Development 122, 1769-1780 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV2047

Complex expression of the *zp-50* pou gene in the embryonic zebrafish brain is altered by overexpression of *sonic hedgehog*

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SUMMARY

We report the characterization of the zebrafish *zp-50* class III POU domain gene. This gene is first activated in the prospective diencephalon after the end of the gastrula period. During somitogenesis, *zp-50* is expressed in a very dynamic and complex fashion in all major subdivisions of the central nervous system. After one day of development, *zp-50* transcripts are present in the fore- and midbrain in several distinct cell clusters. In the hindbrain, *zp-50* expression is found in two types of domains. Correct *zp-50* expression in the ventral fore- and midbrain requires genes known to be involved in dorsoventral patterning of the zebrafish CNS. Transcripts of the *sonic hedgehog* (*shh*) gene encoding an intercellular signaling molecule are detected in the forming diencephalon shortly prior to the appearance of *zp-50* mRNA. Correct expression in this region of both *shh*, and *zp-50*, requires a functional *cyclops* (*cyc*) locus: *shh* and *zp-50* transcripts are likewise absent from the ventral rostral brain of mutant *cyc*$^{-/-}$ embryos. Injection of synthetic *shh* mRNA into fertilized eggs causes ectopic *zp-50* expression at more dorsal positions of the embryonic brain. The close spatial and temporal coincidence of expression in the rostral brain, the similar response to the *cyc*$^{-}$ mutation, and the ectopic *zp-50* expression in the injection experiments all suggest that *zp-50* may directly respond to the reception of the Shh signal.

Key words: forebrain, midbrain, hindbrain, rhombomeres, dorsoventral patterning, *cyclops*, *axial*, *pax-2*, *hlx-1*

INTRODUCTION

The establishment of the dorsoventral axis of the neural plate is one of the earliest patterning events during the ontogeny of the vertebrate CNS. This process has been particularly well studied in the posterior neural tube (reviewed by Yamada et al., 1993; Placzek, 1995). The mesodermal cells of the notochord are emitting inductive signals causing the formation of the floor plate in the overlying ventral spinal cord. The floor plate itself acquires inductive potential, and both the notochord and floor plate contribute to the formation of motoneurons at a ventrolateral position in the neural tube. Homologs of the *Drosophila hedgehog* (*hh*) gene, notably *sonic hedgehog* (*shh*), have been suggested to encode signalling molecules apparently involved in these inductions (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993). *shh* is expressed in the notochord and subsequently in the floor plate, right at the time when these tissues display their inductive potential. These inductive interactions have been reproduced in tissue culture using recombinant Shh protein (Roelink et al., 1994; Marti et al., 1995; Roelink et al., 1995; Tanabe et al., 1995).

Analysis of the *cyclops* mutation in zebrafish has suggested that patterning of the rostral brain involves similar signalling events as those acting more posteriorly, even though there is no underlying notochord at such an anterior position. In *cyclops* (*cyc*) mutants the floor plate is absent (Hatta et al., 1991) and major parts of the ventral forebrain are missing (Hatta et al., 1994; Patel et al., 1994). In agreement with a common patterning mechanism at anterior and posterior positions, *shh* is also strongly expressed in the ventral forebrain of wild type, but not in the corresponding region of *cyc*$^{-/-}$ embryos (Krauss et al., 1993). In the rostral brain, *shh* has been shown to induce Isl-1^{+} neurons in the ventral chick forebrain, and dopaminergic neurons in the rat and chicken midbrain (Ericson et al., 1995; Hynes et al., 1995; Wang et al., 1995). In the zebrafish, overexpression of *shh* leads to ectopic expression of the *nk2.2* and *axial* genes in dorsal parts of the fore- and midbrain (Krauss et al., 1993; Barth and Wilson, 1995). *shh* appears thus to encode an important patterning signal along the entire neural plate. However, the precise identity of the cell types induced by *shh* is determined by factors dependent on the position of the responding cells along the anteroposterior axis (reviewed by Lumsden and Graham, 1995).

Many POU genes are widely expressed in the CNS of both vertebrates and invertebrates (reviewed by Wegner et al., 1993). These genes encode transcription factors with a DNA-binding motif of about 150 amino acids consisting of an N-terminal POU-specific domain separated by a non-conserved linker from a special type of homeodomain (reviewed by Verrijzer and van der Vliet, 1993; Herr and Cleary, 1995). Mammalian POU genes with a class III type POU domain are expressed in distinct but overlapping patterns in many areas of the CNS (reviewed by Alvarez-Bolado et al., 1995). Class III

POU genes appear to have important roles in establishing and/or maintaining the identity of the cells in which they are expressed. The class III POU gene *xlpou1* has been shown to be able to directly neuralize uncommitted ectodermal cells in *Xenopus* (Witta et al., 1995), and the mammalian *brn-2* gene is responsible for determining specific neuronal lineages in the hypothalamus (Nakai et al., 1995; Schonemann et al., 1995).

In a search for genes with a probable role in brain patterning, we screened the zebrafish genome for POU domain containing genes. We report here the sequence and expression pattern of a class III POU gene called *zp-50*. The dynamic and complex pattern of *zp-50* expression in different regions of the CNS is compatible with the notion that POU genes may play important roles in determining regional identity within the zebrafish brain. The analysis of the *zp-50* expression pattern in the rostral brain suggests that this gene is subject to regulation by the initial mechanism determining dorsoventral polarity. We find that a prominent early *zp-50* expression domain in the ventral forebrain is missing in $cyc^{-/-}$ embryos which also lack *shh* expression in this region. In the ventral fore- and midbrain of wild-type embryos, *zp-50* RNA accumulates shortly after that of *shh*, and both transcripts are distributed very similarly. Based on the temporal and spatial similarities of the expression patterns of the two genes, and on the shared dependence on a functional *cyc* gene, we propose a functional link between *shh* and *zp-50* expression. This notion is supported by injections of synthetic *shh* mRNA which lead to ectopic *zp-50* expression in dorsal regions of the diencephalon and in the midbrain. *zp-50* might thus be a target gene responding directly to the Shh dorsoventral patterning signal. *zp-50* may thereby play a role in the translation of this signal into positional or cell type identity.

MATERIALS AND METHODS

Cloning and sequence analysis of *zp-50*

5×10^5 plaques of a neurula λZAPII library (prepared by R. Riggleman and K. Helde, a kind gift from D. J. Grunwald) were screened with a 260 bp PCR fragment containing parts of the *zp-50* POU region. 3 clones repeatedly hybridized to replica filters. Their inserts were subcloned into BSSK− by in vivo excision. Restriction analysis indicated that two of the clones were identical. The third isolate contained an internal fragment in addition to a short rearrangement. Nested deletions were generated and the complete sequence of both strands was determined. By partial sequencing of isolates of a genomic zebrafish library (Stratagene) we obtained additional sequence information including the putative aminoterminus.

Fish stocks and maintenance

Fish were kept essentially as described by Westerfield (1993). For most experiments we used a wild-type strain from West Aquarium (Bad Lauterburg, Germany). Ages are given in hours postfertilization (hpf) based on standard developmental stages at 28.5°C (Kimmel et al., 1995). A stock bearing the *cyclops*, cyc^{b16} allele (Hatta et al., 1991) was kindly provided by P. Haffter.

In situ hybridization

To relate the position of *zp-50* gene activity to the expression domains of marker genes we applied a 2 color in situ hybridization procedure based on RNA probes labeled with either digoxigenin or fluorescein (Hauptmann and Gerster, 1994). RNA probes specific for the *zp-50* genes were prepared by T7 RNA polymerase after *Spe*I linearization of a BSSK− subclone containing the 1131 bp *Bgl*II/*Acc*I fragment with the 3′ part of the *zp*-50 cDNA. This probe excludes the conserved POU domain as well as the poly(A) tail. For double hybridizations we used RNA probes prepared either from full-length or the longest available cDNAs of the zebrafish *hlx-1* (Fjose et al., 1994), *pax[zf-b]*(most probably the homolog of mammalian *pax-2*; Krauss et al., 1991), *axial* (Strähle et al., 1993), and *krx-20* genes (Oxtoby and Jowett, 1993).

Immunohistochemistry

We used the BCIP/NBT and Fast Red dyes for a novel combination of in situ hybridization with immunohistochemistry. This way we obtained a better contrast between the two dyes than when using peroxidase to detect the primary antibody. Embryos were first hybridized with the *zp-50* probe and stained with NBT/BCIP. Incubation for 10 minutes in 0.1 M glycine-HCl, pH 2.2, 0.1% Tween-20 removed the alkaline phosphatase conjugated anti-digoxigenin antibody. The embryos were subsequently blocked and incubated overnight with an antibody against acetylated α-tubulin (Piperno and Fuller, 1985; Sigma, diluted 1:2000), or with the zn-12 antibody (Trevarrow et al., 1990; a gift from M. Westerfield, diluted 1:5000). Samples were then developed with a sheep α-mouse F_{ab} fragment conjugated to alkaline phosphatase (Boehringer Mannheim, diluted 1:600) and Fast Red staining for about 20 minutes.

RNA injection experiments

Synthetic capped *shh* mRNA was produced by in vitro transcription of *Bam*HI linearized p64TZshh (Krauss et al., 1993; a gift from J.-P. Concordet and P. Ingham) using SP6 RNA polymerase and the mMessage mMachine kit (Ambion). RNA was injected at concentrations between 25 and 160 ng/μl in a buffer containing 100 mM KCl, 10 mM sodium phosphate, pH 7.3, 30 μM spermine, 70 μM spermidine, 1% phenol red. Injection needles were prepared on a Narishige PN-3 needle puller and sharpened on a BV-10 micropipette beveller (Sutter Instrument Co.). Injections were carried out on 1- to 4-cell stage embryos immobilized in an agarose holding device according to a design by E. Weinberg, as described by Westerfield (1993).

RESULTS

Cloning of *zp-50*

To identify and isolate POU genes in the zebrafish we carried out consecutive PCR reactions on genomic DNA using two nested sets of degenerate primers directed against conserved regions of POU domains. The majority of the subcloned products showed the expected length of ~260 base pairs (T. G., unpublished data). One clone (*ZP-50*) encoding a partial class III POU domain was used to screen a cDNA library prepared from 9- to 16-hour old zebrafish embryos.

The sequence of the isolated *zp-50* cDNA is 2759 base pairs long (Fig. 1A). Northern blot analysis showed a single transcript of about 2.9 kb (not shown). Considering its very short poly(A) tail this indicates that the cDNA clone is nearly full length. However, the MATTA (or MATAA) motif conserved at the N termini of all other vertebrate class III POU proteins was not encoded by this cDNA clone. Partial sequencing of genomic clones (C. Bornmann and T. G., unpublished data) extended the known sequences at the 5′ end by 126 nucleotides (Fig. 1A). The open reading frame was thus enlarged by five codons encoding precisely the missing MATTA motif. The open reading frame of the combined sequence is 368 amino acids long. The ATG codon of the MATTA motif shows a reasonable match to the consensus translation start signal and is preceded by a stop codon 4 triplets upstream. Thus we

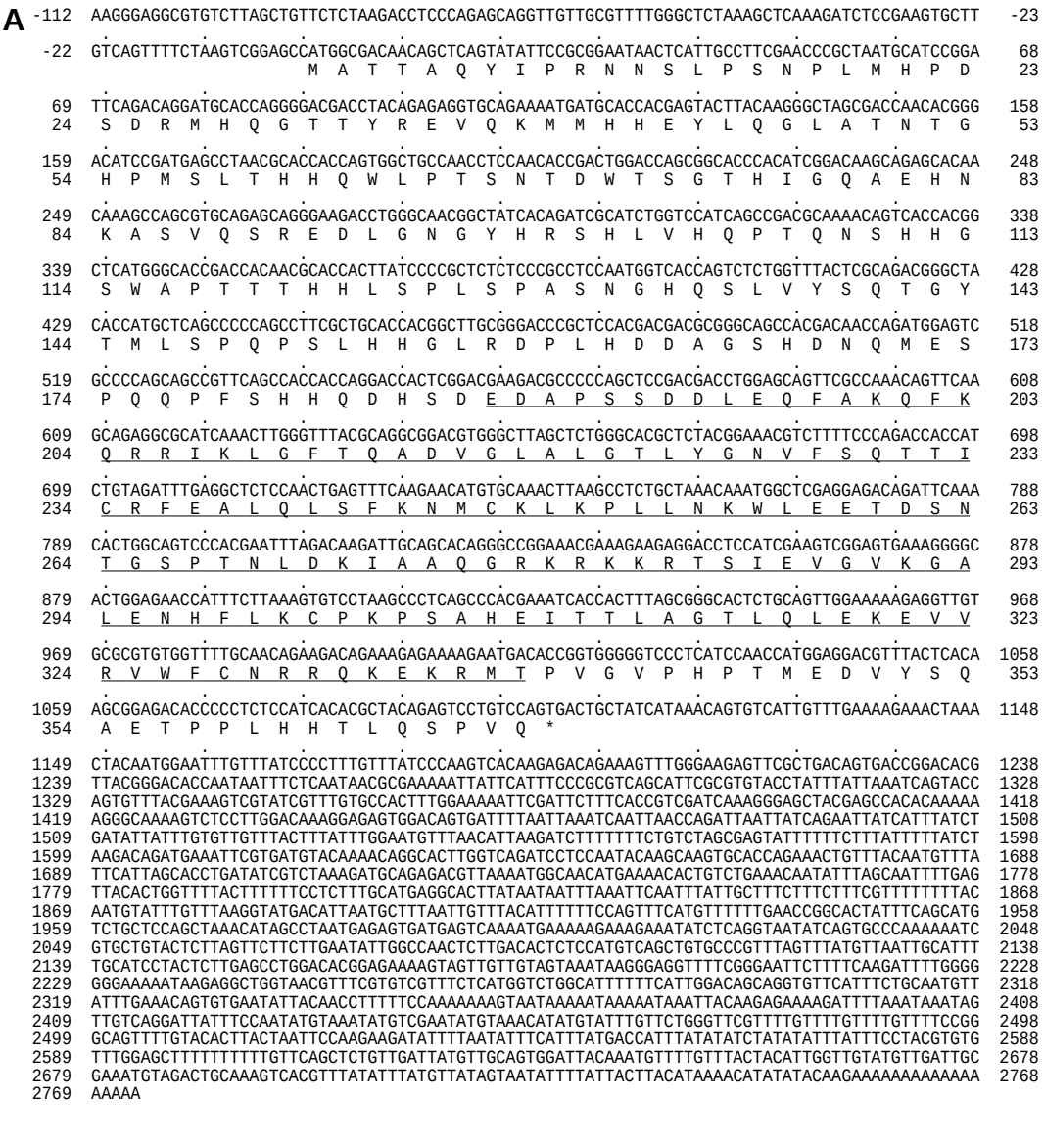
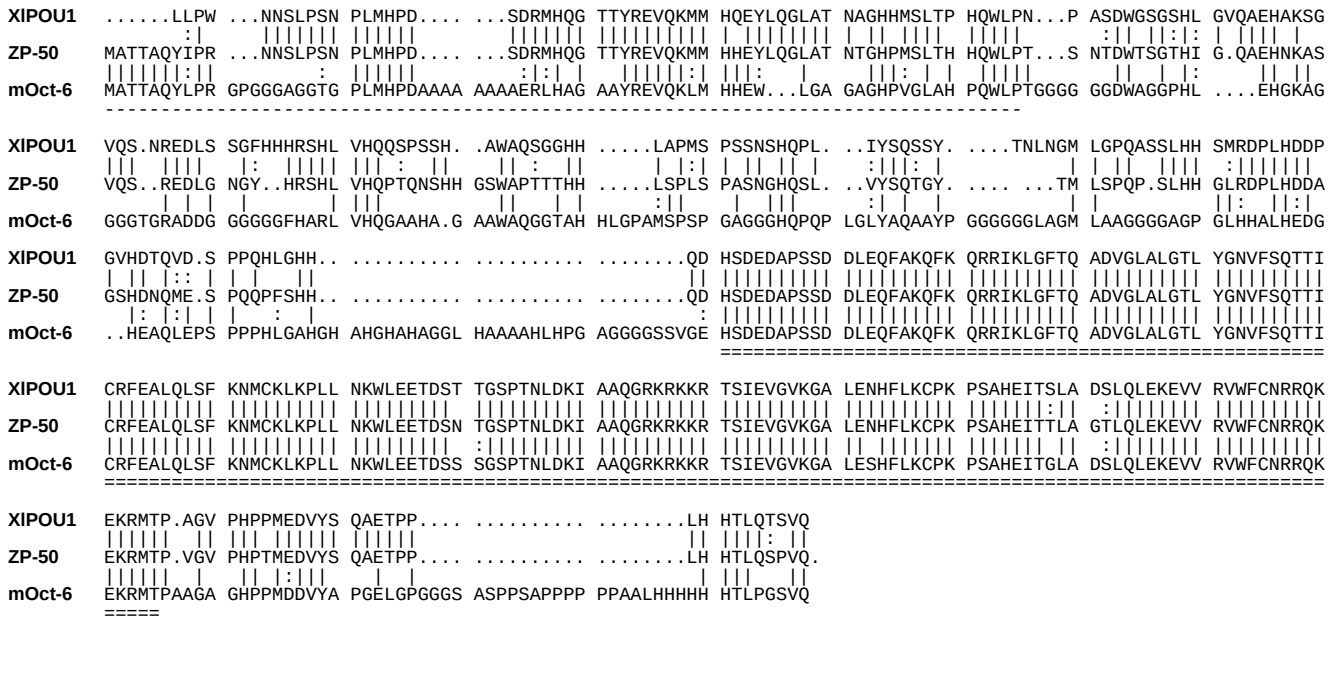


Fig. 1. (A) Nucleotide sequence of the *zp-50* cDNA and 5′ genomic sequences (EMBL accession X96422). The break point between genomic and cDNA sequences is at position +15. 3′ of this position there is an overlap of approx. 100 identical bp between genomic and cDNA sequences. The predicted amino acid sequence of the ZP-50 protein is indicated below the nucleotide sequence. The POU domain is underlined. (B) Sequence comparison of ZP-50 with its two closest relatives, XLPOU1 and Oct-6. Identity between two sequences is indicated by |; conservative exchanges by :. The POU domain is indicated by double underlining.



assume that this ATG is most likely the in vivo translation initiation site. A 152 amino acids class III POU domain is found in the C-terminal half of the predicted protein (Fig. 1A).

The *xlpou1* gene from *Xenopus* (Agarwal and Sato, 1991) shows high homology to *zp-50* over the entire length of the reading frame (Fig. 1B). Disregarding a few minor gaps, 301 of 363 amino acids are identical and an additional 16 similar amino acids are found at corresponding positions. Two domains show particularly high sequence conservation: the POU domain exhibits 97.4% sequence identity. In addition, an N-terminal 58 amino acid region shows an above-average conservation of 89.7% sequence identity.

Northern blot analysis of different embryonic stages (not shown) indicates that expression of *zp-50* starts between 10 and 12 hpf. Transcript levels are highest between 2 and 3 days after fertilization and subsequently drop about five-fold. In adult tissues, *zp-50* mRNA was detected only in the brain. In all stages or tissues with detectable *zp-50* expression, we observed only a single transcript of approx. 2.9 kb.

Expression in the fore- and midbrain

The sites of *zp-50* gene expression during embryogenesis were determined by in situ hybridization. Expression of *zp-50* is first detected after the completion of epiboly in a transverse stripe in the anterior half of the neural plate (Fig. 2A). 2 color in situ hybridization of *zp-50* and *pax-2* shows that this initial *zp-50* domain is located anterior to *pax-2* expression in the posterior midbrain (Krauss et al., 1991) (Fig. 3A). The two expression domains are separated by a gap. This earliest site of *zp-50* expression is thus probably located within the primordium of the posterior diencephalon.

Around the 1 somite stage, a second *zp-50* expression domain appears along the presumptive ventral forebrain, extending from the anterior tip of the neural keel towards the border between the fore- and midbrain primordia (Fig. 2B). During somitogenesis *zp-50* expression is also detected in more posterior regions of the midbrain which contribute to the tegmentum. This *zp-50* domain is separated by a narrow gap from the *pax-2*-expressing cells in the border region between the mid- and hindbrain (Fig. 3B) as seen in dorsal views of later stages (Fig. 3C).

At the 20 somite stage, the contiguous ventral expression domain in the fore- and midbrain has begun to resolve into several cell clusters showing intense *zp-50* expression (Fig. 2D). The rostralmost patch of cells with strong *zp-50* expression is detected at the anterior tip of the CNS of 26 and 34 hpf embryos (Fig. 2E,F). These cells are located medial to the optic stalk which expresses *pax-2* in the 1-day old embryo (Fig. 3D). Double labeling experiments combining *zp-50* in situ hybridization with immunostaining of axons by a mono-

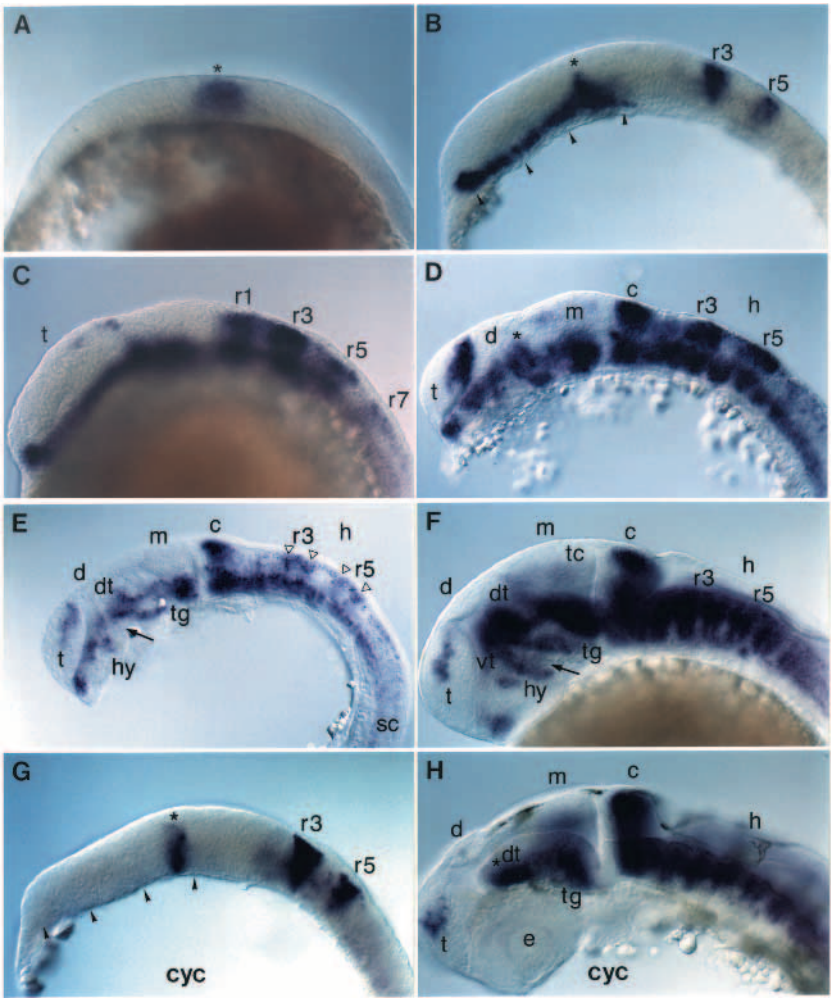


Fig. 2. In situ hybridization analysis of *zp-50* expression during embryogenesis. All pictures are side views with the anterior part of the embryos oriented towards the left. In some cases the yolk has been removed for better visibility. Wild-type (A-F) and *cyclops* mutant (G,H) embryos were hybridized with a *zp-50* probe. (A) bud (10 hpf). The asterisk marks the earliest expression domain in the forming posterior diencephalon (dorsal thalamus). (B) 5 somites (12 hpf). Rhombomeres r3 and r5 are indicated, as well as the early transverse *zp*-50 expression domain in the diencephalon (asterisk). The longitudinal expression domain in the ventral forebrain (arrowheads) is absent in $cyc^{-/-}$ embryos (cf. G). (C) 10 somites (14.5 hpf). Alternating high levels of *zp-50* expression are found in odd-numbered rhombomeres r1, r3, r5 and r7. Expression begins in the telencephalon. (D) 20 somites (19 hpf). The asterisk marks the initial *zp*-50 expression domain in the dorsal thalamus. The initially contiguous longitudinal expression along the forebrain is subdivided into several domains. (E) 26 hpf. Open triangles indicate the lateral arches in rhombomeres r3 and r5. The lower part of these arches coincide with rhombomere boundaries. The arrow points to the forming ventral flexure. (F) 34 hpf. Hindbrain expression is observed in paired stripes along the rhombomere boundaries. (G) 5 somite $cyc^{-/-}$ embryo. *zp-50* is expressed in rhombomeres r3 and r5 and the transverse domain in the posterior diencephalon (asterisk). Arrowheads indicate missing *zp-50* expression in the ventroanterior brain. (H) 30 hpf $cyc^{-/-}$ embryo. Diencephalic *zp-50* expression is found only in the dorsal thalamus in the area derived from the initial expression site visible after the bud stage (asterisk). c, cerebellum; d, diencephalon; dt, dorsal thalamus; e, eye; h, hindbrain; hy, hypothalamus; m, mesencephalon; sc, spinal cord; t, telencephalon; tc, tectum; tg, tegmentum; vt, ventral thalamus.

clonal antibody against acetylated tubulin (Piperno and Fuller, 1985) show that the axons of the postoptic commissure (Chitnis and Kuwada, 1990; Wilson et al., 1990) surround the outside of this rostralmost *zp-50* domain (Fig. 3E). Additional *zp-50* expression sites in the anterior diencephalon include two cell clusters of the ventral thalamus from which staining starts to spread ventrally into the forming hypothalamus (Fig. 2E,F). The anterior cell cluster is located close to the position where the telencephalic (or supraoptic) axon tract (Chitnis and Kuwada, 1990; Wilson et al., 1990) meets the tract of the postoptic commissure in the ventral diencephalon (Fig. 3G).

Two narrow strings of *zp-50*-expressing cells extend caudally from the strong expression site in the posterior diencephalon (dorsal thalamus) (Fig. 2E,F). The more dorsally located of these cell strings connects with a strong *zp-50* expression site in the midbrain tegmentum. The strong *zp-50* expression sites in the tegmentum and in the dorsal thalamus are separated by the tract of the posterior commissure (Fig. 3G) (Chitnis and Kuwada, 1990; Wilson et al., 1990). The more ventral *zp-50*-expressing cell string stretches caudally from the dorsal thalamus into the ventral tegmentum and ends in the vicinity of the nucleus of the medial longitudinal fascicle (nucMLF; Chitnis and Kuwada, 1990; Wilson et al., 1990; Fig. 3G). The two *zp-50* extensions from the dorsal thalamus into the mesencephalon therefore enclose the nucMLF from both the dorsal and the ventral side.

The extent of *zp-50* expression in the telencephalon changes rapidly. *zp-50* expression is detected from the 10 somite stage on (Fig. 2C). During somitogenesis, the expression domain covers a relatively large portion of the dorsal telencephalon (Fig. 2D). However, this domain becomes confined to a smaller patch of cells at the beginning of the second day of develop-

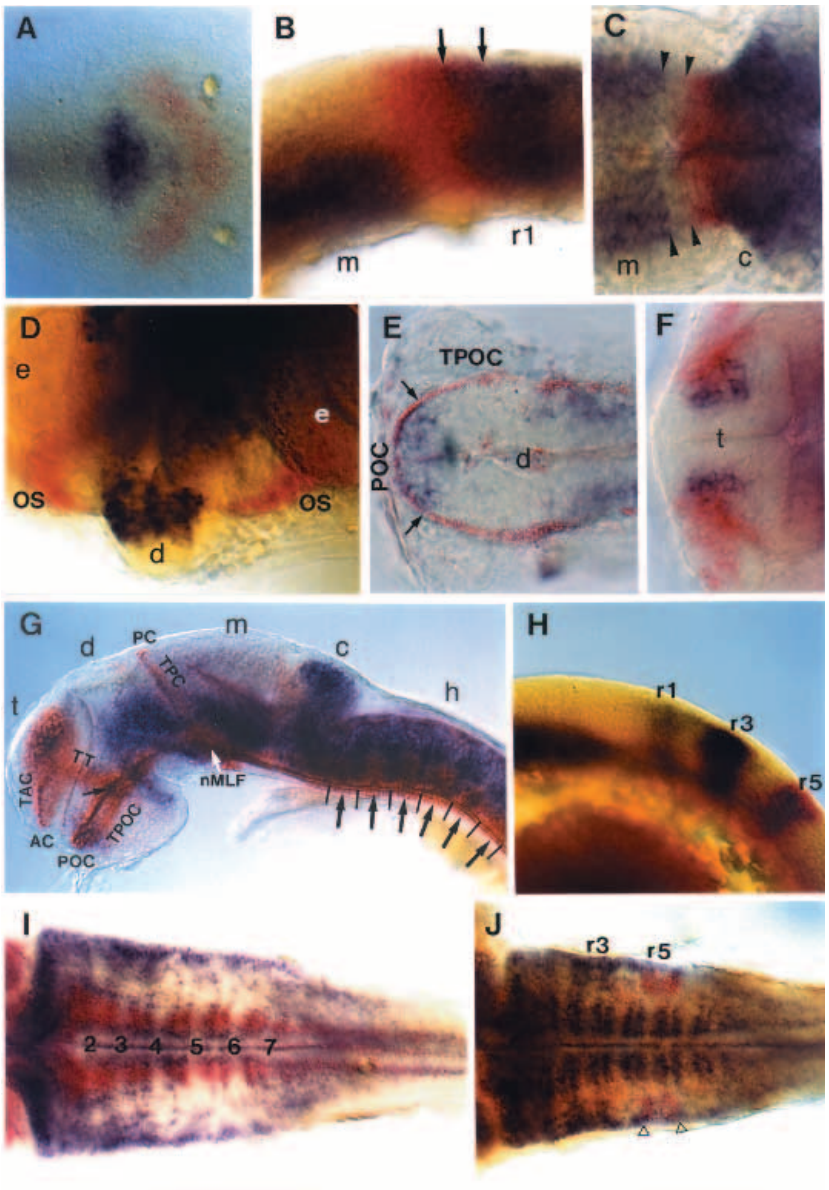


Fig. 3. Characterization of *zp-50* expression by doublelabeling. In all side and dorsal views anterior is to the left. (A-D) 2 color in situ hybridization for *zp-50* (purple) and *pax-2* transcripts (red). (A) Dorsal view of flat mounted bud stage embryo with *zp-50* staining in the posterior diencephalon and *pax-2* expression along the future midbrain/hindbrain junction. (B) Side view of the mid-/hindbrain border region of a 10 somite stage embryo. Arrows indicate a zone of overlapping *zp-50* and *pax-2* expression. (C) Dorsal view of 26 hpf embryo. *zp-50*-expressing cells in the tegmentum are separated by a gap of 2-3 cell diameters (arrowheads) from the *pax-2* domain at the midbrain/hindbrain boundary. (D) Frontal view of 27 hpf embryo: *pax-2* stains the optic stalks emerging next to the anteriormost position of diencephalic *zp-50* expression. (E) Dorsal view of 34 hpf anterior diencephalon hybridized with *zp-50* (purple). Axons are immunostained with an antibody to acetylated tubulin (red). The anteriormost *zp-50* expression site is surrounded (arrows) by the axons of the postoptic commissure and its associated longitudinal tract (TPOC). (F) Dorsal view of 36 hpf telencephalon hybridized with *zp-50* (purple). The *zp-50*-expressing cells are more medial but partially overlap with neural cells positive for the zn-12 epitope (red) projecting axons into the tract of the anterior commissure. (G) Side view of 34 hpf embryo hybridized with *zp-50* (purple). Axons are stained with the acetylated tubulin antibody (red). The eyes have been removed. The small arrow in the anterior diencephalon indicates *zp-50* expression located in the immediate vicinity of the junction between the telencephalic (or supraoptic) axon tract with the tract of the postoptic commissure. The white arrow shows the nucMLF which is surrounded ventrally, anteriorly and dorsally by the *zp-50* expression domains. Large arrows in the area of the hindbrain indicate reticulospinal neurons in the rhombomere centers flanked by *zp-50*-expressing double stripes at the interrhombomeric boundaries. (H) Side view of a 5 somite embryo double labeled with *zp-50* (purple) and *krx-20* (red) expressed in rhombomeres r3 and r5. (I) Dorsal view of 36-40 hpf hindbrain hybridized with *zp-50* (purple) and *hlx-1* (red) showing similar expression domains along medial rhombomere borders. The numbers indicate the corresponding rhombomere centers. (J) Dorsal view of 36-40 hpf hindbrain doublestained for *zp-50* (purple) and *krx-20* (red). At this stage, *krx-20* is only strongly expressed in r5 whose borders are indicated by open triangles. AC, anterior commissure; c, cerebellum; d, diencephalon; e, eye; h, hindbrain; m, mesencephalon; nMLF, nucleus of the medial longitudinal fascicle; OS, optic stalk; PC, posterior commissure; POC, postoptic commissure; r1, r3, r5, rhombomeres 1, 3 and 5; t, telencephalon; TAC, tract of the anterior commissure; TPC, tract of the posterior commissure; TPOC, tract of the postoptic commissure; TT, telencephalic (or supraoptic) tract.

ment (Fig. 2E,F). The *zp-50*-expressing cells in the telencephalon are located in the olfactory bulb, medially to the dorsal ending of the tract of the anterior commissure (Chitnis and Kuwada, 1990; Wilson et al., 1990). This is shown by staining axons of *zp-50* hybridized embryos with the zn-12 monoclonal antibody directed against an HNK-1-like epitope expressed on some of the earliest neurons in zebrafish (Fig. 3F; Trevarrow et al., 1990).

Expression in the hindbrain

zp-50 expression in the forming hindbrain is first detected in two transverse stripes at the five somite stage (Fig. 2B). Double hybridizations with *zp-50* and the zinc finger gene *krx-20* (Oxtoby and Jowett, 1993) maps the two domains of *zp-50* expression to rhombomeres r3 and r5 (Fig. 3H). Whereas *krx-20* is expressed in nearly all cells of these two rhombomeres, *zp-50* expression is found only in a subset of cells. *zp-50* expression is initially more intense in r3 and the number of *zp-50*-expressing cells is higher in r3 than in r5. Around the 10 somite stage, additional expression is found in a narrow stripe anterior to r3 at a distance corresponding to the width of about one rhombomere (Figs 2C, 3H). This *zp-50* domain partially overlaps the *pax-2* expression (Fig. 3B) which at this early stage covers not only the primordium of the posterior midbrain but also the anterior hindbrain (Hauptmann and Gerster, 1995). The partial overlap of *zp-50* with *pax-2* expression, as well as the spacing with respect to r3, suggest that this later arising *zp-50* domain is located at the position of r1. Caudal to r5, *zp-50* is expressed in r7 and in a gradient of decreasing intensity along the spinal cord (Fig. 2C). As a result, *zp-50* shows an alternating pattern of particularly strong expression in odd-numbered rhombomeres between the 10 and the 14 somite stages.

By the 20 somite stage, intense *zp-50* expression has extended to all rhombomeres (Fig. 2D). Hindbrain structures strongly express *zp-50* at two different dorsoventral levels. One type of domain is located dorsally within the rhombomeres and is particularly pronounced in r3 and r5. Strong staining is also found more anteriorly in the anlage of the cerebellum. Around 26 hpf the dorsal expression domains are transformed into dorsolateral arches that are open on the ventral side (Fig. 2E). The position of the ventral parts of the arches correlate well with the interrhombomeric boundaries (Fig. 2E, cf. also 3I,J). The other domain type that becomes clearly visible from around the 20 somite stage onwards, is located more ventromedially and exhibits similar expression levels in each rhombomere (Fig. 2D).

At 34 hpf the more medial hindbrain clusters of *zp-50*-expressing cells have formed a series of transverse columns of cells, generating a repeated pattern of paired stripes (Fig. 2F). This type of pattern strongly resembles the hindbrain expression of the *hlx-1* gene which has been shown to localize along the rhombomere borders (Fjose et al., 1994). By 2 color in situ hybridizations we find that *hlx-1* is strongly expressed at positions similar to those of the medial *zp-50*-expressing cells, whereas the more dorsolateral 'arches' express only *zp-50*, but not *hlx-1* (Fig. 3I). This suggests that the paired *zp-50* stripes are located along the borders of individual rhombomeres. Two additional observations support this. The reticulospinal neurons located in the rhombomere centers (Trevarrow et al., 1990) are flanked on either side by the paired *zp-50* stripes (Fig. 3G). Furthermore, we find that *krx-20* expression localizes in r5 at an anteroposterior level precisely between the transverse *zp-50* double stripes (Fig. 3J). Therefore, we conclude that the *zp-50*-expressing cell columns are located next to the anterior and posterior borders of each rhombomere. Consistent with this interpretation, there is only a single stripe at the posterior end of r7 (Fig. 3J).

Zp-50 expression in *cyclops* mutant embryos

Embryos homozygous for a mutation in the *cyclops* locus are severely perturbed in the dorsoventral patterning of their CNS (Hatta et al., 1991, 1994). Since *zp-50* starts to be expressed very early in a longitudinal domain in the ventral diencephalon we have investigated the effect of the cyc^{b16} mutation on *zp-50* expression. Analysis shows that *zp-50* expression is absent from the ventral diencephalon of 5 somite stage $cyc^{-/-}$ embryos (cf. Fig. 2G with 2B). The defect is restricted to the ventral part of the forebrain since *zp-50* expression is unaffected in the more dorsally located forebrain domain derived from the initial transverse expression stripe seen in Fig. 2A.

At 30 hpf, several *zp-50* expression regions are absent in $cyc^{-/-}$ embryos. The missing domains include the rostral cell cluster medial to the optical stalk, the two bands reaching from the ventral thalamus towards the hypothalamus, and the ventral string extending from the dorsal thalamus into the mesencephalon (cf. Fig. 2H with 2F). Presumably, these cells expressing *zp-50* in wild-type embryos are derivatives of the initially contiguous ventral diencephalic domain which is observed during early somitogenesis. In contrast, the more dorsally located major expression domains of the posterior diencephalon and the mesencephalon are still detected in $cyc^{-/-}$ embryos, although they are fused along the midline. The dorsal expression domain in the posterior diencephalon is thus most probably derived from the transverse diencephalic stripe observed already at the beginning of somitogenesis and not abolished by the cyc^{b16} mutation (cf. Fig. 2A and B with G).

Misexpression of *sonic hedgehog* ectopically activates *zp-50* expression

The zebrafish *sonic hedgehog* (*shh/vhh-1*) gene is involved in dorsoventral patterning of the neural plate (Krauss et al., 1993; Roelink et al., 1994). Similar to the situation with *zp-50*, it has been reported that *shh* expression is undetectable in the forming anterior head region of $cyc^{-/-}$ embryos (Krauss et al., 1993). *shh* transcripts normally accumulate at this position around the 100% epiboly stage (Krauss et al., 1993; data not shown). The longitudinal *zp-50* expression domain in the ventral diencephalon can be observed 1 to 2 hours after the first appearance of *shh* RNA. We compared the expression of *shh* and *zp-50* side by side and by 2 color in situ hybridization at the 5 and 10 somites stages (Fig. 4A-C, data not shown). The expression domains show extensive similarities in the area of the ventral fore- and midbrain. Even in 1-day old embryos we find, in the ventroanterior brain, a very close proximity of the transcript distributions of these two genes (Fig. 5A). These findings suggest a possible functional link between *shh* and *zp-50* expression.

To test the possibility of a functional relationship more directly we injected synthetic *shh* mRNA into fertilized eggs between the 1 and 4 cell stages. In a large majority of embryos, the injected *shh* RNA caused defects as recently reported

(Barth and Wilson, 1995; Macdonald et al., 1995). Morphologically easily visible malformations occurred mostly in the head region: after 1 day the affected embryos showed a reduction of the third ventricle, and eye development was strongly perturbed (not shown). Embryos injected with control mRNAs encoding either the bacterial *lacZ* gene or a mutated *shh* copy carrying a frame shift did not show any obvious defects (not shown).

Analysis of *zp-50* expression around the tailbud stage did not show premature *zp-50* expression in *shh*-injected embryos (Table 1). However, aberrant *zp-50* transcription was observed in the vast majority of injected embryos fixed at the 5 (not shown) and the 10 somite stages. The longitudinal *zp-50* expression in the forming ventral diencephalon is expanded dorsally compared to uninjected control embryos (Fig. 4C,D). In many cases, the extent of *zp-50* expression appears to fill the entire space of the anterior diencephalon up to the optic recess. Ectopic *zp-50* expression is also dramatically evident in the area of the dorsal thalamus and in the prospective midbrain at dorsal positions which usually never express this gene. *zp-50* expression is moreover altered in the hindbrain and the rhombomeric organization is not easily visible anymore. In contrast to the ectopic activation by *shh* in the diencephalon, *zp-50* expression is severely reduced or entirely undetectable in the forming telencephalon of *shh*-injected embryos (Table 1).

Analysis of the expression of other marker genes shows that *shh* overexpression does not perturb patterning of the anterior head in general. The homeodomain-containing *hlx-1* gene is expressed during early somitogenesis in the mesoderm of the prechordal plate underlying the rostral brain rudiment and possibly in the ventralmost midline tissue of the brain (Fjose et al., 1994) (Fig. 4G,I). *hlx-1* expression in this domain is not apparently altered in injected embryos (Fig. 4H,J). Therefore, the Shh signal seems to affect only the anterior neuroectoderm but not the mesoderm beneath it. However, *hlx-1* expression emerging around the 10 somite stage in the mid- and hindbrain is shifted dorsally in *shh*-injected embryos (Fig. 4H). The anterior end of the regular *axial* expression domain in the neural keel is located in the posterior diencephalon (Strähle et al., 1993). Similar to the results of Krauss et al. (1993) we do find at the 5 and 10 somite stages dorsal extensions of *axial* expression in the posterior diencephalon and in the midbrain of *shh*-injected embryos (Fig. 4F). The fact that the *zp-50* and *axial* domains are unaltered in their anteroposterior extent indicates that *shh* overexpression modifies primarily dorsoventral patterning rather than per se disrupting the formation of the anterior brain.

Although the *zp-50* expression domains found normally in the anterior diencephalon can also be inferred in 1-day old injected embryos, the expression sites anterior to the ventral flexure are clearly extended in a dorsal direction (Fig. 5C). Widespread ectopic *zp-50* expression is furthermore observed in the dorsal thalamus and in the midbrain. *axial* expression is ectopically activated by *shh* in a very similar region (Krauss et al., 1993; Barth and Wilson, 1995): expression is dorsally extended at the rostral end of the regular *axial* domain. This extension lies just anterior to the major *zp-50* domain in the dorsal thalamus which is presumably derived from the earliest site of *zp-50* expression and is expanded dorsally in injected embryos (Fig. 5E,F). Increased expression of both *zp-50* and *axial* is observed posterior to this position. However, 2 color in situ hybridization analysis suggests that individual cells within this area usually express only either *zp-50* or *axial*. We generally observe that considerably more cells within this domain aberrantly express *zp-50* than *axial*. In contrast to ectopic activation elsewhere, *zp-50* expression is drastically reduced or is entirely undetectable in the telencephalon of the majority of 1-day old *shh*-injected embryos (Fig. 5C,E,F; Table 1).

Table 1. Ectopic *zp-50* expression after *shh* mRNA injection

Stage	*shh* injected embryos 25 ng/µl	*shh* injected embryos 50 ng/µl	Uninjected siblings
Beginning of somitogenesis			
No staining	8	ND	4
Weak transverse stripe in primordium of dorsoposterior diencephalon	1	ND	4
Total	9		8
Mid-somitogenesis			
Ectopic expression in diencephalon and mesencephalon, disorganized hindbrain expression, complete lack of expression in telencephalon	23	24	0
Ectopic expression in diencephalon and mesencephalon, disorganized hindbrain expression, reduced expression in telencephalon*	13	6	0
Ectopic expression in diencephalon and mesencephalon but relatively normal expression in hindbrain and telencephalon	0	1	0
Embryos strongly deformed, no structures can be identified	1	3	1
Apparently normal expression pattern	0	0	34
Total	37	34	35
1 day after fertilization			
Ectopic expression in diencephalon and mesencephalon, disorganized hindbrain expression, complete lack of expression in telencephalon	74	37	0
Ectopic expression in diencephalon and mesencephalon, disorganized hindbrain expression, reduced expression in telencephalon	13	8	0
Quite normal expression pattern, except for disturbances in the hindbrain	1	0	0
Embryos strongly deformed, no structures can be identified	0	2	0
Apparently normal expression pattern	0	2	66
Total	88	49	66

In one experiment embryos were collected just after the bud stage (beginning of somitogenesis). Embryos from several injection series were fixed between the 10 and 15 somite stages (midsomitogenesis), or on the morning after injection (1-day old). Fixed embryos were analyzed by *zp-50* in situ hybridization. Embryos were then classified into different categories according to the alterations in *zp-50* expression.

*In embryos at these early stages it is difficult to unequivocally decide whether the staining is really located in the presumptive posterior telencephalon or whether the staining represents ectopic expression in dorsal regions of the forming posterior diencephalon.

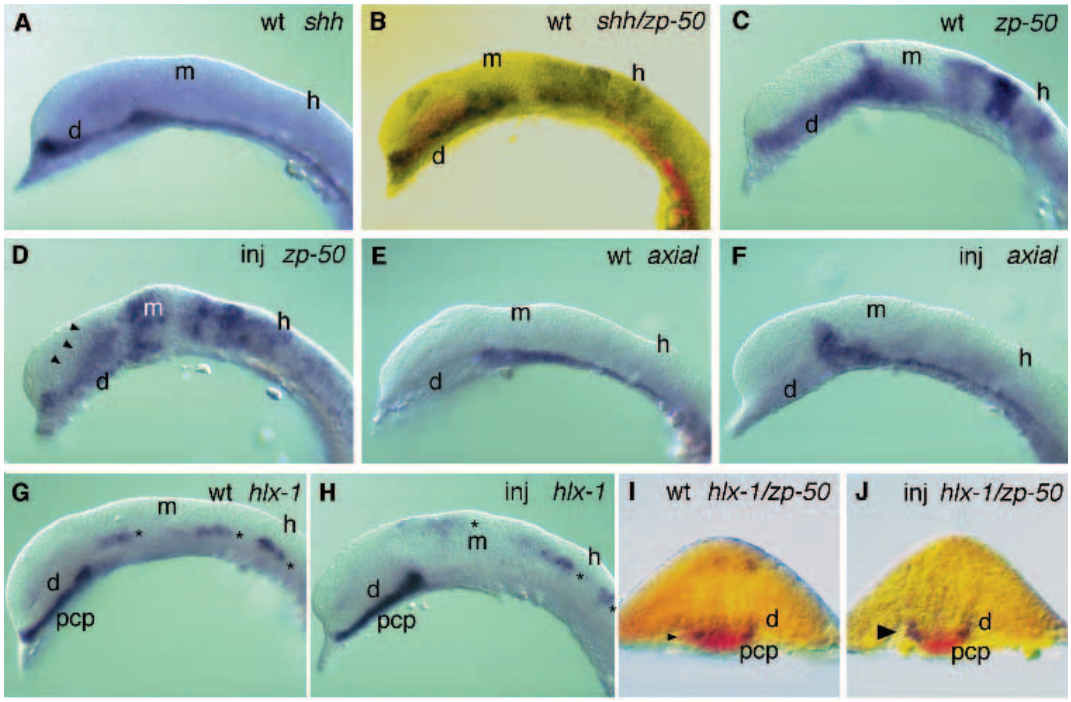


Fig. 4. Alterations of gene expression in 10-12 somite stage *shh*-injected embryos. Embryos injected (inj) with 25 ng/µl *shh* RNA (D,F,H,J) or control embryos (A,B,C,E,G,I) fixed at the 10-12 somite stage were analyzed by in situ hybridization. (A) *shh* staining in the ventral CNS. (B) The longitudinal expression domains of *shh* (red) and *zp-50* (purple) occupy very similar territories in the ventral diencephalon. (C,D) *zp-50* expression is dorsally expanded in the ventral diencephalon and the midbrain of *shh*-injected embryos. The beginning expression in the telencephalon (cf. B) is reduced in injected embryos. (E,F) *axial* expression is extended dorsally in the posterior diencephalon. (G,H) *hlx-1* expression in the prechordal plate and the ventralmost midline ectoderm is not significantly altered by *shh* overexpression. However, expression (asterisks) in the mid- and hindbrain is shifted to more dorsal locations. (I,J) Crossections through the middiencephalon of double-labeled embryos show the dorsal expansion (arrowheads) of *zp-50* expression (purple), whereas *hlx-1* expression in the mesoderm and in the ventralmost ectodermal tissue is unaltered (red). d, diencephalon; h, hindbrain; m, mesencephalon; pcp, prechordal plate.

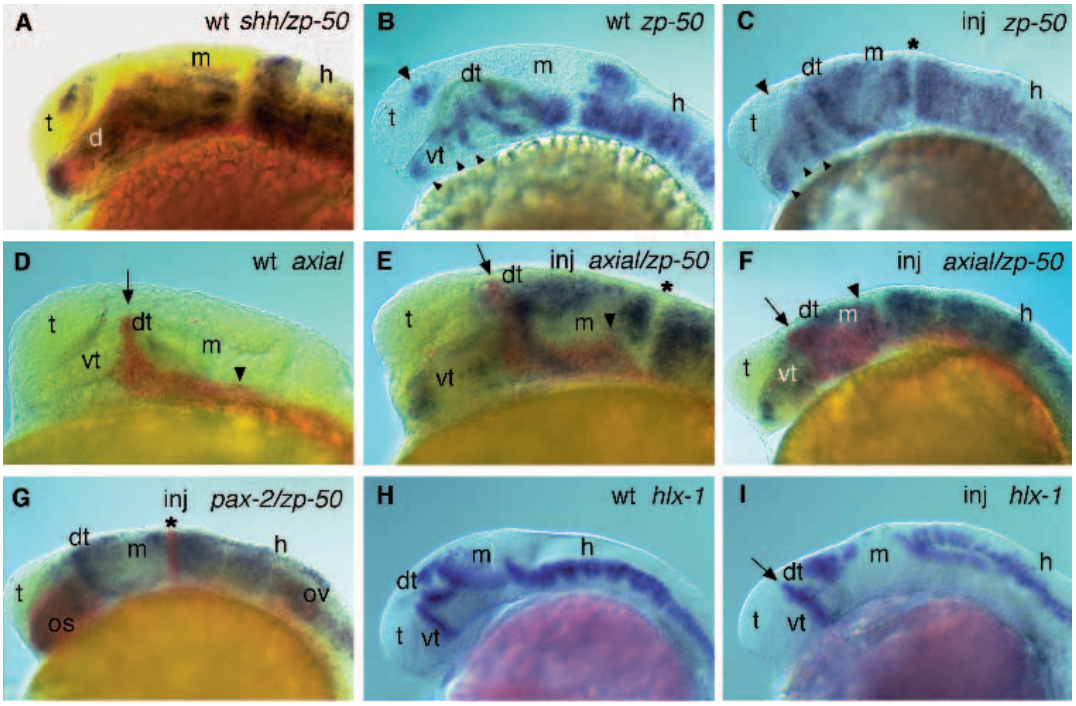


Fig. 5. Ectopic gene expression in 1-day old *shh*-injected embryos. Embryos injected with capped *shh* RNA at 25 or 50 ng/µl (inj; C,E,F,G,I) or controls (wt; A,B,D,H) were analyzed by in situ hybridization. Embryos were fixed at 24-26 hpf (A-G) or at 30 hpf (H,I). (A) Double hybridization with *zp-50* (purple) and *shh* (red) shows close proximity of expression in the ventroanterior brain. (B,C) *shh* injection causes disorganized and elongated *zp-50* expression domains in the ventroanterior diencephalon (small arrowheads). Prominent ectopic expression is visible in the dorsal thalamus and the midbrain. No aberrant *zp-50* expression is observed along the midbrain/hindbrain boundary of injected embryos (asterisk). *zp-50* expression is reduced in the telencephalon of injected embryos (large arrowheads). (D) Uninjected embryo stained for *axial*. (E,F) Two different examples of *shh*-injected embryos double labeled for *zp-50* (purple) and *axial* (red). Besides ectopic *zp-50* expression, *axial* expression shows in these embryos a dorsal expansion of the finger-like domain in the dorsal thalamus (arrow). The relatively modest *axial* overexpression in the midbrain seen in E is more frequently seen than the more widespread expression in the midbrain tegmentum (arrowhead) visible in F. (G) *shh*-injected embryo double labeled for *zp-50* (purple) and *pax-2* (red). *pax-2* expression in the otic vesicle and in the *zp-50*-free gap at the midbrain/hindbrain boundary (asterisk) is not obviously affected by *shh* misexpression. However, *pax-2* expression is dramatically widened in the optic stalk (cf. Fig. 3D). (H,I) Injection of *shh* RNA eliminates several *hlx-1* expression domains in the midbrain. Residual *hlx-1* midbrain expression is shifted dorsally like the expression domains in the hindbrain. d, diencephalon; dt, dorsal thalamus; h, hindbrain; m, mesencephalon; os, optic stalk; ov, otic vesicle; t, telencephalon; vt, ventral thalamus.

Despite the perturbations of gene activity in most brain regions, *zp-50* expression does not appear to be dramatically altered in other parts of the brain. Overexpressed Shh has apparently no effect on the gap of *zp-50* expression found in wild-type embryos at the border between the mid- and the hindbrain. Double in situ hybridization demonstrates that the position of this gap roughly corresponds to the position of *pax-2* expression at the midbrain/hindbrain boundary of wild-type embryos (Figs 5G, 3C). *pax-2* expression is not obviously affected by ectopic Shh either at this position or in the otic vesicle. In contrast, the distribution of *pax-2* transcripts is widened in the area of the optic stalk (Fig. 5G) as recently reported (Macdonald et al., 1995).

In 30 hpf injected embryos, *hlx-1* is now primarily expressed in the CNS (Fjose et al., 1994). Analysis of *hlx-1* expression in *shh*-injected embryos shows a dorsal elongation of the anteriormost expression domain in the posterior ventral thalamus (Fig. 5I). The different expression domains in the dorsal thalamus and midbrain are fused and located at very dorsal positions. The expression stripes in the hindbrain are not clearly distinguishable anymore and they are shifted dorsally. Therefore, our combined analysis of the *zp-50*, *axial* and *hlx-1* expression during early somitogenesis and after the first day of embryogenesis suggests that overexpression of *shh* leads to widespread ventralization of the entire brain.

DISCUSSION

zp-50 contains a class III POU domain

We have isolated a cDNA of the zebrafish *zp-50* gene which encodes a POU domain of the class III type. Genes containing this particular type of POU domain have been found in insects and vertebrates (reviewed by Verrijzer and van der Vliet, 1993; Wegner et al., 1993). These genes share at least 89% protein sequence identity within their POU domains. *zp-50* is predominantly expressed in the central nervous system, similar to many other POU genes, in particular those containing a class III POU domain, which have been found to be expressed in various cell types of the CNS (reviewed by Wegner et al., 1993). The DNA-binding potential of the POU motif has been experimentally verified for class III POU domain genes in the cases of *oct-6* and *brn-4*. The encoded proteins bind specific DNA sequences and act as transcriptional regulators by either stimulating or repressing gene expression (Monuki et al., 1990; Suzuki et al., 1990; He et al., 1991; Mathis et al., 1992; Meijer et al., 1992). Given the strong conservation among their POU domains (Fig. 1B), it is reasonable to assume that all other class III POU proteins, including *zp-50*, have the ability to bind specifically to DNA and that they function by regulating tissue specific gene expression in the brain and spinal cord.

The predicted protein sequences of the *zp-50* and the *Xenopus xlpou1* (Agarwal and Sato, 1991) genes are 82.9% identical over their entire length. Both *zp-50* and *xlpou1* are expressed first in the CNS at the onset of neural plate formation in apparently equivalent structures of the embryonic brain. A minor difference, reported by Agarwal and Sato (1991) is that *xlpou1* is expressed in the eyes of tadpoles whereas we have not detected *zp-50* expression in embryos of comparable stages. Furthermore, on northern blots we have observed a single *zp-50* transcript, whereas two transcripts of different sizes were reported for *xlpou1*. Nevertheless, the similarities in sequence and neural expression patterns strongly suggest that *zp-50* and *xlpou1* are functional homologs involved in the regulation of similar developmental pathways in fishes and frogs.

Within the POU domain, *zp-50* displays 96.0% amino acid sequence identity with the mammalian *oct-6* (*SCIP/tst-1*) gene. This degree of homology is slightly higher than that found for other mammalian class III genes. Furthermore, the *zp-50* N-terminal domain, which is particularly conserved between *zp-50* and *xlpou1* (90% sequence identity over 51 amino acids), also exhibits considerable similarity to the corresponding region of the murine *oct-6* gene. Disregarding two short gaps and a short run of alanine residues in *oct-6*, the homology is above 60%. The functional significance of this N-terminal domain remains unclear. In contrast to *zp-50* and *xlpou1*, however, murine *oct-6* is expressed very early during embryogenesis. Furthermore, the *oct-6* expression pattern in the rodent brain (Suzuki et al., 1990; Alvarez-Bolado et al., 1995) shows no obvious similarities to that of *zp-50* and *xlpou1* in fishes and frogs. Although the *zp-50* and *xlpou1* genes may share some relationship with *oct-6*, the sequences and the expression patterns are too diverged to consider them direct homologs of mammalian *oct-6*.

zp-50 is expressed in a very complex and dynamic pattern in the developing brain

Expression of *zp-50* in various regions of the zebrafish CNS starts very soon during the formation of the neural anlagen. Thus it is possible that *zp-50* is an early component of the genetic programs leading to progressive differentiation and regionalization of the brain. *zp-50* expression is observed at many different locations of the forming CNS. The order of appearance of the various domains is controlled in an intricate temporal fashion. We observe that several *zp-50* expression domains are located close to forming axon tracts. As noted by Macdonald et al. (1994) the juxtaposition of gene expression boundaries and sites of axonogenesis may indicate a causative link between axon guidance and the function of such regulatory genes. Besides a possible involvement in the formation of axon tracts, the ZP-50 transcription regulator might be contributing to the regionalization of the CNS in general by establishing the identity of various groups of cells. This could be achieved by combinatorial interactions between the transcription factors encoded by *zp-50* and other differentially expressed regulatory genes.

Hindbrain expression of *zp-50* is first detected in rhombomeres r3 and r5. A short time after the initial expression of *zp-50* in r3 and r5, high level expression in the hindbrain spreads to r1 and to a lesser extent to r7. This predominant expression in all odd-numbered segments mirrors the recently proposed pair-rule-like arrangement of rhombomeres in the vertebrate hindbrain (reviewed by Krumlauf et al., 1993; Wilkinson, 1993). *zp-50* is one of the first molecular markers reflecting, at least in a transient fashion, such an alternating expression pattern over the entire length of the hindbrain. From the 20 somite stage on, *zp-50* is expressed with equal intensity in all rhombomeres in bilateral cell clusters close to the midline. These clusters form, at 34 hpf, a series of paired stripes located on both sides of the interrhombomeric borders similar to those observed with the *hlx-1* gene (Fjose et al.,

1994). *zp-50* may therefore be involved in the generation and/or maintenance of a functional subdivision of rhombomeres into border and center regions. Such subdivisions have been described by Trevarrow et al. (1990) at a cellular level: curtains of glia cells separate the commissural neurons developing along the rhombomere borders from the rhombomere centers which contain reticulospinal neurons.

zp-50 is subject to regulation by *shh*

Induction processes establishing dorsoventral polarity have been studied in considerable detail on a cellular level for posterior regions of the neural tube (reviewed by Yamada et al., 1993; Placzek, 1995). However, much less information has been available about the anteriormost regions of the brain and whether similar mechanisms are involved. In more posterior areas of the embryo, *axial* is expressed initially in the axial mesoderm and subsequently in the floor plate of the ventral neuroectoderm (Strähle et al., 1993). The encoded transcription factor presumably plays a major role in specifying the identity of floor plate cells since targeted misexpression of *HNF3β*, the murine *axial* homolog, causes the ectopic induction of floor plate marker genes in transgenic mice (Sasaki and Hogan, 1994). Ectodermal *axial* expression is induced by the mesodermally derived Shh signal and may in turn activate *shh* expression in the floor plate (Strähle and Blader, 1994).

In the anteriormost areas of the brain the situation must be different because *axial* is not expressed there. Nevertheless, *shh* might play an important role in the patterning of the forebrain since it is expressed also in the anterior neuroectoderm from the 100% epiboly stage on (Krauss et al., 1993). Expressing *shh* can lead to induction of particular Isl-1$^+$ neurons and to alterations of *nk2.2* expression in the forebrains of chicken and zebrafish, respectively (Barth and Wilson, 1995; Ericson et al., 1995). The *cyclops* gene is required for the correct specification of the floor plate and its absence also leads to massive defects in the ventral forebrain (Hatta et al., 1991; Hatta et al., 1994; Patel et al., 1994). In *cyc*$^{-/-}$ mutant embryos, *shh* expression is undetectable both in the anterior and the posterior neuroectoderm (Krauss et al., 1993). These observations all suggest that similar mechanism are involved in dorsoventral patterning over the entire length of the neural keel.

Analysis of *zp-50* expression in *cyc*$^{-/-}$ embryos showed no detectable expression in the ventral domains of the fore- and midbrain as seen in wild-type embryos. Since *shh* is similarly affected by the *cyc* mutation, we have compared the expression of these two genes in wild-type embryos. Whereas *shh* is first expressed in the anterior neuroectoderm around 100% epiboly (Krauss et al., 1993), low levels of *zp-50* appear around the 1-2 somite stages. Comparisons of the location of their sites of expression show extensive overlaps in the area of the presumptive ventral fore- and midbrain. This similarity of expression is detectable already during early somitogenesis and can still be seen 1 day after fertilization. Even by the use of 2 color in situ hybridization it is hard to determine at the single cell level how precisely the *zp-50* and *shh* expression domains overlap. However, it has been shown that Shh itself or an Shh-dependent secondary signalling molecule can act over the distance of several cell diameters (Fan et al., 1995; Hynes et al., 1995; Marti et al., 1995; Roelink et al., 1995). The joint dependence on a functional *cyc* locus, the relatively short time span between the appearance of transcripts of the two genes, and the similar extent of their expression domains suggest that the activation of the *zp-50* gene in the initial longitudinal domain in the ventroanterior brain might be a direct nuclear response to the reception of the signal of the *shh* morphogenic system.

Injection of synthetic *shh* mRNA into zebrafish eggs causes perturbation of *zp-50* expression in several regions of the brain. Widespread ectopic *zp-50* expression is observed in dorsal positions of the posterior diencephalon and in the midbrain. This area seems to be particularly prone to alterations of gene expression patterns by *shh*. Investigations of *shh* effects on other zebrafish genes (*axial* and *nk2.2*) have shown that those genes are also ectopically activated preferentially in the posterior diencephalon and in the midbrain (Krauss et al., 1993; Barth and Wilson, 1995). Furthermore, we do find strong ectopic *zp-50* expression in the rostral diencephalon where the normally visible expression domains are distorted and expanded dorsally. Similarly, the *hlx-1* expression domains in the brain are shifted to more dorsal positions. All these disturbances of normal gene expression correspond to a ventralization of the corresponding brain regions. This interpretation is consistent with the proposed role of *shh* to organize patterning of the ventral CNS. In contrast to the ectopic expression observed elsewhere, the *zp-50* expression domain in the dorsoposterior telencephalon is strongly reduced in *shh*-injected embryos. In line with the interpretation of a general ventralization of the anterior brain by ectopic Shh, this finding can easily be explained by assuming a respecification of the *zp-50*-expressing dorsal telencephalon to cells of a more ventral identity which normally do not transcribe *zp-50*.

The prechordal plate mesoderm underlying the anterior brain does not appear to be altered by overexpressing *shh*. Furthermore, we have not observed any inappropriate *zp-50* transcripts in the border region between the mid- and the hindbrain. The absence of ectopic *zp-50* activation in regions next to domains showing a strong response to Shh could indicate the existence of negatively acting factors inhibiting signal propagation. Similarly, *pax-2* expression is not apparently altered at the midbrain/hindbrain boundary and in the otic vesicle although an expansion of expression can be seen in the optic stalk. Factors limiting the range over which a signal like Shh exerts its effect may thus be an important mechanism in the patterning of different tissues.

Furthermore, positively acting factors probably exist which modulate the transmission and reception of the Shh signal. The target tissue has to reach a state of competence in order to respond to the incoming signal. This is evident from the observation of induction of different types of Isl-1$^+$ neurons in the chick forebrain which depends on the precise location of the responding tissue along the anteroposterior axis (Ericson et al., 1995). The requirement to reach a state of competence is also demonstrated by our observation that *zp-50* is not prematurely activated in *shh*-injected embryos, although it is reasonable to assume that prior to the completion of gastrulation the injected *shh* mRNA is translated and the resulting protein is autoproteolytically cleaved. During patterning of the neuroectoderm, cells differ in their response to Shh. Although both *axial* and *zp-50* are ectopically activated in *shh*-injected embryos, individual cells in the posterior diencephalon and midbrain

generally do not coexpress both genes. It must thus be assumed that cells have to decide which of the two genes they are going to express. The decision between the expression of either *axial* or *zp-50* seems not to depend on the local Shh concentration but might be determined by intrinsic factors. Understanding the functioning of such determinants will expand our insights into the mechanisms of embryonic brain patterning.

We appreciate Caroline Bornmann's excellent technical assistance. We thank Robert Riggleman, Kathryn Helde, and David Grunwald for the cDNA library, Monte Westerfield for antibodies, Trevor Jowett, Denis Duboule, Anders Fjose, Uwe Strähle, Jean Paul Concordet and Philip Ingham for DNA constructs, and Pascal Haffter for providing initial batches of *cyc* embryos. We thank Andreas Vogel and Philipp Spaniol for comments on the manuscript. T. G. is a START fellow of the Swiss National Science Foundation. This work was supported by the Swiss National Science Foundation and the cantons of Basel-Stadt and Basel-Landschaft.

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(Accepted 19 March 1996)