A Zebrafish Forebrain-Specific Zinc Finger Gene Can Induce Ectopic dlx2 and dlx6 Expression

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Identification of the earliest forebrain-specific markers should facilitate the elucidation of molecular events underlying vertebrate forebrain determination and specification. Here we report the sequence and characterization of fez (forebrain embryonic zinc finger), a gene that is specifically expressed in the embryonic forebrain of zebrafish. Fez encodes a putative nuclear zinc finger protein that is highly conserved in Drosophila, zebrafish, Xenopus, mouse, and human. In zebrafish, the expression of fez becomes detectable at the anterior edge of the presumptive neuroectoderm by 70% epiboly. During the segmentation period, its expression is completely restricted to the rostral region of the prospective forebrain. At approximately 24 h postfertilization, fez expression is mostly confined to the telencephalon and the anterior–ventral region of the diencephalon. Although fez expression is present in one-eyed pinhead (oep) and cyclops (cyc) zebrafish mutants, the pattern is altered. Forced expression of fez induces ectopic expression of dlx2 and dlx6, two genes involved in brain development. Knockdown of fez function using a morpholino-based antisense oligo inhibited dlx2 expression in the ventral forebrain. Our studies indicate that fez is one of the earliest markers specific for the anterior neuroectoderm and it may play a role in forebrain development by regulating Dlx gene expression. © 2001 Academic Press

Key Words: zebrafish; forebrain; zinc finger protein.

INTRODUCTION

Neural development of vertebrates is initiated during gastrulation with the induction and patterning of the neural plate. It is proposed that dorsal mesendoderm first provides “activation” signals that specify the entire neural plate to an anterior fate. Signals from posterior mesoderm then transform a part of the neural plate into a more posterior fate corresponding to hindbrain and spinal cord. According to this model, embryonic forebrain is the first tissue to be determined during neural development (Brewster and Dahmane, 1999; Kolm and Sive, 1997; Saxen, 1989). Although the morphologic events of vertebrate forebrain formation have been well depicted (Rubenstein et al., 1994), molecular mechanisms underlying the induction, patterning, and organization of the embryonic forebrain remain unclear. Explant and transplant experiments in Xenopus and zebrafish indicate that the inductive and patterning signals are transmitted to the anterior neural tissue both vertically from the underlying mesendoderm and horizontally within the neuroectoderm (for review, see Brewster and Dahmane, 1999). Additionally, anterior endoderm of Xenopus (Bouwmeester et al., 1996; Bradley et al., 1996) as well as the extraembryonic visceral endoderm of mouse contain inductive signals for forebrain (Thomas and Beddington, 1996; Varlet et al., 1997).

At the molecular level, studies from Xenopus have identified a number of dorsal mesendoderm-derived molecules, such as Noggin, Chordin, and Follistatin (Piccolo et al., 1996; Xu et al., 1995; Zimmerman et al., 1996), that can induce anterior neural fates. Within the presumptive forebrain, several genes, including members of Lim, Otx, Emx, Dlx, and Six gene families, have important roles in forebrain specification and patterning. The functions of these genes have been analyzed genetically in mice through a targeted gene inactivation strategy. For example, disruption of either Lim1 (Shawlot et al., 1999) or Otx2 (Acampora et al., 1995) leads to the failure of forebrain/midbrain formation, whereas a double mutation in Dlx1 and Dlx2 induces defects in telencephalon (Anderson et al., 1997a,b).

Many homologs of the forebrain-related genes have been isolated from zebrafish, an organism suitable for embryological and genetic studies (Haftet et al., 1996). Large-scale
mutagenesis has isolated more than a thousand mutations affecting embryonic development. Analyses of expression patterns of the forebrain-related genes in wild-type and mutants with neuronal defects have revealed new information regarding forebrain induction and specification. For instance, it has been shown that expression of opl, a gene restricted to forebrain during early neural development, appeared normal in oep mutant embryos, where the prechordal plate fails to form (Grinblat et al., 1998). Using in vitro explant assays, it was further demonstrated that forebrain specification begins during gastrulation earlier than what had been previously predicted. In addition, it is believed that a wider area of dorsal mesendoderm, along with the most anterior row of cells in the prospective neuroectoderm, is required for zebrafish forebrain induction (Grinblat et al., 1998; Houart et al., 1998). However, the molecular events underlying this process are undetermined. The identification of early genes that are restricted to anterior neuroectoderm should help elucidate these events.

Here we report the sequence and characterization of a zebrafish gene, termed fez after its Xenopus homolog (Matsuo-Takasaki et al., 2000), which is specifically expressed in the embryonic forebrain throughout development. Fez encodes a putative nuclear zinc-finger protein and can activate ectopic expression of dlx2 and dlx6. Inhibition of fez function caused specific reduction of dlx2 expression in the ventral forebrain. Since fez has Drosophila, Xenopus, mouse, and human homologs and shares similar expression patterns in Xenopus and mouse, we propose that fez is a highly conserved key player that mediates very early events in forebrain formation.

MATERIALS AND METHODS

Identification of fez

We performed a systematic search for novel tissue-specific genes that have unique expression patterns during early zebrafish embryogenesis by using whole-mount RNA in situ hybridization. A number of tissue-specific cDNA libraries were constructed using mRNA of cells purified from transgenic zebrafish embryos expressing green fluorescent protein (GFP) in specific tissues by fluorescence-activated cell sorting. Partial sequences of individual clones from these libraries were obtained and used to search the Genbank database. Those clones with novel sequences were selected for whole-mount RNA in situ hybridization. Fez was identified as one of the cDNA clones with a forebrain-specific expression pattern. We originally called our gene foreheadin (as presented in the Zebrafish Development and Genetics meeting. Cold Spring Harbor Laboratory, April 2000) and renamed it fez to be consistent with the Xenopus nomenclature (Matsuo-Takasaki et al., 2000). Searching the public Genbank databases identified the Drosophila, Xenopus, mouse, and human homologs of zebrafish fez.

Constructs

The original clone containing fez cDNA was subcloned into pCRII (Invitrogen), allowing in vitro transcription of both sense and antisense RNA. The construct pCMV-fez/GFP was generated by ligating the 5′ untranslated region and coding sequence of fez in frame to the enhanced green fluorescent protein (EGFP) reporter gene using the vector pEGFP-N2 (Clontech). To facilitate preparation of capped mRNA, the fez/GFP fusion cassette and fez cDNA alone were inserted into the multiple cloning region of pXT7 that has 5′ and 3′ untranslated regions derived from the Xenopus major beta-globin gene to generate the construct pXT7-fez/GFP and pXT7-fez, respectively.

In Vitro Transcription and mRNA and DNA Injection

pXT7-fez/GFP and pXT7-fez were digested with NdeI at the 3′ end and capped mRNA was synthesized in vitro using the mMESSAGE mMACHINE Kit (Ambion). After purification as recommended by the manufacturer, mRNA was dissolved in nuclease-free 0.1 M KCl at a concentration of approximately 100 μg/ml. RNA was injected into one cell of 16-cell stage embryos. For each construct, RNA injection was performed 2–4 times and the data were pooled. pCMV-fez/GFP DNA construct was digested with Afl II at the 3′ end, purified, and injected into one-cell stage embryos as previously described (Meng et al., 1999a,b).

Whole-Mount RNA in Situ Hybridization

For single-probe staining, digoxigenin-UTP-labeled sense and antisense RNA for fez, dlx2, and dlx6 were generated by in vitro transcription and used for in situ hybridization. For double-probe staining, the antisense fez RNA probe was labeled with fluorescin-UTP and developed with Fast red (Roche Molecular Biochemicals, Cat. # 1496549), which produces a red color. The dlx2 probe was labeled with digoxigenin-UTP and developed with purple AP substrate (Roche Molecular Biochemicals, Cat. # 1442074), which produces a dark purple color. Whole-mount RNA in situ hybridizations were performed as described previously (Meng et al., 1999b; Thissie et al., 1993). To correlate fez/GFP expression and dlx2 induction, individual live embryos injected with the fez/GFP construct were photographed, the expression pattern was documented using the dorsal shield as a reference point, and digital images were stored in the computer. Approximately 30 of these photographed embryos were individually fixed and processed for in situ hybridization. Ectopic expression pattern of dlx2 was then matched with the GFP images.

Morpholino-Based Antisense Oligo

Antisense morpholino oligo of fez was ordered from Gene Tools, LLC (Corrallis, OR). The following sequence was used: 5'-GGTGTGAGCATCGACCAGCCGCCCC-3'. Sequence complementary to the predicted start codon is underlined. The oligo was dissolved in 0.1 M KCl as 1 mg/ml working solution and injected into 1–2 cell stage embryos at approximately 2 ng per embryo. A higher concentration of oligo appeared to cause nonspecific defects. For coinjection, approximately 20 pg of fez/GFP mRNA per embryo was used for GFP expression analysis and 10 pg for rescue experiment.
A Protein Sequence

MLTPSVVEVASSLPLETVMCSPRLDDRSGATAAPKSLAFSIDRIMSKTSEFKAAAAEERESEGKKTVGCLSCFIPCH
IPIQFSDYDLQAKALMNYESFWMKNFPRGACTSAAMCTNGVCSDAKAGIKSIVLPGFTKVKPIQVIHALMPA
NGSLCCFNYLDSAYQSELSGHLFSSAIAINSQQAISAHQKLHLLLENAKLACSPFKFFPQYPFHEHLPGQLDQ
IVRENHLTEKNGVAKSHTNNCSDDGKFKNFTCEVCVRFTAHYKLTRMPVFHTGARPFVCKGCGBKGFQASTL
CRHKIHTQEFHKCNQCGKAFNRSSTLTHIRHAYKPFVCFPCGKFBQKCHNKHLYSNEGKQYKCSICS
KAFBQYLYNLTFFHMTDNDOEPTCAGKGFCLFNLKHKRHLKHDANLCLSGNLDSSRQHN

B Zinc Finger Alignment

C

FIG. 1. Sequence analysis of fez. The zebrafish fez cDNA has a major open reading frame encoding a putative protein of 438 amino acids (A). Fez has Drosophila, Xenopus, mouse, and human homologs with six highly conserved zinc fingers (B). Each of the six zinc fingers is underlined and the identical residues are shaded. When a Fez/GFP fusion protein is expressed in zebrafish embryos, the green fluorescence is mainly located in the nuclei of the GFP-positive cells (C). This embryo is at approximately 1000 cell stage. GenBank Accession Number of fez: AF281076.
RESULTS

Fez Encodes a Highly Conserved Putative Zinc Finger Protein

We identified fez through RNA whole-mount in situ hybridization screens for genes with tissue-specific expression patterns during zebrafish embryonic development. The zebrafish fez cDNA clone, selected because of its forebrain-specific expression pattern, has an insert of 1691 bp with a major open reading frame encoding a putative polypeptide of 438 amino acids (Fig. 1A). The predicted polypeptide has six C2–H2-type zinc fingers, which are commonly seen in...
the DNA-binding domains of many transcription factors (Klug and Schwabe, 1995).

Searching Genbank by using either the nucleotide or the predicted amino acid sequence of zebrafish fez led to the identification of homologs from Drosophila, Xenopus, mouse, and human. Each homolog contains a highly conserved zinc finger domain (Fig. 1B). The zinc fingers of zebrafish fez share 88, 94, 95, and 95% identity to that of Drosophila, Xenopus, mouse, and human homologs, respectively. To visualize the subcellular localization of Fez, we generated reagent expression construct by fusing fez to the EGFP reporter gene and injected mRNA encoding the fusion protein into one-cell stage embryos. As shown in Fig. 1C, green fluorescent fusion protein was observed in the nuclei of embryonic cells. These results indicate that fez encodes a highly conserved nuclear protein.

Fez Expression Is Restricted to Prospective Neuroectoderm and Forebrain

To reveal the temporal and spatial expression pattern of fez during embryogenesis, we performed whole-mount in situ hybridization. Fez expression first became detectable at approximately 70% epiboly stage. At 80% epiboly, the expression domain appeared as a half-egg-shaped patch in the most anterior part of the prospective neural plate along the rostral–caudal axis (Figs. 2A and 2B). Sections of these embryos showed that the expression was restricted to cells in the ectoderm (Fig. 2K). At the tail bud stage, expression in the prospective forebrain region was seen as a raised dorsal circle bisected by a ventral stripe along the rostral–caudal axis (Figs. 2A and 2C). From 70% epiboly to tail bud stage, the half-egg-shaped expression domain of fez underwent a morphological change that resulted in an arch facing the opposite direction. During the period in which the first five somites are formed, the raised circle fused into a single dorsal stripe (Figs. 2E and 2F). From 15-somite to 24-h postfertilization, fez expression was mostly confined to the telencephalon and the anterior–ventral region of diencephalon (Figs. 2G–J, and 2L). After 24-h postfertilization, the level of fez expression decreased and by approximately 48 h, only weak expression was detected in the ventral region of the forebrain (data not shown). The sense probe of fez did not give any detectable staining (data not shown).

Expression of fez in Zebrafish Lacking the Prechordal Mesoderm

It is believed that the underlying prechordal mesoderm and anterior notochord play certain roles in the induction and patterning of forebrain and midbrain. A number of zebrafish mutants with defects in both prechordal mesoderm and forebrain have been identified (Brand et al., 1996; Jiang et al., 1996; Schier et al., 1996). The zebrafish oep mutation has defects in endoderm, prechordal plate, and ventral neuroectoderm formation because of a mutation in an EGF-related membrane-bound ligand protein (Schier et al., 1997; Zhang et al., 1998). To investigate whether the expression of fez is affected by the oep mutation, mutant embryos from a heterozygous cross were hybridized with a fez antisense RNA probe. Compared to the wild-type siblings (Fig. 3G), a different expression pattern could be seen at the tail bud stage and oep mutants did not develop the midline stripe that expresses fez (Fig. 3A). In wild type, this stripe occupies a ventral location and appears overlying the prechordal plate. At 8-somite stage, the expression of fez in the dorsal circle along the rostral–caudal axis of the forebrain collapsed and failed to fuse (Figs. 3B and 3C), remaining as a single dorsal circle in the oep mutant embryos. We also examined expression in cyc mutant embryos, in which a mutation in the nodal-related protein Dr2 leads to a loss of medial floor plate and defects in ventral forebrain development (Feldman et al., 1998; Rebagliati et al., 1998). Similarly, we noticed that the ventral stripe that should be positive for fez expression was absent in cyc mutant embryos (Fig. 3D). Unlike oep, the dorsal portion of fez-expressing domain is appeared normal and fused into a single dorsal stripe at the 8-somite stage as observed in the wild-type (Figs. 3E, 3F, 3H, and 3I). The arc of fez expression appeared broader in oep and cyc mutant embryos at tail bud stage. This may be caused by disorganization of cells that presumably should have occupied the expression domain of the ventral stripe.

Overexpression of fez Induces Ectopic dlx2 and dlx6 Expression

The vertebrate Dlx family comprises at least eight members in zebrafish and six in mammals (Stock et al., 1996). Mice lacking both Dlx1 and Dlx2 develop defects in ventral forebrain with abnormal subcortical telencephalon (Ander- son et al., 1997a,b). In zebrafish, dlx1, 2, 4, and 6 are mainly expressed in multiple domains of the telencephalon, diencephalon, and hypothalamus of the embryonic forebrain (Akimenko et al., 1994; Ellies et al., 1997). In neuronal tissues, dlx2 is expressed earlier than other dlx members and has been shown to be required for the induction and maintenance of Dlx4/5 and Dlx6 expression in mouse and zebrafish (Zerucha et al., 2000). Dlx2 appears to bind to a highly conserved enhancer sequence located in the Dlx4/5 and Dlx6 intergenic region, suggesting a cross-regulation between Dlx genes. However, it is not known what other factors regulate the expression of Dlx genes. To determine if fez is involved in the regulation of dlx2 and dlx6 expression, we injected a plasmid DNA construct containing the fez-coding sequence under the control of a CMV promoter into one-cell stage embryos. The injected embryos were examined for dlx2 and dlx6 expression between shield and 5-somite stages, at which they are normally not yet expressed. We found that approximately 15% of the fez DNA-injected embryos showed ectopic expres-
sion of dlx2 and dlx6 (Figs. 4A and 4B), whereas uninjected control embryos did not express either of the genes (Fig. 4C). The expression pattern of dlx2 and dlx6 in the injected embryos was spotty, possibly due to the mosaic distribution of the injected DNA. In order to determine whether the induced dlx2 expression coincided to the location of injected fez construct, we used a fez/GFP fusion protein to monitor the distribution of the injected DNA. Individual injected embryos were first imaged for GFP expression (Fig. 4D) and subsequently fixed for in situ hybridization with a dlx2 probe (Fig. 4E). The results show that dlx2 expression is indeed localized in regions of DNA injection. However, it appeared that only a portion of the GFP positive cells had ectopic expression of dlx2. This was confirmed by two-color double in situ hybridization using probes for fez and dlx2. As shown in the inset of Fig. 4E, ectopic expression of dlx2 (black) is located in the same cells that express the injected fez (red), suggesting Fez is acting cell autonomously. Those cells that expressed fez but did not induce dlx2 expression were often located deep in the embryos (data not shown) and likely represented nonectoderm cells. Additionally, not all cells expressing fez on the surface of the embryos showed induction of dlx2, suggesting certain cofactor(s) may also be needed in the ectoderm tissues. The frequency of ectopic induction of both dlx2 and dlx6 by Fez appeared low. To determine if this induction could be increased at a later stage when dlx2 is normally expressed, we examined dlx2 expression in the injected embryos by whole-mount in situ hybridization at 16-somite stage. We observed ectopic expression of dlx2 mostly on the yolk surface (Fig. 4F) but the frequency of induction remained approximately same.

We also injected in vitro synthesized mRNA encoding Fez or Fez/GFP fusion protein into zebrafish embryos. However, when zebrafish embryos were injected at the 1–2 cell stage with fez mRNA at a concentration of approximately 20 pg per embryo, all the embryos died before or during gastrulation. To overcome this problem, mRNA was injected into one cell of 16- or 32-cell stage embryos. This mosaic approach increased embryo survival and allowed the examination of dlx2 expression after gastrulation stages. As expected, we observed ectopic expression of dlx2 for both types of mRNA (Fig. 4G).

Either injecting mRNA or the plasmid DNA construct, pEGFP, encoding GFP alone did not induce ectopic dlx2 expression (data not shown). Additionally, we found that injection of the same fez DNA construct did not induce any ectopic bf-1 or emx1 expression in zebrafish (data not shown). These results suggest the induction of dlx2, although at a low frequency, is specific.

**Knockdown of fez Function**

Morpholino-based antisense oligos have recently been shown to effectively block gene function in zebrafish (Na-sevicius and Ekker, 2000) and Xenopus (Heasman et al., 2000). We designed a fez antisense morpholino oligo corresponding to its 5’ ATG region and performed a series of microinjection studies. We first coinjected this oligo with a synthetic mRNA encoding the Fez/GFP fusion protein or GFP alone as a control (10 pg per embryo for each mRNA). We found that the fez antisense morpholino oligo specifically blocked Fez/GFP fusion protein synthesis (Figs. 5A and 5B) but had no effect on GFP alone (data not shown). This experiment also allowed embryos injected at the 1-cell stage with 20 pg fez mRNA to survive beyond gastrulation, suggesting alleviation of the catastrophic effect described in the previous section. We then injected wild-type embryos with the fez antisense morpholino oligo and fixed them for in situ hybridization with the zebrafish dlx2 probe. As shown in Figs. 5G, 5H, and 5I, injection of approximately 2 ng per embryo resulted in reduction of dlx2 expression in the ventral forebrain as compared to uninjected controls (Figs. 5F and 5I). However, most of the other dlx2 expression domains remained relatively normal (Figs. 5I and 5J). Close-up comparison of dlx2 and fez expression in zebrafish forebrain indicated that the expression of the two genes mostly overlapped in the ventral region (Figs. 5C, 5D, 5E, and 5F). More than 80% of the injected embryos (N > 200) showed the phenotype, and injection of five different morpholino oligos specific to other zebrafish genes did not reduce dlx2 expression (data not shown), indicating that the reduction of dlx2 in ventral forebrain induced by blocking the synthesis of Fez was specific and effective. Additionally, coinjection of approximately 10 pg Fez/GFP mRNA, as described above, allowed rescue of the morpholino-induced reduction of dlx2 expression (N = 40; >60% of the embryos were rescued). Concentration of fez antisense morpholino oligo was a critical factor. By injection of 3 ng of the oligo per embryo, the expression of dlx2 in the ventral forebrain became almost undetectable by whole-mount in situ hybridization, and the dlx2 expression domain dorsal to the ventral forebrain was also often reduced (Fig. 5H). Further increases in the concentration of oligo started to cause nonspecific developmental defects in the whole embryos.

**DISCUSSION**

We have performed functional studies on the zebrafish fez gene, which is specifically expressed in the earliest stages of forebrain development. We have shown that mutants containing mutations in genes involved in pre-chordal mesoderm and forebrain development have altered fez expression. In addition, we have shown that overexpression of fez results in the ectopic expression of dlx2 and dlx6. Finally, we have demonstrated that knockdown of Fez function results in reduction of dlx2 expression specifically in the forebrain.

Fez is highly conserved among such species as Drosophila, Xenopus, mouse, and human. Expression of these ho-
FIG. 3. Analysis of fez expression in zebrafish mutants. Expression of fez is present in both oep (A–C) and cyc (D–F) mutant embryos, but the ventral stripe that bisects the dorsal circle fails to form at tail bud stage in the mutants (A and D) as compared to the wild-type embryo (G). At the 8-somite stage, the midline stripe that is normally localized at the ventral location in wild-type embryos is absent in both mutants (arrows, B and E; anterior view, C and F side view) when compared to the wild-type embryos (H and I). In oep mutants, the dorsal portion expressing fez fails to fuse and collapses to a more ventral location (C). For the cyc mutants, the dorsal expression domain appears normal and has fused into a single stripe (E) as seen in wild-type (H). (A, D, and G) Tail bud stage. (B, C, E, F, H, and I) 8-somite stage. (A–I) Dorsal to the right. (C, F, and I) Anterior to the top. (A, B, D, E, G, and H) Anterior view. (C, F, and I) Side view. Scale bar: 175 μm.

FIG. 4. Induction of ectopic dlx6 and dlx2 expression by fez. Injection of a plasmid DNA construct containing coding sequence for Fez or Fez/GFP fusion protein under the control of the CMV promoter into one-cell stage embryos can induce ectopic expression of dlx6 (A) and dlx2 (B) as early as the shield stage at which the uninjected controls (C, stained with dlx2) do not express either of the genes. Distribution of the injected plasmid DNA (D) expressing Fez/GFP is in the same region expressing ectopic dlx2 (E). The spatial relationship between fez/GFP and dlx2 expression was also examined by simultaneously staining the same embryos with both probes using two
different color substrates (inset in E). Dlx2 expression (dark purple) is in the same cells expressing fez (red, white arrowheads). The inset also shows a group of cells expressing fez that are not positive for Dlx2 (white arrow). Injection of fez mRNA into one cell of 16 or 32 cell stage embryos also induces ectopic expression of Dlx2 (F, arrows). Induction can also be obtained using mRNA encoding Fez-GFP fusion protein (data not shown). At 16-somite stage, ectopic expression of Dlx2 is mostly located on the yolk surface (arrows in G). Scale bar: A: 300 μm; B: 430 μm; C: 395 μm; D–G: 185 μm.
mologs appears to be specific to forebrain. While this paper was in preparation, the sequence and expression pattern of the Xenopus homolog of fez was reported (Matsumo-Takasaki et al., 2000). Matsumo-Takasaki et al. also analyzed Fez expression patterns in mouse using a mouse EST clone. A highly conserved expression pattern similar to that of zebrafish is seen in both mouse and Xenopus, suggesting that fez plays a role in forebrain formation during vertebrate embryogenesis. More recently, Hashimoto et al. (2000) also reported zebrafish fez (called fezl) sequence and expression patterns. In zebrafish, the expression of fez is restricted to early anterior neuroectoderm and continues to be specific for the forebrain. To the best of our knowledge, this is the earliest neuroectoderm- and forebrain-specific gene in zebrafish. Other genes that are expressed at the same time or earlier than fez in the anterior neuroectoderm are either coexpressed in mesendoderm or become less specific to forebrain later during embryogenesis, such as six3 (Kobayashi et al., 1998) and opl (Grinblat et al., 1998), respectively.

In oep and cdc mutants, it appeared that the transformation from a single cell mass expressing fez into the structure of a dorsal circle bisected by a ventral stripe is blocked between 80% epiboly and tail bud stages. Specifically, the ventral stripe is not formed. Our observations in both oep and cdc mutants support the notion that signals from the underlying prechordal plate may be critical for patterning the ventral forebrain but are not required for induction and determination of the entire embryonic forebrain. It will be interesting to determine what signals are required to initiate fez expression during the late gastrulation stage. We have isolated the genomic locus of fez from zebrafish and will define cis-acting elements important for regulation of fez expression through transgenic zebrafish approaches.

We have shown that ectopic expression of both dlx2 and dlx6 can be induced by forced expression of fez. This induction acts cell autonomously since expression of dlx2 and fez is located in the same cells. However, the frequency of inducing ectopic expression of either dlx2 or dlx6 in zebrafish embryos by Fez is fairly low. Two possibilities acting in concert may account for this. First, additional cofactors may be needed to induce dlx expression and they may be present only in certain cells. The cofactor(s) interacting with Fez are most likely expressed in a subset of ectoderm since we observed most of the induction of dlx2 expression on the surface of the injected embryos, and many deep cells positive for Fez failed to induce ectopic expression of dlx2. Second, since massive ectopic expression of fez is detrimental to embryos, only highly mosaic embryos will survive long enough to allow induction. Thus, under these circumstances, one would expect to observe only a low frequency of ectopic expression of dlx2 or dlx6 induced by Fez. Ectopic expression of dlx2 by Fez also appears stage specific since an increase is not observed at the stage when dlx2 is normally expressed. It is conceivable that differentiated cells at a later embryonic stage are no longer potent for induction of dlx2 expression by Fez. Nonetheless, we believe that the ectopic activation of dlx2 or dlx6 is specific because injection of the same fez DNA construct had no effect on bfl-1 or emx1 expression (data not shown). Our findings suggest that a unique combination of Fez and other factors at certain stages of cellular development may be important for determining subcellular lineages of embryonic forebrain.

The antisense morpholino oligo of fez inhibited dlx2 expression most notably in the ventral forebrain where expression of fez and dlx2 overlap. This further supports the fact that Fez plays a specific role in regulating regional dlx2 expression. Since dlx2 can in turn regulate other dlx gene family members, Fez may be involved in patterning the early embryonic forebrain through activating the cascade of dlx genes. Several potential homeodomain-binding sites have been found in the promoter region of Dlx2 in mouse (Liu et al., 1997; McGuinness et al., 1996). The intergenic region between dlx5 and dlx6 was found to contain highly conserved sequences (from zebrafish to mouse) required for the concurrent expression of dlx5 and 6 (Zerucha et al., 2000). A similar situation was observed for dlx1 and 2 (Zerucha et al., 2000). However, trans-acting factors that interact with these sequences have not been identified. Fez may well be a candidate for such a trans-acting factor. Further studies should help elucidate the function of both the fez and dlx gene families during forebrain development.

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