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Developmental Expression Pattern

Expression of *FoxP2* during zebrafish development and in the adult brain

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ABSTRACT Fox (forkhead) genes encode transcription factors that play important roles in the regulation of embryonic patterning as well as in tissue specific gene expression. Mutations in the human *FOXP2* gene cause abnormal speech development. Here we report the structure and expression pattern of zebrafish *FoxP2.* In zebrafish, this gene is first expressed at the 20-somite stage in the presumptive telencephalon. At this stage there is a significant overlap of *FoxP2* expression with the expression of the *emx* homeobox genes. However, in contrast to *emx1, FoxP2* is not expressed in the pineal gland or in the pronephric duct. After 72 hours of development, the expression of zebrafish *FoxP2* becomes more complex in the brain. The developing optic tectum becomes the major area of *FoxP2* expression. In the adult brain, the highest concentrations of the *FoxP2* transcript can be observed in the optic tectum. In the cerebellum, only the caudal lobes show high levels of *Foxp2* expression. These regions correspond to the vestibulocerebellum of mammals. Several other regions of the brain also show high levels of *Foxp2* expression.

KEY WORDS: *Emx, forkhead, FoxP2, homeobox, speech, telencephalon, zebrafish*

Forkhead proteins are important transcriptional regulators that are involved in pattern formation during vertebrate development as well as in tissue specific gene expression and tumorogenesis (Accili and Arden, 2004, Carlsson and Mahlapuu, 2002, Dirksen and Jamrich, 1992, Dirksen and Jamrich, 1995, El-Hodiri *et al.*, 2001, Erickson, 2001, Kaufmann and Knochel, 1996, Lai *et al.*, 2001, Lai *et al.*, 1990, Lehmann *et al.*, 2003, Li and Vogt, 1993, Tseng *et al.*, 2004).

FOXP2, a member of the *Foxp* subfamily of Fox genes, is the only gene shown to be involved in speech and language development in humans (Bruce and Margolis, 2002, Enard *et al.*, 2002, Fisher *et al.*, 1998, Katoh, 2004, Lai *et al.*, 2001, Lu *et al.*, 2002, Saleem *et al.*, 2003, Shu *et al.*, 2001, Wang *et al.*, 2003, Zhang *et al.*, 2002). Mutations in this gene result in impaired linguistic and grammatical skills that, together with diminished control of complex face and mouth movements, lead to disrupted speech (Hurst *et al.*, 1990, Vargha-Khadem *et al.*, 1998). A recent study showed expression of *FoxP2* in the entire adult brain of birds and crocodiles (Haesler *et al.,* 2004). In this paper, we provide information about the isolation, sequence and expression pattern of zebrafish *FoxP2* during development and in adult brain.

Foxp2 is somewhat of an unusual protein in that it contains a forkhead and zinc finger domain. PCR and degenerate primers were used to isolate a cDNA fragment of the zebrafish *FoxP2* gene that encodes both of these domains of the protein. After sequencing the isolated PCR fragment, we found that our sequence has a high homology to several zebrafish EST fragments in the GenBank database. Figure 1 shows the comparison of the deduced amino acid sequence of the zebrafish to the mouse and human FOXP2 protein. This comparison shows that the FoxP2 protein is highly conserved during evolution. Not surprisingly, the similarities between the zebrafish, mouse and human FOXP2 protein are highest in the forkhead and the zinc finger domain. However, the overall conservation of amino acids in the entire protein between zebrafish and human is unusually high, greater than 80%. The most notable difference between these three proteins is in the poly-glutamine region. While the human and mouse FOXP2 contain as many as 50 glutamines in two adjacent poly-glutamine regions, the zebrafish FoxP2 contains only nine. The functional significance of this difference is not known, but the expansion of poly-glutamine stretches of proteins has been identified as the cause of several neurodegenerative diseases in humans (for review see (La

Abbreviations used in this paper: EST, expressed sequence tag; PCR, polymerase chain reaction.

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Spada and Taylor, 2003).

Expression of FoxP2 *during embryogenesis*

Expression of *FoxP2* begins in zebrafish at 20-somites in the dorsal telencephalon (Fig. 2A, C). When we compared the *FoxP2* expression pattern to the expression pattern of other genes transcribed at this stage, we found that *FoxP2* expression is very similar to the expression pattern of the *emx* homeobox genes (Kawahara and Dawid, 2002, Morita *et al.*, 1995). However, in contrast to *emx1* (Kawahara and Dawid, 2002), *FoxP2* does not show expression in the pineal gland, in pronephric duct and the urogenital opening (Fig. 2B). The expression of *FoxP2* in the dorsal telencephalon is partially overlapping with that of *emx1.* A double *in situ* hybridization demonstrates that *emx1* expression at the 20-somite stage is limited to a subdomain of *Foxp2* expression (Fig. 2D). Expression of *Foxp2* becomes more complex in the brain after 48 hours of development. At 7 days post fertilization, there is expression of *FoxP2* in several domains throughout the entire brain (Fig. 3A). The highest levels of *FoxP2* expression can be observed in the presumptive optic tectum.

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          1
zFoxP2    MMQESANETISNSSMSQNGMSSLSSQLDAGSRDGRSSGETSSEVSAVELLHLQQQQALQA
mFoxp2    MMQESVTETISNSSMNQNGMSTLSSQLDAGSRDGRSSGDTSSEVSTVELLHLQQQQALQA
hFOXP2    MMQESATETISNSSMNQNGMSTLSSQLDAGSRDGRSSGDTSSEVSTVELLHLQQQQALQA
          61
zFoxP2    ARQLLLQQPGSGLKSPKNNDKQRPLQVPVSVAMMSPQVITPQQMQQILQQQVLSPQQLQA
mFoxp2    ARQLLLQQQTSGLKSPKSSEKQRPLQVPVSVAMMTPQVITPQQMQQILQQQVLSPQQLQA
hFOXP2    ARQLLLQQQTSGLKSPKSSDKQRPLQVPVSVAMMTPQVITPQQMQQILQQQVLSPQQLQA
          121
zFoxP2    LLQQQQAVMLQQQHLQEFYKKQQEQLHLQLLQQQ--------------------------
mFoxp2    LLQQQQAVMLQQQQLQEFYKKQQEQLHLQLLQQQQQQQQQQQQQQQQQQQQQQQQQQQQQ
hFOXP2    LLQQQQAVMLQQQQLQEFYKKQQEQLHLQLLQQQQQQQQQQQQQQQQQQQQQQQQQQQQQ
          181
zFoxP2    -----------HPGKQAKEQQQQQQ----LAAQQLVFQQQLLQMQQLQQQQHLLNMQRQG
mFoxp2    QQQQQQQQQQQHPGKQAKEQQQQQQQQQ-LAAQQLVFQQQLLQMQQLQQQQHLLSLQRQG
hFOXP2    QQQQQQQQQQQHPGKQAKEQQQQQQQQQQLAAQQLVFQQQLLQMQQLQQQQHLLSLQRQG
          241
zFoxP2    LLSMPPGPGQPTLPGQTLPPAGLSPAELQQLWKDVTASHTMEDNGMKHSGLDLSTNNNTS
mFoxp2    LISIPPG--QAALPVQSLPQAGLSPAEIQQLWKEVTGVHSMEDNGIKHGGLDLTTNNSSS
hFOXP2    LISIPPG--QAALPVQSLPQAGLSPAEIQQLWKEVTGVHSMEDNGIKHGGLDLTTNNSSS

          301                                          Zinc Finger
zFoxP2    TTSTSNPKASPPITHHSMSNGQSPALNNRRESSLHEETAVSHSLYGHGVCKWPGCESICD
mFoxp2    TTSSTTSKASPPITHHSIVNGQSSVLNARRDSSSHEETGASHTLYGHGVCKWPGCESICE
hFOXP2    TTSSNTSKASPPITHHSIVNGQSSVLSARRDSSSHEETGASHTLYGHGVCKWPGCESICE
          361
zFoxP2    DFGQFLKHLNNEHALDDRSTAQCRVQMQVVQQLEIQLSKERERLQAMMAHLHMRPSEPKP
mFoxp2    DFGQFLKHLNNEHALDDRSTAQCRVQMQVVQQLEIQLSKERERLQAMMTHLHMRPSEPKP
hFOXP2    DFGQFLKHLNNEHALDDRSTAQCRVQMQVVQQLEIQLSKERERLQAMMTHLHMRPSEPKP
          421
zFoxP2    SPKPLNLVSSVTMSKNLPSISPPNLPQTPTTPTAPVTPLSQMPQVPNVLSPANVPSMGAM
mFoxp2    SPKPLNLVSSVTMSKNMLETSPQSLPQTPTTPTAPVTPITQGP---SVITPASVPNVGAI
hFOXP2    SPKPLNLVSSVTMSKNMLETSPQSLPQTPTTPTAPVTPITQGP---SVITPASVPNVGAI
          481
zFoxP2    RRRHTDKYSMALSSEIAPNYEFYKNADVRPPFTYATLIRQAIMESSDRQLTLNEIYSWFT
mFoxp2    RRRHSDKYNIPMSSEIAPNYEFYKNADVRPPFTYATLIRQAIMESSDRQLTLNEIYSWFT
hFOXP2    RRRHSDKYNIPMSSEIAPNYEFYKNADVRPPFTYATLIRQAIMESSDRQLTLNEIYSWFT

          541          Forkhead domain
zFoxP2    RTFAYFRRNAATWKNAVRHNLSLHKCFVRVENVKGAVWTVDEMEYQKRRSQKITGSPTLV
mFoxp2    RTFAYFRRSAATWKNAVRHNLSLHKCFVRVENVKGAVWTVDEVEYQKRRSQKITGSPTLV
hFOXP2    RTFAYFRRNAATWKNAVRHNLSLHKCFVRVENVKGAVWTVDEVEYQKRRSQKITGSPTLV
          601
zFoxP2    KNLPSSLGYGAALNASLQAALAETTLPLLGNPGLMNSASAMMGASPPVMMSGSPTGLLQG
mFoxp2    KNIPTSLGYGAALNASLQAALAESSLPLLSNPGLINNAS---------------SGLLQA
hFOXP2    KNIPTSLGYGAALNASLQAALAESSLPLLSNPGLINNAS---------------SGLLQA
          661
zFoxP2    TTHEELNGTLDHLDTNGHSSPGYS--PHTHLPPIHVKEEPLNMEDEDCPMSLVTTANHSP
mFoxp2    V-HEDLNGSLDHIDSNGNSSPGCSPQPHIHS--IHVKEEPVIAEDEDCPMSLVTTANHSP
hFOXP2    V-HEDLNGSLDHIDSNGNSSPGCSPQPHIHS--IHVKEEPVIAEDEDCPMSLVTTANHSP
          721
zFoxP2    ELDDDRELEEGNLSEDLE
mFoxp2    ELEDDREIEEEPLSEDLE
hFOXP2    ELEDDREIEEEPLSEDLE
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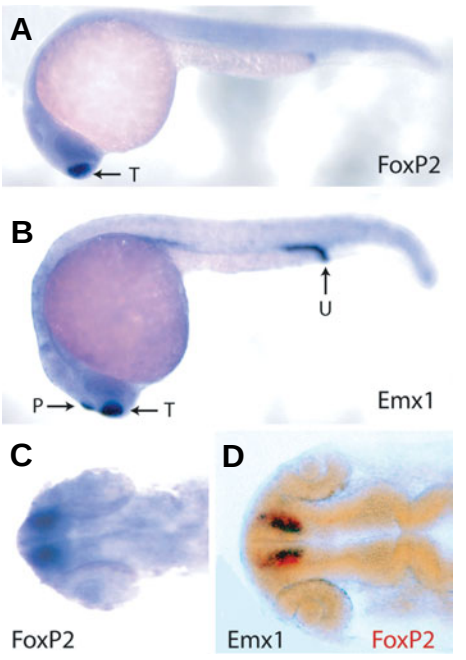
Fig. 1. Amino acid sequence comparison between zebrafish FoxP2 protein and its orthologues in mouse and human. *Identical amino acids are in bold. The absence of residues at the corresponding region is indicated by dashes. The zinc finger and the forkhead domain are underlined.*

Expression of FoxP2 *in the adult brain*

In the adult brain, the periventicular gray zone of the optic tectum shows very high levels of *FoxP2* expression (Fig. 3B, D and E). However, specific regions in the ventral forebrain and the hypothalamus display high levels of *FoxP2* transcripts as well. The ventral telencephalon (Fig. 3B, most anterior expression domain) and the preoptic area show high levels of *FoxP2* transcripts (Fig. 3B, C). There is strong expression in the periventricular pretectum and weaker expression in the dorsal thalamus and ventral posterior tuberculum (Fig. 3D). The rostral cerebellum does not express this gene (Fig. 3E, F), while the caudal lobe of cerebellum does (Fig. 3G). The caudal lobe of cerebellum corresponds to the vestibulocerebellum of mammals. The cerebellar *FoxP2* expression is in a conspicuous band of seemingly large cells, possibly corresponding to Purkinje cells. Specific cells in the superior reticular nucleus show distinct expression of *FoxP2,* as do some cells in the medial octavolateralis nucleus, which is the primary sensory nucleus for the lateral line (Mueller *et al.,* 2004).

The functional significance of the temporospatial expression of *FoxP2* in zebrafish neural tissue and for that matter in other species, is yet to be elucidated. There is a great shift of expression between the initial expression of *FoxP2* in the dorsal telencephalon and the widespread, but region-specific expression in the adult brain. The significance of this shift is not understood, but it is likely that *FoxP2* plays a different role during the early development of the brain than it does later in the differentiated neuronal cells. If the expression of *FoxP2* is any indication of its sites of function, then *FoxP2* is clearly involved in several aspects of brain development and function unrelated to language formation. There are many simmilarities of *FoxP2* expression in the brains of birds and crocodiles (Haesler *et al.,* 2004) when compared to that of zebrafish, e.g., ventral telencephalon (possibly striatum), optic tectum, torus semicircularis/inferior colliculus, cerebellum, dorsal thalamus and hypothalamus. It is the challenge for the future to determine the significance of *FoxP2* expression in the different



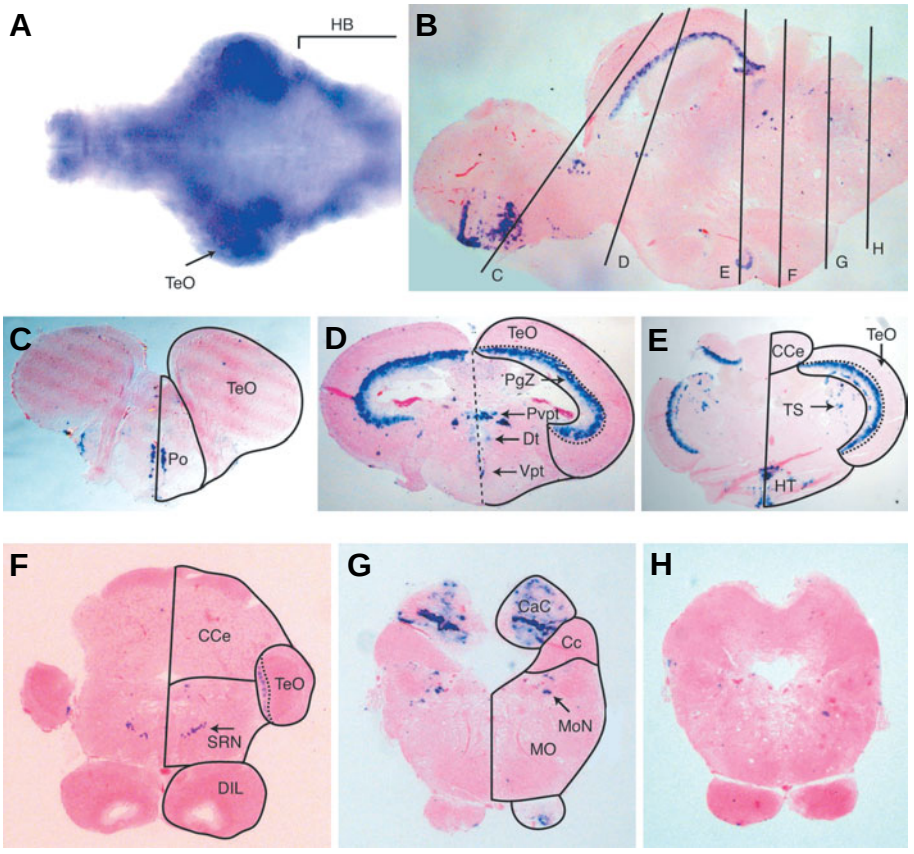


Fig. 2. (Left) Expression of *FoxP2* during zebrafish embryogenesis. (A) *Lateral view of* in situ *hybridization of* FoxP2 *probe to a 20-somite zebrafish embryo. The expression is in the dorsal telencephalon (arrow).* **(B)** *Lateral view of* in situ *hybridization of* Emx1 *probe to a 20-somite zebrafish embryo. Expression is in the dorsal telencephalon (T), pineal gland (P) and the urogenital opening (U).* **(C)** *Dorsal view of the head region from a 20-somite zebrafish embryo hybridized with a* FoxP2 *probe.* **(D)** *Double* in situ *hybridization of a* FoxP2 *probe (red) and* Emx1 *(black) to the head region of a 20-somite zebrafish embryo. Dorsal view.*

Fig. 3. (Right) Expression of *FoxP2* in the zebrafish brain. (A) *Dorsal view of* in situ *hybridization of* FoxP2 *probe to the isolated brain from a 7 day-old zebrafish. The isolated brain was opened along its dorsal axis and flattened. Anterior is to the left.* **(B)** *Sagittal section of a brain from a 3 months old zebrafish hybridized with a* FoxP2 *probe. Vertical lines indicate the positions of cross sections in images (C - H). Cross sections, hybridized with a* FoxP2 *probe, through the* **(C)** *telencephalon,* **(D)***optic tectum,* **(E)** *optic tectum, cerebellum and hypothalamus,* **(F)** *cerebellum,* **(G)** *caudal lobe of the cerebellum and the medulla oblongata. Arrow in (F) indicates the expression in the superior reticular formation. Arrow in (G) indicates expression in the medial octavolateralis nucleus.* **(H)** *A section caudal to (G) shows no expression of* FoxP2. *Abbreviations: Cc, cerebellar crest; CaC, caudal lobe of cerebellum; CCe, corpus cerebelli; DIL, diffuse nucleus of the inferior hypothalamic lobe; Dt, dorsal thalamus; HB, hindbrain; HT, hypothalamus; MO, medulla oblongata; MoN, medial octavolateralis nucleus; PgZ, periventicular gray zone of the optic tectum; Po, preoptic area; Pvpt, periventricular pretectum; SRN, superior reticular nucleus; TeO, optic tectum; TS, torus semicircularis; Vpt, ventral posterior tuberculum.*

brain areas.

While this manuscript was under review, a paper by (Bonkowsky and Chien, 2005) described expression of *FoxP2* during early stages of zebrafish development. These authors have demonstrated that there is a widespread expression of *FoxP2* in the neural system of zebrafish during the first three days of development.

Experimental Procedures

Isolation of zebrafish FoxP2

We used PCR and degenerate primers to isolate a cDNA fragment of the *FoxP2* gene encoding the forkhead and the zinc finger domain. The PCR reaction was carried out on reverse transcribed cDNA generated from 9-16 day old zebrafish embryos according to manufacturer's instructions. Degenerate primers encoding the following peptides were used: FoxP2-F: HGVCKW; FoxP2-R: -HKCFVRV

After sequencing the isolated PCR fragment, we found several EST fragments with high homology in the GenBank database (BQ617568; BQ783717).

Whole mount in situ *hybridization and histology*

Whole mount *in situ* hybridization was performed according to (Harland, 1991). For double *in situ* hybridization we followed the protocol of (Hauptmann and Gerster, 1994). For sections, the zebrafish brain was embedded in paraffin and 7 micrometers sections were made for *in situ* hybridization. Sections were dewaxed in xylene, rehydrated and hybridized with a digoxigenin-labeled probe. After the chromogenic reaction, the sections were

counterstained with hematoxylin and eosin.

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