shown), \textit{six3}-overexpressing embryos exhibited modified expression of subregion-specific marker genes in the CNS during later embryogenesis. During somitogenesis, \textit{lim5} mRNA forms a tightly delimited stripe in the diencephalon in normal embryos (Toyama et al., 1995a), which moved posteriorly in \textit{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 \textit{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 \textit{six3} led to an enlarged rostral \textit{otx2}-negative region and a shortened \textit{otx2}-positive region (Fig. 8C,D). The domain of \textit{shh} expression in ventral regions of the brain (Krauss et al., 1993; Ekker et al., 1995) showed only slight expansion after \textit{six3} mRNA injection (Fig. 8E,F). In the case of \textit{emx2}, which is expressed both dorsally in the telencephalon and ventrally in the hypothalamus (Morita et al., 1995), overexpression of \textit{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 \textit{Islet-1} expression in the spinal chord and \textit{shh} expression in the floor plate and notochord (data not shown).
Furthermore, no obvious changes were observed in \textit{lim3} or \textit{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 \textit{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.

\textbf{\textit{six3} overexpression induces enlargement of the optic stalk}

Because evolutionary considerations implicate the \textit{six3} gene in visual system development, we tested \textit{pax2} expression in \textit{six3} mRNA-injected embryos. During segmentation, \textit{pax2} is expressed primarily in the optic stalk, midbrain-hindbrain boundary and otic vesicles (Krauss et al., 1991). Of these major expression sites, \textit{pax2} staining in the prospective optic stalk at the 10-somite stage was enlarged in \textit{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 \textit{pax2} staining, was located ventrally because of the bending of the rostral forebrain (Fig. 9C). In contrast, the optic stalk in \textit{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 \textit{six3} mRNA-injected embryos (Fig. 9E-H), indicating that \textit{six3} is involved in optic stalk formation. Though the region of \textit{pax2}-negative cells in the retina remained essentially unchanged, the extension of \textit{pax2}-positive regions caused an enlargement of the retina.

\section*{DISCUSSION}

\textit{Six3} is highly conserved among zebrafish, mouse and chicken

The amino acid sequence of the zebrafish \textit{six3} protein is highly similar to mouse \textit{Six3} and chicken \textit{cSix3} (Fig. 1). We suggest that zebrafish \textit{six3} is the ortholog of mouse \textit{Six3} and chicken \textit{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 \textit{six3}, mouse \textit{Six3} and chicken \textit{cSix3} (Oliver et al., 1995a; Bovolenta et al., 1998); these genes are

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{Disorganized head formation in \textit{six3} mRNA-injected zebrafish embryos. 20 pg of globin (A,C,E,G) or \textit{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 \textit{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 \textit{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 \textit{a}-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 \textit{six3} mRNA-injected embryos. Additional abbreviations: ot, otic vesicle; tg, trigeminal ganglion. Scale bar, 200 \textmu m.}
\end{figure}
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 *Six*-related and a *Six*-related gene in its genome. In this context, it is possible that there is another *Six*-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* 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

---

**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.

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

---

**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 BFL1, 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’Toosa, 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
REFERENCES


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