

Establishing a Positional Information Assay For Brain Ventricle Mutants and
Investigating the Choroid Plexuses in Zebrafish

by

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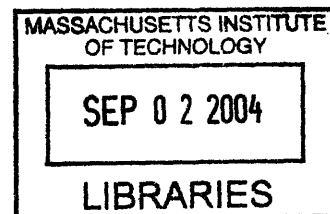


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ABSTRACT

The process by which the neural tube expands into three brain ventricles can be understood through genetic mutant analysis. Within the framework of a characterization of zebrafish mutants with brain ventricle phenotypes, I have developed an assay that looks for evidence of compromised gene expression patterns. I have shown that a cocktail of *krox20*, *pax2a*, *shh*, and *zic1* antisense RNA probes hybridizes to domains in the developing brain that reflect anterior, posterior, dorsal, and ventral axis specification. In addition, I have investigated the choroid plexus (CP) cells lining the brain ventricles in the zebrafish. Though we were unable to clearly identify the CP in the adult brain, we did identify two homologues in zebrafish of a conserved gene expressed in CP of vertebrates. We found that one of these genes, *Drcpl1b*, was expressed from tailbud into early larva stage. Further, *Drcpl1b* is expressed in neurula stage embryos in the anterior neural plate. Through these studies, we established an assay to analyze positional identity of cells in the neural tube and discovered a potential choroid plexus marker, shown its expression time course, and outlined its early expression pattern in the zebrafish.

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CHAPTER I: CHARACTERIZATION OF ZEBRAFISH MUTANTS WITH BRAIN VENTRICLE PHENOTYPES

INTRODUCTION

The morphogenesis of brain ventricles begins within the anterior region of the neural tube, where the epithelia at three distinct locations along the anterior-posterior axis of the future brain expand into vesicles. These develop into the continuous fore-, mid-, and hindbrain ventricles that are filled with cerebrospinal fluid (CSF). Classical literature suggests a role for ventricles in buoying the brain within the skull and serving together as a drainage route for brain metabolites (Davson and Segal, 1996). More recent literature suggests that the ventricles form a circulatory system in the brain that, in addition to removing waste, carries nutrients and chemical signals (Nilsson et al., 1992). It is likely that the ventricles serve other, unknown functions; for example, an extensive system of both dendrites and axons of unknown function project into the ventricular cavities (Vigh and Vigh-Teichmann, 1998). Further, it is known that changes in brain ventricle structure are associated with severe disorders such as schizophrenia, autism, and hydrocephaly (Brambilla et al., 2003; Harrison, 1999; Rolf et al., 2001).

Brain ventricle formation is essential for neuronal differentiation, and either an increase or a decrease in ventricular volume inhibits neurogenesis (Desmond and Jacobson, 1977). The initial ventricles change shape and become, in mammals, the final lateral ventricles lying to the telencephalon, the third ventricle in the diencephalon, the Aqueduct of Sylvius in the midbrain, and the fourth ventricle in the hindbrain. Like mammals, zebrafish have four ventricles in the adult: the telencephalic ventricle, the diencephalic ventricle, two tectal ventricles, and a rhombencephalic ventricle. Though

the appearance of these four ventricles varies greatly across vertebrates, the expansion of the neural tube into fore-, mid-, and hindbrain ventricles in early development is conserved among the vertebrates (Desmond and Jacobson, 1977; Schier et al., 1996).

It seems likely that the expansion of the tube to make brain ventricles is an active process, requiring information within cells of the neural tube and in their surrounding environment to drive morphological changes. Several cell biological processes are likely to contribute to ventricle formation. These include cell death, proliferation, and shape changes. Brain ventricle morphogenesis also requires that cells lie in specific domains along the anterior-posterior (A-P) and dorsal ventral (D-V) axes, and that they maintain epithelial polarity within a sheet of cells to facilitate proper cell-cell contact and communication.

We can uncover the signals required to orchestrate this initial expansion by characterizing genetic mutants with aberrant brain ventricles. Another graduate student, Laura Anne Lowery, and I have collaborated on this project. She began collecting from previous zebrafish mutant screens multiple mutant fish with abnormal brain ventricle phenotypes (Jiang et al., 1996; Schier et al., 1996) and together we have screened through heart ventricle mutants (Warren et al., 2000), initially imaging their brain ventricle abnormalities. Many heart mutants fail to properly make a tube structure, and we have found several also have abnormal brain ventricles. We viewed the ventricle phenotypes using a technique developed by Laura Anne Lowery; we injected a Texas-Red conjugated dextran into the hindbrain ventricles of zebrafish embryos at stage 20-22 hours post fertilization (20-22 hpf), just as the ventricles have opened (Fig. 1). The dye diffused through the ventricular cavities of the expanded neural tube, and fluorescence and light

microscopy were used to image brain ventricle shape. This assay has permitted us to verify previously identified brain ventricle mutants, as well as identify new brain ventricle mutants during shelf screening.

Laura Anne Lowery began the mutant characterization, analyzing cell death and cell proliferation patterns during vesicle expansion, later appearance of known axon tracts, and cellular movements and shape changes. My role in particular in this characterization was to design a cocktail of *in situ* probes against patterning genes. This cocktail would then be used to assay the degree of patterning along the anterior-posterior (A-P) and dorsal-ventral (D-V) axes in mutants with brain ventricle phenotypes. In this chapter, I ask the following:

1. What gene expression patterns convey information about proper A-P and D-V axes in early zebrafish development?
2. In what proportions should the different probes be combined to give a clear signal of all gene expression patterns?

RESULTS

Identifying patterning genes for characterization

Cells within the developing embryo establish regional identity as they acquire their cellular fate. The regions that contribute to cell identity are characterized by gene expression patterns; some of the best defined patterning genes emerged in early embryogenesis of *D. melanogaster* (Nusslein-Volhard and Wieschaus, 1980). In vertebrates as well, expression of many genes establishes the anterior and posterior, dorsal and ventral, and midline and lateral axes, setting up a sort of Cartesian coordinate system in the body plan. Gene expression patterns that identify regions can be seen at early stages of development; in *X. laevis*, the restricted expression of *fhx5* and *opl* (*zic1*) during early gastrulation in presumptive neuroectoderm defines the A/P axis (Gamse and Sive, 2001). The early set-up of the axes is later refined, and this is reflected in the subdivisions of the developing CNS, in particular the neural tube.

We would like to know if, in the brain ventricle mutants, cells of the neural tube lack complete axis specification as reflected in patterning gene expression. Our assay aims to identify aberrant positional information within cells involved in the ventricle expansion process. To accomplish this, we have chosen to use *in situ* hybridization, a process by which a gene's mRNA transcripts can be identified and labeled, to analyze patterning gene expression in the developing nervous system. The identification of transcripts is accomplished by hybridization of a labeled antisense probe.

The expansion of the neural tube in three distinct locations to form vesicles begins around at the 22 somite stage (Fig. 1C). We have chosen to assay gene expression patterns at an earlier stage, at 15-16 somites, when the neural rod has formed but not yet

cavitated. By this point in development, any obvious abnormality in axis specification will be apparent. As well, at this stage, many positional gene markers have clear boundaries of expression. Thus, visualization of pattern abnormalities will be easier to see in the mutant characterization.

The genes we want to look at are those that establish A-P and D-V polarity in the central nervous system (CNS) of the zebrafish. We wanted to select a small number of genes to reduce the complexity of cocktail production, yet we wanted genes that would relay anterior, posterior, dorsal, and ventral positional information. Among several potential genes, we have chosen four: *krox20*, *zic1*, *pax2a*, and *sonic hedgehog (shh)*. *krox20* encodes a zinc-finger transcription factor, and is expressed in rhombomeres 3 and 5 (r3 and r5) of the hindbrain (Oxtoby and Jowett, 1993), giving information about posterior brain specification. *pax2a*, a member of the highly conserved *pax* family of transcription factors, is expressed at the midbrain hindbrain boundary (MHB), as well as the otic vesicle and optic stalk (Lun and Brand, 1998). Its expression in the MHB outlines the division between the more anterior midbrain and the hindbrain that lies caudal to it. *shh*, a signaling molecule with homology to *D. melanogaster* segment polarity gene *hedgehog (hh)*, is expressed in the ventral cells of the neural tube, called the floor plate, as well as in the notochord that lies ventral to the tube (Krauss et al., 1993), serving as a label for ventral CNS cells. *zic1* is an *odd-paired like* protein containing five zinc fingers and is expressed dorsally from the telencephalon back into the rhombencephalon (Rohr et al., 1999).

Quantification and testing of *krox20*, *pax2a*, *shh*, and *zic1* probes

After identifying the genes ideal for assaying patterning in our mutants, we synthesized large quantities (between 0.2 to 1.0 mg) of RNA probe for each individual gene. To get such large quantities, we amplified the amount of starting DNA and adjusted the protocols accordingly (see Materials and Methods), as well as pooled together separate reactions containing the same gene probe. These pooled RNA probes were then run on agarose gels after denaturation (Fig. 2) and compared to known quantities of RNA in the simultaneously run RNA ladder (see Materials and Methods).

To make the probes work together as a cocktail, we first tested them out individually in appropriately staged embryos to discover their properties: the strength of the signals, the amount of background, and the approximate concentration of probe needed to get the best quality expression pattern.

Testing the *krox20*, *pax2a*, *shh*, and *zic1* probes was all done in 15-16ss embryos, the chosen stage of development for the patterning assay in the brain ventricle mutant characterization. We found that relatively small concentrations of *krox20* probe in hybridization buffer (1.3 ng/ μ l) gave a strong signal with little background (Fig. A). An equally small concentration of *zic1* probe in hybridization buffer (1.3 ng/ μ l) gave a strong signal (Fig. 3B), but extended incubation time in the color reaction step gave more background (data not shown). The *pax2a* probe required a higher concentration (12.0 ng/ μ l, Fig. 3C.), and too showed more background with extended color reaction time (not shown). The *shh* probe worked best with a concentration of 8.0 ng/ μ l (Fig. 3D.), and maintained relatively low background in longer reaction times.

***In situ* with four-probe cocktail in wild type**

We tested the combination of all four probes in a monochromatic *in situ* reaction in 15-16ss wild type embryos. The final mass ratio of *krox20* : *zic1* : *pax2a* : *shh* probes in the cocktail was 1.0 : 1.0 : 9.0 : 6.0. The embryo showed an expression pattern that summed all four individual expression patterns (Fig. 4). *krox20* labeled r3 and r5, *pax2a* labeled the MHB, as well as the otic placode and optic stalk faintly, *zic1* labeled throughout the dorsal brain, and *shh* labeled the ventral neural tube, its anterior most expression reaching into the forebrain.

DISCUSSION

We have shown here that a cocktail of four gene probes can be used to identify axis specification in zebrafish embryos during late somitogenesis. The readout in the embryo of the *krox20*, *zic1*, *pax2a*, and *shh* probe combination is a sum of their individual expression patterns. Prior characterization of genetic mutants with patterning defects has been done using RNA probes singly (Hoyle et al., 2004; Kikuta et al., 2003; Rohr et al., 1999; Wiellette and Sive, 2003), and a mutant screen in this lab (Wiellette et al., submitted) used a cocktail with *shh*, *tbx6*, *krox20*, *dlx3*, *pax2a*, and *rx3*. Confirming what we have found, this cocktail required relatively more *pax2a* and *shh* RNA probe in the overall mix.

This creation of an in situ cocktail probe will allow rapid characterization of patterning gene expression in the mutants. Since the large pools of individual gene probes have all been tested for complete hybridization, the same concentrations will be used in all mutant analysis, eliminating probe differences from experiment to experiment. The next step is to utilize the probe and identify brain ventricle mutants with patterning gene misexpression. This information will shed light on what mutants fail to establish the proper axis specification needed for normal morphogenesis of the three initial brain ventricles.

METHODS

Making large quantities of labeled RNA probe:

Standard transcription reactions for 1 µg linear DNA were amplified for 5-15 µg linear DNA. For each 1 µg of DNA, the following were used: 5x transcription buffer (Stratagene), 10x DIG RNA labeling mix (Roche), 40 U RNasin® (Promega) and 100 U T7 RNA polymerase (Stratagene) for *pax2a*, *shh*, and *zic1* or 100 U T3 RNA polymerase (Stratagene) for *krox20*. Reaction time at 37°C was extended to 4-5 h, followed by another 30 min with 1 U DNase/ µg DNA added to each reaction, and then heat inactivated at 65°C for 10 min. The reaction products were then run through a Mini Quickspin® column (Roche) according to manufacturer's instructions.

Quantification of probe

Samples of probes (3µl) in 1 µg /µl Ethidium Bromide and an RNA Buffer of 10x MOPS buffer, formamide, formaldehyde, 50% glycerol, 10% bromo-phenol blue were run on a 1% agarose gel in TAE. 3µl of 1 µg /µl 0.24-9.5 kb RNA ladder (Invitrogen) were run concomitantly to help gauge probe quantities. Each sample was heated to 65°C for 10 min, chilled on ice for 5 min before electrophoresis.

The following was used to determine approximate concentrations of each probe: each of the three most distinct bands in the 0.24-9.5 kb RNA ladder lane contains 0.5 µg RNA. We compared the intensity of individual bands in the RNA probe lanes to the intensity of the RNA ladder bands to get a quantity of RNA, and then divided by 3 µl, the amount of sample run. For instance, we approximated that the *krox20* band contained 0.5 µg RNA/ 3 µl, and thus the concentration of *krox20* RNA probe is 0.17 µg /µl. The following concentrations were calculated for the remaining probes: *zic1* 0.17 µg /µl,

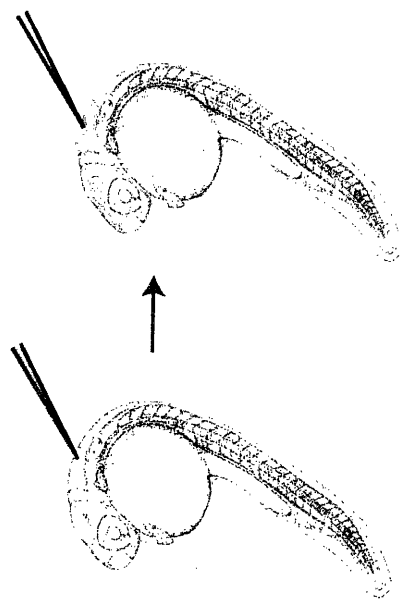
pax2a 1.5 µg /µl, *shh* (newly made probe) 1.0 µg /µl, and *shh* (old probe) 0.33 µg /µl.

The old *shh* probe was discarded after testing it in an *in situ* experiment in wild type, 15-16ss embryos and finding it nonfunctional. The new *shh* probe hybridized successfully in a parallel experiment and was used in the cocktail.

Whole mount *in situ* hybridization with cocktail

In situ hybridization was performed as previously described by Sagerstrom et al., 1996. Specifically, the following conditions were used: fixed 15-16ss embryos were incubated at 22°C in 5 µg/ml Proteinase K (Roche) for 2-3 min. Based on RNA electrophoresis quantification, the following approximate amounts of RNA probe were used in the 250 µl hybridization cocktail: 0.33 µg *krox20* probe, 3.0 µg *pax2a* probe, 2.0 µg *shh* probe, 0.33 µg *zic1* probe. The purple precipitate resulted from a combination of NBT (Roche) and BCIP (Roche).

A Inject Texas-Red conjugated dextran into hindbrain ventricle



Laura Anne Lowery

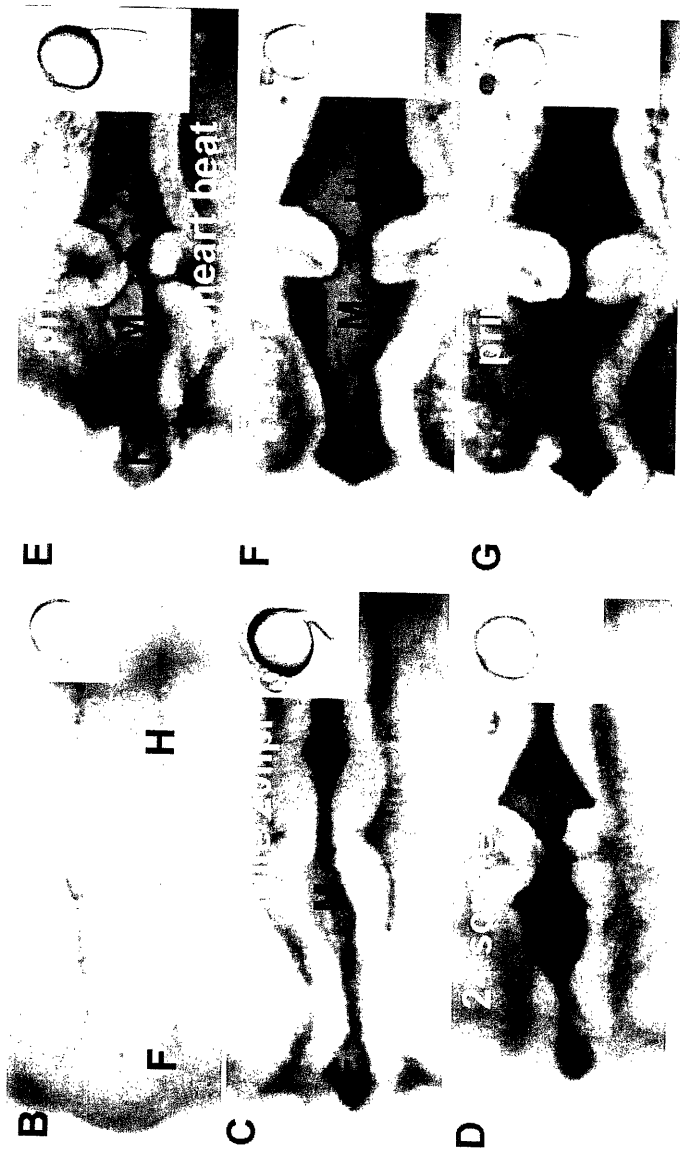


Fig. 1. Brain ventricle morphogenesis in zebrafish embryos. A. Schematic of brain ventricle injection assay. B-G. Dorsal views of embryos at different developmental time points with Texas-Red conjugated dextran dye filling the expanding neural tube. Forebrain ventricle (F), midbrain ventricle (M), and hindbrain ventricle (H).

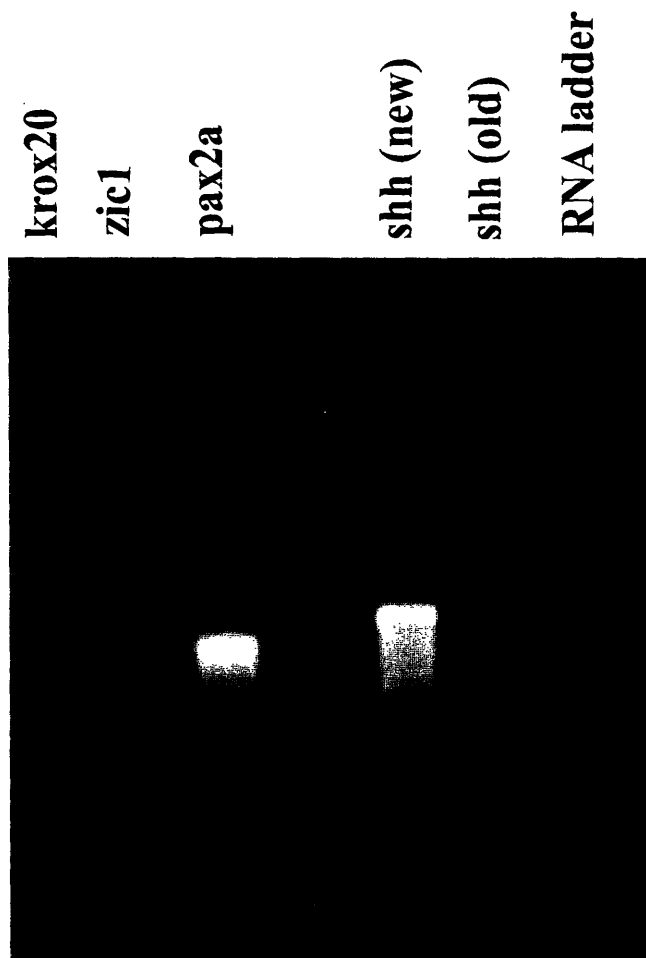


Fig. 2. RNA probe quantification. Each band in RNA ladder contains approximately 0.5 ug/ul RNA. The following concentrations were calculated (see Materials and Methods) for the individual probes: *krox20* 0.17 $\mu\text{g}/\mu\text{l}$, *zic1* 0.17 $\mu\text{g}/\mu\text{l}$, *pax2a* 0.5 $\mu\text{g}/\mu\text{l}$, *shh* (new) 0.5 $\mu\text{g}/\mu\text{l}$, and *shh* (old) 0.33 $\mu\text{g}/\mu\text{l}$.



Figure 3. Expression of individual patterning genes. Whole mount *in situ* hybridization (ISH). 15-16ss embryos, lateral view (A-C), dorso-lateral view (D). (A-D) hybridized with antisense *krox20* (A), *zic1* (B), *pax2a* (C), and *shh* (D) probes.

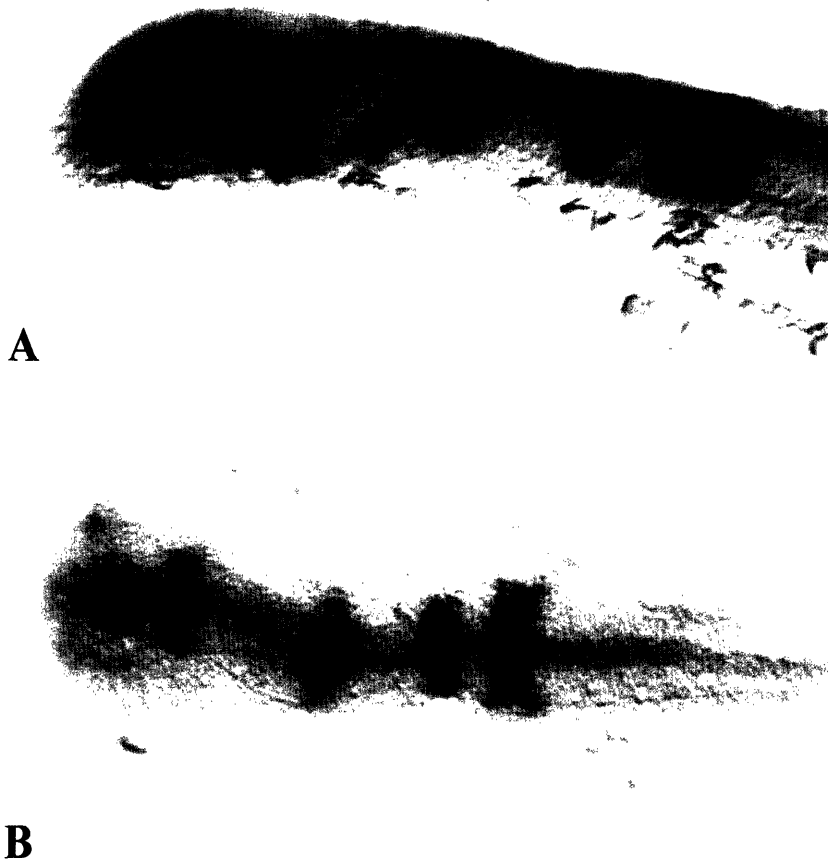


Fig. 4. Expression of *krox20*, *pax2a*, *shh*, and *zic1* in 15-16 somite-stage wild type embryos. Whole mount *in situ* hybridization (ISH) A. Dorsal view of flat-mounted embryo. B. Lateral view of same embryo.

CHAPTER II: IDENTIFICATION OF THE ZEBRAFISH CHOROID PLEXUSES

INTRODUCTION

The brain ventricles make up a system of cavities within the vertebrate brain, and are filled with cerebrospinal fluid. The choroid plexuses secrete this fluid, and comprise cells of the ependymal lining of the brain and closely interdigitate with blood vessels. A choroid plexus is present in all four ventricles of the mammalian brain. In mice, the CP arises within the developing brain shortly following neural tube closure, though the timing of their appearance varies across species (Desmond and Jacobson, 1977; Dziegielewska et al., 2001). CP cells are thought to secrete CSF by week 9 of human gestation (Catala, 1997), as suggested by enzyme activity analysis.

The architecture of the CP is stereotyped throughout vertebrate species (Segal, 2000). The CP establishes the blood-CSF barrier, allowing selective passage of organic substrates from plasma and extracellular fluid and the CSF. The cells of a choroid plexus compose an epithelial sheet, with the apical side facing the ventricle and the basal facing the blood compartment. The CP ependyma differs from non-CP ependyma lining the ventricles in its resemblance to a transporting epithelium and in its proximity to fenestrated capillaries (Speake et al., 2001). The capillaries of the stroma below the CP epithelial cells are contiguous with the fluid bathing the basolateral side of the epithelium. Thus, the tight junctions of the CP epithelium stand as the barrier between the CSF of the ventricles and the blood. Electron microscopy shows further the defining features of the apical side of CP epithelia, the dense, clavate-shaped microvilli and the beating cilia (Nilsson et al., 1992).

An investigation of the development of brain ventricles would not be complete without considering the choroid plexuses. Little is known about the zebrafish choroid plexuses. Research in fish species has been limited to hagfish, in which CP could not be identified (Murray et al., 1975), and in the adult shark (Villalobos et al., 2002). The latter study characterized the structure of shark CP cells, and demonstrated their similarity to mammalian CP cells, as well investigated transepithelial transport across CP cells in the adult. Though there is a void in the literature on CSF secretion and choroid plexus in the teleost, zebrafish blood vessel studies do provide a time point at which CP vascularization begins (Isogai et al., 2001). Fluorescent microspheres were injected into the sinus venosus at day 1, and the zebrafish vascular wiring was observed through confocal microscopy in the living animal for 7 days. The resulting atlas of vasculature revealed the presence of a knot of fluorescing cells in the brain that the authors identify as the choroidal vascular plexus. This knot is present in fish at 60 hours post fertilization (hpf) through 7 days, and not present in fish at 26-28hpf.

Two approaches were employed to identify the CP in the zebrafish. First, we used histology to elucidate the ontogeny of CP cells in zebrafish, as histology in other vertebrates provides us with the stereotyped architecture of CP. Second, we searched for a potential marker of the choroid plexuses in the zebrafish so that we could label CP cells and follow their development during embryogenesis. Several gene candidates were considered, including transthyretin (*ttr*) and a brain lipocalin identified in both *L. dumerili* (Achen et al., 1992) and *X. laevis* (Lepperdinger et al., 1997), in which it is called *Xlcp11*. Further investigation of the literature on *ttr* ruled it out this candidate; transthyretin is expressed in the choroid plexuses of humans (Dickson and Schreiber,

1986), rat (Dickson et al., 1985), pig (Duan et al., 1995), chick (Southwell et al., 1991), and in the brain of turtles (Richardson et al., 1997), but is expressed only in the liver of amphibians and fish (Power et al., 2000; Santos and Power, 1999). The brain lipocalin candidate emerged as a potentially useful marker for choroid plexuses in the zebrafish, as its expression in the choroid plexuses is apparent in *X. laevis*. Further, *Xlcp11* has been suggested to be the amphibian counterpart of transthyretin (Lepperdinger, 2000), as it too binds thyroxine and retinol binding protein (RBP). Thus, based on the highly conserved expression of transthyretin-like proteins in vertebrate choroid plexuses, we consider a zebrafish form of *cp11* an attractive candidate marker for zebrafish CP.

In this chapter, I pose the following questions:

1. What does the choroid plexus look like in the adult zebrafish?
2. Is there a zebrafish homologue of *Xlcp11*? Can this gene serve as a marker for choroid plexus in the developing embryo?

RESULTS

Histological sections of zebrafish brain show possible CP structures.

To date, there is no information on the appearance of the choroid plexuses in the zebrafish. We have sectioned through adult zebrafish heads, making both sagittal (data not shown) and cross-section cuts (Figure 5). We chose the Milligan Trichrome stain to outline different cell types. This particular series of stains labels nuclei red, neurons purple, collagen blue, and erythrocytes orange.

The resulting sections were compared with an atlas of the zebrafish brain (Wullmann et al., 1996) to help identify gross morphological structures. Sections from the anterior-most end of the brain to the posterior end showed all four ventricles; the telencephalic, diencephalic, tegmental, and rhombencephalic ventricles were all present. In the tegmental ventricle we saw clusters of cells with what appeared to be villi facing the CSF and extending into the ventricular space (Fig. 5B, arrow). We suggest that these cells may be CP cells, though our methods of identifying these cells are not complete enough to conclude that they are indeed epithelial and innervated with blood vessels.

***Xlcp11* has two homologues in the zebrafish**

Previous studies in *X. laevis* (Lepperdinger et al., 1997) have identified a gene expressed exclusively in the choroid plexuses of the adult animal, *Xlcp11*. The gene encodes a retinoid binding protein which shares sequence homology with the lipocalin protein family. This family of small sized proteins is characterized by having hydrophobic cavities shaped like calyces; these cavities are binding pockets for small hydrophobic molecules, such as retinoic acid.

Using the *Xlcp11* amino acid sequence (Genbank accession No. S52354) as our query, we performed a BLAST search against the Ensembl zebrafish cDNA database. The results gave one clearly high scoring cDNA candidate, ENSDART00000006682, which we named *Drcpl1a*. Further searches in other databases (see Materials and Methods) revealed an additional EST with 82% identity and 89% similarity in nucleotide sequence with *Drcpl1a*. We named this EST (TC15923) *Drcpl1b*. An alignment of the translation products of these two sequences with the most similar lipocalins from other vertebrates, including frog, cane toad, mouse, and human is shown in Figure 5. We calculated the percentage of similar amino acids between *Drcpl1b* and the other lipocalin proteins and found the following similarity hierarchy: *Drcpl1a* (90.7%), *Xlcp11* (45.6%), *Bmlip* (43.5%), *Mmmup5* (31.5%), and *Hspgds* (24.5%).

***Drcpl1b* is expressed in embryos from 10h to 72h of development.**

The expression pattern of *Xlcp11* in *X. laevis* has been studied from late gastrulation to adulthood. Expression has been shown in *X. laevis* from stage 12 to 25, when expression decreases, and then later in stage 35 and into adulthood. We sought to determine the time course of our *Drcpl1a* and *Drcpl1b* expression in the developing zebrafish. Zebrafish embryos from a range of developmental stages (6h to 72h) were analyzed by a reverse transcriptase-PCR (RT-PCR) assay (see Materials and Methods).

The *Drcpl1a* assay revealed no bands of expected size (600 bp). The only visible PCR product was 200 bp in size, and appeared only at the 55h stage of development. There was clearly cDNA to be amplified, as the same source of cDNA showed amplification with the *β -actin* primers. It is possible that *Drcpl1a* is not expressed in the assayed stages of development, though it is also likely that the primers failed to anneal

specifically to the *Drcpl1a* species if it was present. No conclusions can be drawn about the expression time course of *Drcpl1a* in zebrafish. In the *Drcpl1b* RT-PCR assay, we saw bands of expected size (810 bp) at stages 10h, 20h, 48h, 55h, 60h, and 72h. This data suggests that *Drcpl1b* is expressed from the end of gastrulation into early larval stages in the zebrafish.

***Drcpl1b* is expressed in the anterior neural plate**

X. laevis express *Xlcp11* in the anterior end of the animal pole in late gastrulation embryos. At the early neurula stage, regions adjacent to the cement gland and posterior of the folding plate express *Xlcp11*. At the mid-neurula stage, expression remains in the anterior brain, and narrows in a stripe in the dorsal hindbrain. By tailbud, in addition to the anterior brain expression, *Xcp11* is expressed in what the authors identify as the future anlage of the choroid plexus in the dorsal posterior hindbrain. It is not clear if this is indeed the future choroid plexus.

Though our RT-PCR suggested that the developmental expression of *Drcpl1b* followed a similar time course as *Xlcp11* did in *X. laevis*, we lacked information on what types of cells express *Drcpl1b*. We made an antisense RNA probe for whole mount in situ hybridization from a plasmid (Imageclone # CK028918) containing the *Drcpl1b* cDNA insert. After approximating the concentration of RNA in the probe solution, we assayed embryos for *Drcpl1b* expression at tailbud stage (10hpf), 14ss, 19hpf, and 60hpf. No specific labeling was observed, and background staining was robust (data not shown). We repeated the *in situ*, titrating out the amount of probe in tailbud staged embryos. We found similar results as before, with no specific labeling, in the two experiments with the highest concentration of probe used (2 ng/μl and 4 ng/μl; Fig 8C and D, respectively).

However, we did see cells labeled with the lowest concentration of probe (1 ng/ μ l) in the anterior neural plate (Fig. 8A, B). There are labeled cells at the anterior of the animal, and faint staining at the posterior. Whether this latter staining indicates *drxplb* expression in the posterior of the animal is unclear. In the anterior, the labeling appears as two stripes along the edge of the anterior neural plate (Fig. 8B).

DISCUSSION

The histology in adult zebrafish brains gave us limited information. Though it appears that there may be CP cells jutting into the ventricular space in the tegmentum, we do not have any specific markers or stains to verify the identity of the cells that appear to be CP. Future histological analysis would be more conclusive if antibodies specific to CP cells or blood vessels were used. Further, stains that mark basement membrane could confirm or refute the epithelial identity of these potential CP cells.

Our search for a marker of the choroid plexus has led us to discover a homologue of *Xlcp11* in the zebrafish. The BLAST search pointed us to two genes, both showing homology to previously identified vertebrate brain lipocalin genes. The degree of homology is not high, but this is not surprising. The lipocalin proteins have low sequence homology on the primary sequence level, but they do share highly similar structures (Lepperdinger, 2000). Our similarity analysis has shown, as well, that *Drcp11b* protein is most similar to its orthologue in zebrafish, and is least similar to more distant vertebrate species.

Our RT-PCR experiment shows an expression time course of the *Drcp11b* gene. Sometime between mid gastrulation to the completion of gastrulation *Drcp11b* begins to be expressed, as a PCR product band of 810 bp is not present at shield stage (6hpf) but is at tailbud (10hpf). This expression appears to continue through somitogenesis and into the beginning of the larval stage (72 hpf). Expression has been shown in anterior neural cells in *X. laevis* from stage 12 to 25, when expression decreases. Later, around stage 35, additional expression is seen in what the authors identify as the anlage of the future choroid plexus (Lepperdinger et al., 1997). Like the frog, zebrafish may have a dip in

expression between late gastrulation and 20hpf. A more thorough time course with quantitative RT-PCR could resolve the question of whether or not *drcpl1b* has continuous expression from 10hpf to 72hpf. The RT-PCR data is inconclusive for the *Drcpl1a* gene; there are currently no plasmids of this gene on which to test our primers.

We have also shown that the expression pattern of *Drcpl1b* is similar to that of *Xlcp11* (Lepperdinger et al., 1997) at the neurula stage. Both show expression at the anterior edges of the neural plate. Our titration experiment shows that *in situ* hybridization with the *Xlcp11* probe works best at a low concentration. This information will permit further use of the probe in later stages of zebrafish development. In particular, we would like to look for expression of *Drcpl1b* during the stages of ventricle expansion, from 18hpf to 22hpf. We do not know if clusters of cells in the neural tube facing the expanded vesicles have differentiated into CP cells. The presence or absence of *Drcpl1b* expression along the tube could give us clues as to whether or not a defined CP has emerged in the developing brain yet. Further, *Drcpl1b* *in situ* experiments in later stages of development could help us identify when and where the choroid plexuses appear in the zebrafish.

METHODS

Histology

Adult zebrafish heads were fixed for at least 24 h in Bouin's Solution. Fixed tissue was washed over the course of a day in 50% Ethanol (EtOH), and stored overnight in 70% EtOH. Dehydration washes included 2, 1h washes in 95% EtOH, followed by 2x 1h washes in 100% EtOH. Fixed heads were then cleared in 3, 45 min. changes in xylene (1 at 22°C and two at 60°). To the last xylene soak, an equal amount of melted paraffin was added and let soak for 30 min in 60°. This solution was removed and replaced with fresh paraffin for 1 h at 60°. The animal was then removed from the final soak and embedded in a dish with fresh paraffin, oriented, and cooled at 22°C for several hours. Sections were cut in 0.9 µm sections and mounted onto charged glass slides. Paraffin was removed in a dehydration series beginning with xylene to EtOH to 1X PBS. Slides were stained with Milligan's TriChrome, and then immediately dehydrated back into xylene. Slides were then coated in a thin layer of Permount, and a slide cover added atop. Sections were visualized on a Nikon microscope.

BLAST search

To find the zebrafish homologue of *Xlcp11*, we performed a BLAST (Washington University) search using the Ensembl Zebrafish Database. The query sequence was the protein sequence of *Xlcp11* (GenBank Accession No. S52354), and we searched the zebrafish EST cDNA database using tblastn to compare our protein query against nucleotide sequence databases dynamically translated in all reading frames. Our threshold for significance was e-value <10, and our scoring matrix set at the default for Ensembl. The highest scoring candidate was a cDNA EST ENSDART00000006682

(gene: ENSDART00000007476, protein: ENSDARP00000023712), a sequence we named *Drcplla*.

Additionally, we performed another BLAST search (Washington University) using The Institute for Genomic Research (TIGR) Zebrafish Gene Index. We used the same query sequence as previously identified, and searched the zebrafish cDNA EST database using tblastn. Our threshold for significance was e-value <10, and our scoring matrix set at the default for TIGR. Two EST sequences emerged with clearly high scores (972 and 807 respectively): TC152924 and TC152923. The second of them, TC152923, shared 100% nucleotide sequence similarity with the previously found *Drcplla*, and thus these sequences are the same. We named TC152923 *Drcpllb*.

RT-PCR

RNA was isolated from embryos at stages 6h, 10h, 20h, 48h, 55h, 60h, and 72h using Trizol (Invitrogen, Carlsbad, CA), following the manufacturer's instructions, precipitated using 4M LiCl, and treated with DNase (Promega, Madison, WI) to remove DNA contamination. First strand cDNA was synthesized with Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Primers used in PCR were: *cplla*: 5'-CAAACGTCTTCACAACGAAGAG-3' and 5'-GGAGTAGTTCGCTTCTGTTT-3'; *cpllb*: 5'-TCTCTCCCCTCTGCTTTTGA-3' and 5'-CACCAGACTCTGCCTTGTGA-3'; β -*actin*: 5'-TATCCACGAGACCACCTTCAACTCC-3' and 5'-CTGCTTGCTGATCCACATCTGCTGG-3'. The PCR reactions contained 10x Hotstar Buffer (Qiagen), 10 mM dNTP mix, 20 mM each primer, and 2.5 U HotStar Taq Polymerase (Qiagen). The PCR cycle program began with 15 min 95°C and cycled through 45 sec 94°C, 45 sec 55°C, and 1 min 72°C 37 times. The PCR

program ended with 10 min 72°C and cooled to 4°C. Resulting DNA was run out on a 1% agarose gel and stained with Ethidium Bromide.

Whole mount *in situ* hybridization with *cpl1b*

Drepl1b probe was made in the following way: a Zebrafish IMAGE cDNA clone (IMAGE ID No. 7055904) containing a TC152923 insert was cut with EcoRI, the DNA purified through phenol: chloroform extraction, and DIG-labeled RNA probed transcribed using T7 RNA polymerase (Stratagene) in the previously described reaction conditions.

Samples of probes were run as described previously in this text and quantified according to the same method described for the patterning gene probes.

In situ hybridization followed the protocol described in Sagerstrom et al, 1996. Specifically, the following conditions were used: fixed embryos at gastrula stage were not permeabilized after methanol incubation, 10-18ss embryos incubated at 22°C in 5 µg/ml Proteinase K (Roche) for 2-3 min, and 24, 48, and 60 h embryos at 22°C in 10 µg/ml Proteinase K for 5, 8, and 15 min respectively.

Based on RNA electrophoreses, the first *in situ* experiments in embryos staged 10hpf-60hpf used probe at a concentration of 5 ng/µl in hybridization buffer. The second experiment, in which probe was titrated out in embryos at 10hpf, used probes at a concentration of 1 ng/µl, 2 ng/µl, and 4 ng/µl in hybridization buffer. The purple precipitate resulted from a combination of NBT (Roche) and BCIP (Roche).

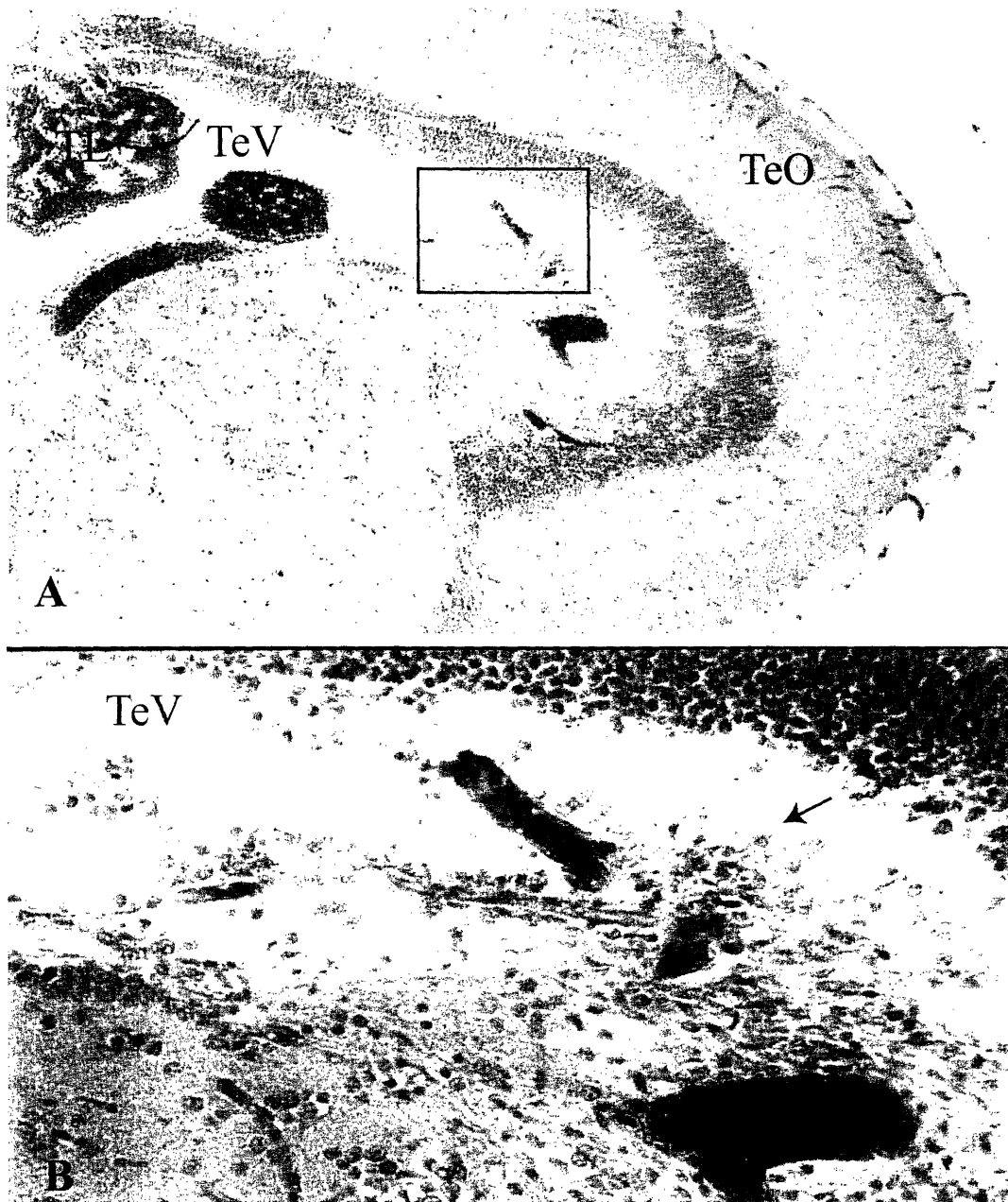


Fig. 5. Sections of adult zebrafish brain. A. Cross section through tectum. B. Magnification of boxed section in A. Arrow points to cells with villi facing the ventricle. Red stain labels nuclei, purple labels neurons. Tectum opticum (TeO), tectal ventricle (TeV), torus longitudinalis (TL).

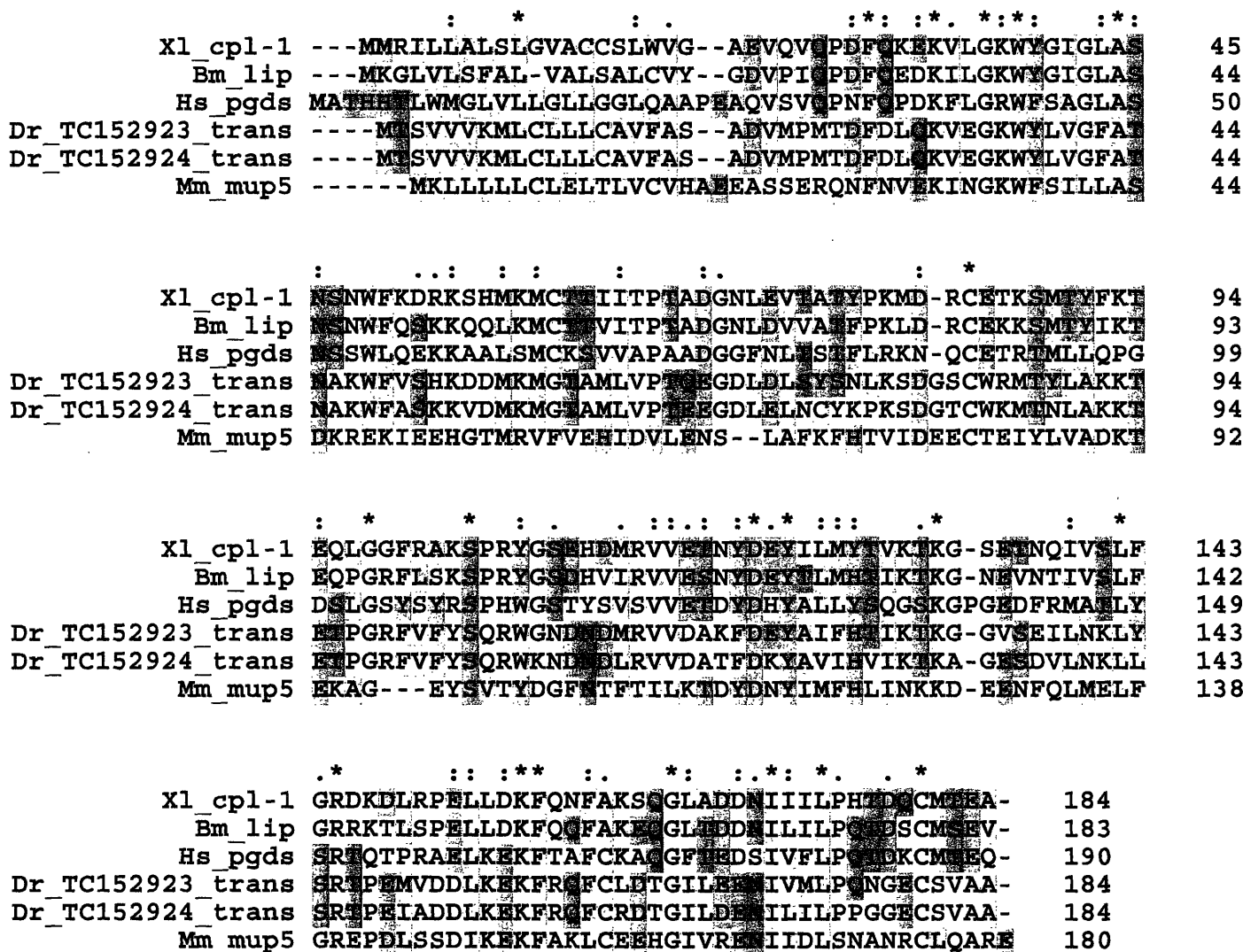


Fig. 6. Multiple sequence alignment. Homologous lipocalin protein sequences have been aligned, along with the translation products of putative zebrafish lipocalin-like prostaglandin D2 synthase homologue cDNAs. Hyphens (-) represent gaps that were introduced to maximize similarities. Identical amino acid matches are denoted with an asterisk (*), highly similar amino acids with a colon (:), and weakly similar amino acids with a period (.). Colors denote type of amino acid as defined by the ClustalX (1.81) program. Xlcp11 (Genbank accession No. S52354), *X. laevis* choroid plexus lipocalin; Bmlip (Q01584), *B. marinus*; Hspgds (AAA36494), *H. sapiens* prostaglandin D2 synthase; DrTC152924 (tentative annotation: similar to GP19911827), *D. rerio* lipocalin-type prostaglandin D synthase like protein (a); DrTC152923 (BAB88223.1), *D. rerio* lipocalin-type prostaglandin D synthase-like protein (b); Mmmup5 (P11591), *M. musculus* major urinary protein 5.

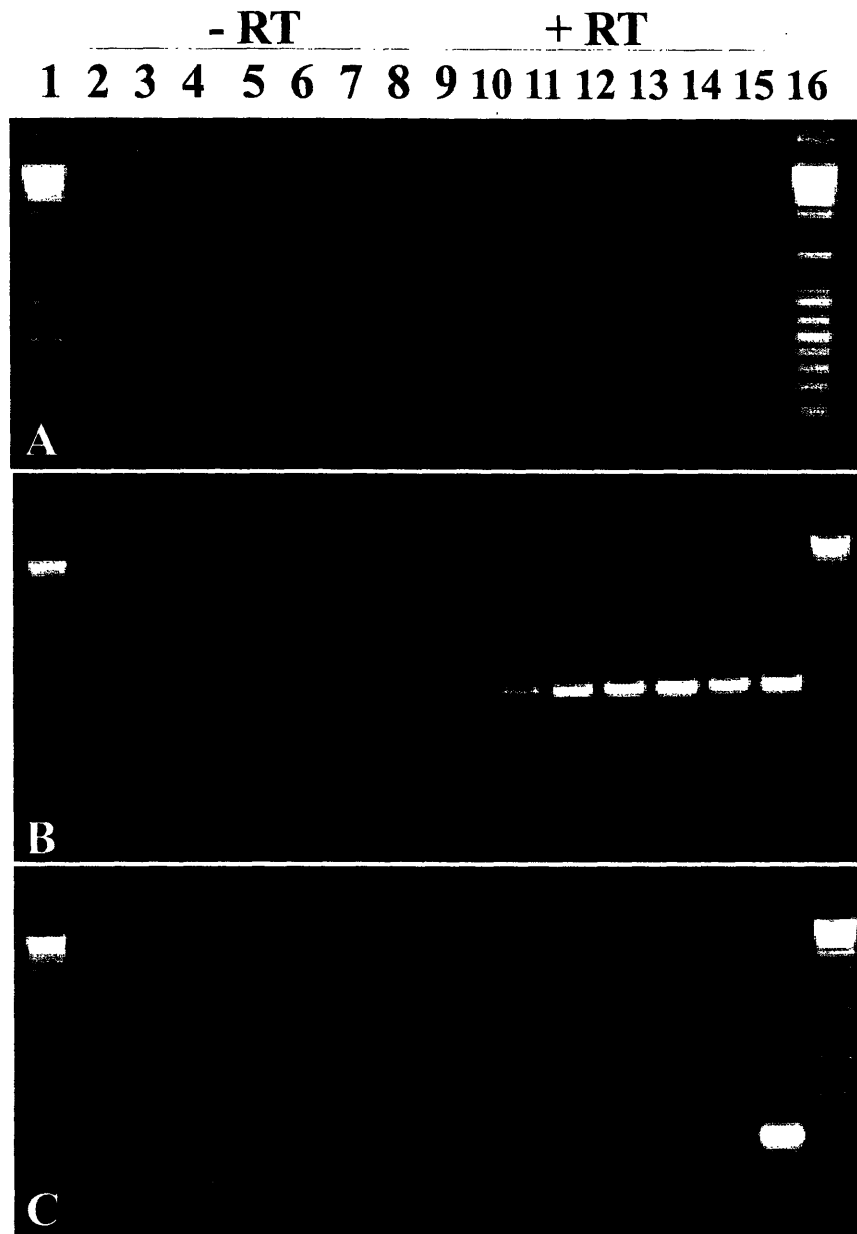


Fig. 7. RT-PCR analysis of *Drcpl1a* and *Drcpl1b* expression throughout a developmental time course. RNA was extracted from staged embryos and were assayed by reverse transcriptase-PCR (RT-PCR) for gene expression at stages 6h (lanes 2,9), 10h (lanes 3,10), 20h (lanes 4,11), 48h (lanes 5,12), 55h (lanes 6,13), 60h (lanes 7,14), and 72h (lanes 8,15). Lanes 1 and 16 contain 1kb Plus DNA Ladder. Lanes 2-8 in A-C contain samples without RT added, lanes 9-15 in A-C contain samples with RT added. A. *Drcpl1a* assay. B. *Drcpl1b* assay. C. β -actin assay.

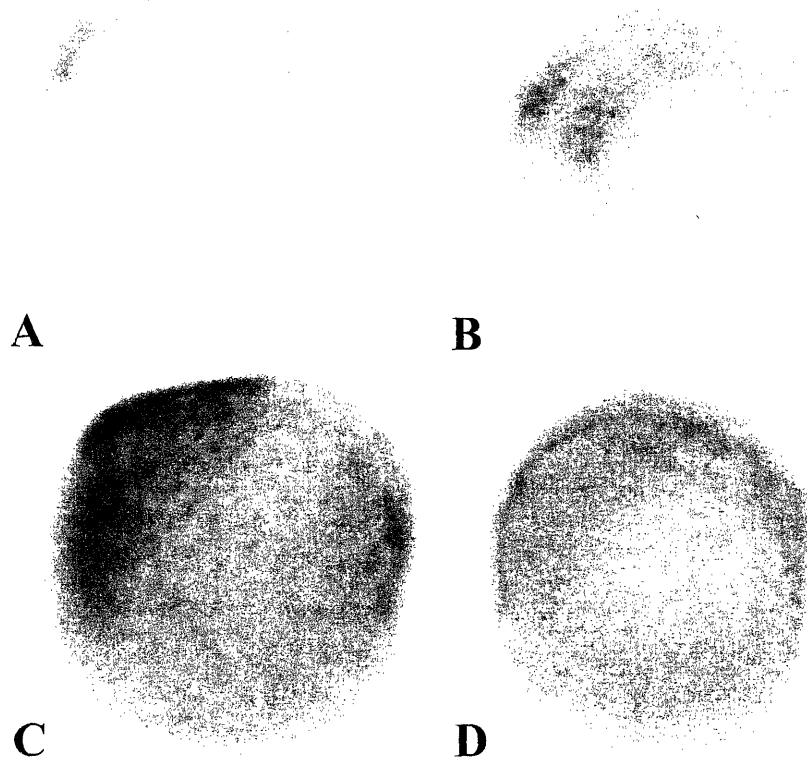


Fig. 8. Expression of *Drcpl1b* in 10hpf stage embryos. Whole mount *in situ* hybridization (ISH); lateral view (A, C, D), dorso-lateral view (B). (A, B) hybridized with 1 ng/μl probe in hybridization buffer, (C) with 2 ng/μl probe in hybridization buffer, and (D) 4 ng/μl probe in hybridization buffer.

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