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Expression Of POU Domain Genes In Zebrafish Development

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Karuna Sampath

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ABSTRACT

Development in higher eukaryotes requires the sequential activation of a large number of transcription factors. One family of developmental regulators is the homeodomain family, found in plants and animals alike. These proteins are the products of "homeobox" genes. A subset of homeobox genes is the POU domain group of genes. POU domain genes encode sequence-specific DNA-binding developmental regulators found from worms to humans.

Using the polymerase chain reaction and cDNA cloning, four POU domain-containing sequences were isolated from the zebrafish, Danio rerio. The expression patterns of the four genes, Brn1, Brn1.1, Brn2a, and Brn3, were determined by reverse transcription-polymerase chain reaction and whole mount in situ hybridization. Brn1 is expressed during neurulation in developing embryos. In adult zebrafish, Brn1 in expressed in the brain and the eyes. Brn1.1 is expressed in early cleavage, and declines during gastrulation. Later in development, embryos show expression of Brn1.1 in the eyes and brain. Expression of Brn1.1 in adult zebrafish is restricted to the ovary, eyes, and brain. The Brn2a transcript is expressed during gastrulation, with increasing levels later on in development. In adults, Brn2a expression is strongest in the brain. Brn3 shows a complex pattern of expression, with a large transcript detected in early embryos and the adult ovary. A smaller transcript is expressed in

hatching larvae and in the adult eyes, gill, brain, and testes.

All four Brain genes are expressed in the developing and adult nervous sytems, but not restricted to these tissues. These genes are similar in their expression patterns to that of their the mammalian counterparts. It is likely that the Brain genes exert specific roles in the determination of various cell types in the embryonic and adult nervous systems in the zebrafish.

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INTRODUCTION

Development in higher eukaryotes involves the transformation of a single celled zygote into a complex multicellular organism. During this process, several precisely timed and highly structured cellular events take place. These events are regulated by a network of genes, whose products determine the establishment of the structure of the organism. During the past decade or so, a combination of embryological techniques and elegant genetic analyses of development in the fruit fly, Drosophila, and the worm, Caenorhabditis elegans, have led to the identification of several such regulatory factors (Nüsslein-Volhard and Weischaus, 1980; St.Johnston and Nüsslein-Volhard, 1992; Burglin et al., 1989). The sequential activation of a large number of these factors is required for the establishment of the body plan as well as other aspects of development. Many of these factors or related factors have been identified in more complex organisms such as the frog, mouse, humans, and some plants as well. The evolutionary conservation of regulatory factors and the similarities in the mechanism of their action through phyla reiterates the significance of these molecules in embryonic development.

Transcription Factors in Development

Many regulatory factors mediate their effects by controlling gene expression at the level of transcription. A large number of transcription factors are expressed during development in higher eucaryotes (He et al., 1989). Several of these share specific DNA-binding motifs that are well-conserved through evolution. These include transcriptional regulators containing helix-loop-helix, zinc finger, leucine zipper, and homeodomain DNA-binding motifs.

Homeobox Genes and the Regulation of Gene Expression

The "homeobox" was identified originally in the Drosophila segmentation gene fushi tarazu, and the homeotic genes Antennapedia, and Ultrabithorax (McGinnis et al., 1984; Scott and Weiner, 1984). Mutations in these genes led to alterations in the body plan. Homeobox genes encode transcriptional regulators that share a conserved 60 amino acid DNA-binding motif, the homeodomain. These genes have since been identified in a variety of organisms ranging from hydra, worms, sea urchins, and several vertebrates to plants (Volbrecht et al., 1991; Kenyon, 1994; Lawrence and Morata, 1994; Krumlauf, 1994; Weigel and Meyerowitz, 1994). The mammalian homeobox genes, or HOX genes, are organized in four large complexes in the genome: HOX A, B, C, and D (Krumlauf, 1994). The organization of individual HOX genes within a cluster is very reminiscent of the Drosophila orthologs in the

HOM-C cluster. In addition, similar to the HOM-C genes in Drosophila, there is a correlation between the position of individual Hox genes in a cluster and the spatial and temporal expression patterns of the genes: i.e., genes at the 5' end of a cluster are expressed posteriorly and later on in embryogenesis, whereas the more 3' genes are expressed early on in development and have an anterior domain of expression within the embryo. This collinearity in chromosomal order and expression patterns has led to the suggestion that the ordered arrays of HOX expression could provide a code of molecular values that specify different regional identities in the embryo, much like the Drosophila HOM-C genes (Krumlauf, 1993). In addition, the order of the genes might be important for their regulation. HOX genes are believed to function in the formation of a variety of structures such as the limbs, the skeleton and the nervous system. Targeted disruption of several HOX genes in mice have shown that these genes mediate the formation of the mammalian body plan along the anteroposterior as well as appendicular axes (Krumlauf, 1993). Ectopic overexpression of combinations of HOX genes by dominant overexpression in transgenic mice have shown homeotic transformations of the axial skeleton. These experiments also demonstrated that changes in the expression of a single Hox gene can perturb that of other HOX genes, leading to formation of an alternative structure. Compound mutants in HOX genes suggest that there are dosage-dependent interactions between

different HOX genes (Condie and Capecchi, 1994; Rancourt et al., 1995). For instance, mice with independent targeted disruptions in the paralogous genes hoxa-3 and hoxd-3 had no defects in common. However, hoxa-3$^-$/hoxd-3$^-$ double mutants show strong dosage-dependent interactions between these genes. Some HOX genes can also function as oncogenes, indicating their role in cell proliferation (Condie and Capecchi, 1994). It can be inferred that these genes function at several different levels during embryonic development, and in combination with other genes, which may themselves be homeobox containing genes or other regulatory genes.

POU Domain Genes

Several subsets of homeobox containing genes have been identified whose products have a homeodomain in association with other DNA binding motifs such as the paired domain (Bopp et al., 1988). POU domain genes are one such group that encode sequence-specific, DNA binding, homeodomain proteins that have been found in organisms as diverse as the fruit fly, frog, fish, mouse, worm and man (He et al., 1989; Rosenfeld, 1991). These proteins share a well-conserved 150-160 amino acid POU domain (Herr et al., 1988; He et al., 1989; Rosenfeld, 1991, Wegner et al., 1993). POU domain proteins are thought to function as transcriptional regulators, either activating or repressing target genes (Wegner et al., 1993).

Structure of the POU Domain

The POU domain is bipartite, consisting of a 75-82 amino acid POU-specific domain and a 60 amino acid POU-homeodomain. The two subdomains are joined by a short linker of variable length and sequence. The POU-specific domain is always found in association with a POU homeodomain, and both subdomains are required for sequence-specific DNA binding (Rosenfeld, 1991; Aurora and Herr, 1992). Deletion of the POU specific domain decreases DNA binding affinity a thousand fold whereas deletion of the POU homeodomain completely abolishes binding (Verrijzer et al., 1990). The POU specific domain of Oct-1 is known to have four alpha helices packed to enclose a hydrophobic core. This structure shows striking similarity to the bacteriophage lambda repressor DNA-binding domain. The precise structure of the POU homeodomain is not known, but structural similarity among other divergent homeodomains suggests that the structure of the POU homeodomain is similar to that of other homeodomains and has three alpha helices. The solution structure of the Oct-1 POU domain from multidimensional nuclear magnetic resonance (NMR) data has shown that the complete POU domain consists of two physically linked but separate domains, each related to a different family of helix-turn-helix containing DNA-binding proteins (Assa-Munt et al., 1993).

Classification of POU Domain Genes

On the basis of their primary sequence throughout the POU domain including the linker region and the highly basic cluster of amino acids at the amino terminus of the POU homeodomain, POU domain proteins have been classified into six groups, I-VI (He et al., 1989; Johanssen et al., 1993). The first two of the three alpha-helices of the homeodomain are relatively variable between members of the gene family, whereas the third helix is highly conserved (Wegner et al., 1993). The variable linker that connects the two subdomains shows remarkable conservation within members of a group, suggesting a functional role. The regions outside the POU domain show enormous divergence even between members of the same group of POU domain proteins. Similar to members of other families of transcription factors, several POU domain proteins contain regions rich in specific amino acids, including serine/threonine-rich as in Pit-1, Oct-1 and Oct-2, glutamine rich as in Brn-2, Oct-1 and Oct-2, and glycine/alanine-rich regions as in Tst-1/SCIP/Oct-6 (Rosenfeld, 1991).

Chromosomal Order

Unlike the classical homeobox genes (HOM-C and HOX), the POU domain genes do not appear to be arranged in clusters, several POU domain genes display overlapping expression

patterns similar to several Hox genes. Members within a class are known to be dispersed: Brn 1 and Brn 2, which are class III genes, are localized on mouse chromosomes 1 and 4, respectively. Brn 3.0 is present on mouse chromosome 14, Brn 3.1 on chromosome 18, Brn 4 on chromosome 1, Pit-1 on chromosome 16, Oct-1 on chromosome 1, and Oct-2 is localized on chromosome 7 (Rubenstein and Puelles, 1994). It is possible that these genes diverged very early on in evolution.

Sequence Specific DNA Binding

These proteins bind the consensus octamer motif ATGCAAAT and were initially identified as octamer binding proteins. Some members like Pit-1 and pou(c) have also been shown to bind variant sequences like the TAATGARAT motif (Rosenfeld, 1991; Wegner et al., 1993). The POU homeodomain binds the 3' end of the octamer motif whereas the POU specific domain binds to the 5' end of the consensus sequence. These interactions involve contacts of both subdomains with the major groove of DNA and result in DNA bending. Genetic analysis of targeted deletions in the C. elegans ceh-18 gene also show that each POU subdomain can bind DNA and function independently (Greenstein et al., 1994).

Functional Interactions with Other Proteins

In addition to DNA binding, the POU domain interacts functionally with other proteins, which may themselves be POU

domain proteins or other transcription factors (Wegner et al., 1993). The three original mammalian POU proteins, Oct-1, Oct-2, and Pit-1, have been shown to bind cooperatively as dimers on double-binding sites of appropriate sequences and spacing. In addition to multiplicity of binding sites, sites for POU domain proteins are often in close proximity to other transcription factor binding sites. Pit-1 interacts cooperatively with the thyroid hormone receptor in binding the growth hormone gene promoter. Pit-1 also interacts with the estrogen receptor to mediate transcription of the prolactin gene. It is now known that dimer formation by Pit-1 is dependent on the POU specific domain (Wegner et al., 1993).

Transcriptional Regulation by POU Domain Proteins

POU domain proteins can act as transcriptional activators or repressors of their target genes. Pit-1 is required for the activation of the genes for the trophic hormones, prolactin and growth hormone, in the anterior pituitary gland. In addition, Pit-1 is also required for the differentiation and proliferation of somatotrophic, thyrotrophic and lactotrophic cells in the pituitary. Oct-1 activates the transcription of the ubiquitously expressed histone H1 gene and the snRNA genes. In lymphoid cells, Oct-1 and Oct-2 are required for the activation of immunoglobulin gene expression. Oct-6/Tst-1/SCIP functions as a transcriptional activator of early and late genes of the glial-specific human papovavirus

JC (reviewed in Wegner et al., 1993). Its activation functions have also been demonstrated using synthetic promoters containing an octamer element adjacent to a TATA box. Oct-6/Tst-1/SCIP can function as a transcriptional repressor as well, on the promoter of P_O gene which is a member of the immunoglobulin superfamily expressed in myelinating glia in the peripheral nervous system. Skn-1a activates the cytokeratin K10 promoter in terminally differentiated epidermal keratinocytes. Skn-1i, which is an alternatively spliced form that is unable to bind known target DNA sites, can inhibit Oct-1 dependent activation of the K10 promoter (Wegner et al., 1993). In vitro experiments have suggested that the function of the drifter protein (also known as cf1a) in Drosophila may be regulated by the formation of inhibitory heterodimers with another Drosophila POU domain gene product, I-POU (Treacy et al., 1991, 1992). These distinct positive and negative regulation functions might be employed by POU domain proteins during differentiation and development.

POU Domain Proteins in the Regulation of Development

POU domain proteins are believed to exert developmental functions in early embryogenesis as well as terminal differentiation events in specific cell types (Wegner et al., 1993). Pit-1 in the anterior pituitary functions in regulating prolactin and growth hormone gene expression, and

also determines differentiation of somatotrophs, lactotrophs and thyrotrophs in the pituitary gland (Rhodes et al., 1994). Oct-1 and Oct-2 regulate immunoglobulin gene expression in B lymphocytes (Corcoran et al., 1993). In C. elegans, Unc-86 is required for terminal differentiation of sensory neuroblasts (Finney and Ruvkun, 1990). The ceh-18 gene in C. elegans is required for two distinct aspects of oocyte differentiation, regulation of the oocyte cell cycle and subsequent embryogenesis (Greenstein et al., 1994). In Drosophila, expression of the drifter gene is confined to restricted areas at the cellular blastoderm stage, and later on, in the nervous system, hindgut, and throughout the tracheal system (Johnson et al., 1989; Johnson and Hirsh, 1990; Anderson et al., 1995). Examination of an EMS-induced lethal allele of dfr suggests a role for the dfr protein in the differentiation and migration of tracheal cells and midline glia in the CNS (Anderson et al., 1995). In mammals, several class III and class IV POU domain proteins are expressed in the developing and adult central nervous system. The mammalian Brn2 is CNS specific and is required for establishing neural cell lineages (Fujii and Hamada, 1993). Brn3.2 is involved in specifying terminally differentiated neuronal cell types (Turner et al., 1993).

Expression of POU Domain Genes in the Central Nervous System

An interesting feature of POU domain genes is that with the

exception of the members of group V, the transcripts of all known POU domain genes are expressed in the embryonic nervous system during at least some period of development (He et al., 1989; Rosenfeld, 1991; Wegner et al., 1993). In addition, these genes are expressed in an evolutionarily recent structure, the vertebrate forebrain (Rosenfeld, 1991).

Do these genes exert specific effects in the specification of neuronal cell types? Genetic analysis has revealed that Unc-86 in C. elegans is required for the differentiation of sensory neuroblasts as well as for the specification and maintenance of particular neuronal phenotypes (Finney and Ruvkun, 1990). The Drosophila mitimere gene is required for the specification of a subset of neuroblasts and ganglion mother cells (Bhat and Schedl, 1994). Characterization of the drifter gene has shown that it is required for correct tracheal differentiation and migration of tracheal cells and midline glia in Drosophila (Anderson et al., 1995). The mammalian Brn3 transcripts are expressed in sensory ganglia which are derived from the neural crest (He et al., 1989). Expression of Brn3.2 in the mouse coincides with the appearance of postmitotic, terminally differentiating neurons (Turner et al., 1994). It is also expressed in the developing retina and sensory peripheral nervous system. The Brn 3.0 gene maps close to, and is a candidate gene for mutations on chromosome 14 in the mouse, such as piebald (s) and agitans (ag), which affect neural crest derivatives (Xia

et al., 1993). Several class III and class VI POU genes are also located in close proximity to several mutations that result in abnormal neurological symptoms (Avrahan et al., 1993; Okamoto et al., 1993). The rat Brn 1 and Brn 2 transcripts are expressed in almost all layers of the cerebrum and cerebellum, and their expression can be correlated with the establishment of cortical lamination (Rosenfeld, 1991).

Zebrafish Gene Expression and Development

The zebrafish, Danio rerio, has in recent years, become a popular model for the analyses of vertebrate development (Kimmel, 1989; Hopkins and Rossant, 1991; Nusslein-Volhard, 1994). The short generation time of the fish (2-3 months), and the large numbers of embryos obtained from the fish facilitate genetic analysis. Zebrafish can be maintained and raised inexpensively. The embryos develop rapidly in vitro: The early cell division cycles are about 15 min. each, gastrulation occurs at 5.3 h, neurulation begins at 10 h, and by 24 hours the body plan is apparent. The embryos hatch from their chorions by 3 days. The transparency of the embryos allows detailed observation while they are developing. The movement of cells during gastrulation, the formation of the heart, and the different regions of the brain are some of the processes that can be observed in live embryos. The optical clarity of the embryos is also of value in determining the spatial and temporal patterns of cloned genes by non-

radioactive whole mount in situ hybridization and immuno-histochemical methods.

It is possible to generate transgenic fish by the microinjection of foreign DNA (Stuart et al., 1988). Ectopic overexpression of synthetic RNA molecules can be used to evaluate the function of cloned genes (Takeda et al., 1994). Several mutants have been isolated by conventional chemical and irradiation methods (Kimmel, 1989; Solnica-Krezel et al., 1994, Mullins et al., 1994), which are beginning to enhance our understanding of various aspects of vertebrate development.

Numerous genes that are known to be developmentally significant in other organisms such as Drosophila, Xenopus, and mice have been cloned from zebrafish as well. These genes include members of the HOX, PAX, and engrailed groups of genes (Fjose et al., 1988; Puschel et al., 1993; Ekker et al., 1992). The expression patterns of the zebrafish homologues is very similar to the known patterns in these organisms.

For instance, the engrailed homologues in zebrafish are expressed in the mid-brain hindbrain junction (Ekker et al., 1992). However, some differences do exist; there are three engrailed genes in fish, compared to two in mice. The eng-2 and eng-3 genes are both homologues of the murine en-2 gene (Joyner et al., 1987; Ekker et al., 1992). The pax[zf-b] gene, which is similar in primary sequence to the murine pax-2 gene, also displays a very similar expression domain as its

mammalian counterpart: both genes are expressed in the optic stalk, the otic vesicle, and the nephritic primordia of the respective organisms (Nornes et al., 1990; Krauss et al., 1991). The similarities in expression patterns maintained through different phyla suggests functional conservation of these factors as well. Hence, the zebrafish provides a good system to determine the function of genes that are also present in higher vertebrates.

Since POU domain genes are present in invertebrates and vertebrates alike, it was likely that these genes were present in zebrafish as well. The expression patterns and functions of POU genes isolated from zebrafish could be used to understand the function of the mammalian counterparts. With this in mind, the objectives of this work were to isolate POU domain genes from the zebrafish, and determine their expression patterns.

MATERIALS AND METHODS

Zebrafish Maintenance and Embryo Culture

Adult zebrafish were maintained and bred in an in-house facility according to standard procedures (Westerfield, 1993). Adult males and females were placed in breeding baskets the evening prior to collection of embryos. Embryos were siphoned from the tanks, cleaned in tank water, and staged according to hours postfertilization (h) at 28.5°C or by counting the number of somites (Westerfield, 1993).

Isolation of Genomic DNA

Genomic DNA isolation was essentially as described by Sambrook et al., 1987, with a few modifications. Adult fish were placed in a mortar, quickly frozen in liquid nitrogen, and ground to powder using a pestle, taking care to keep the material frozen in liquid nitrogen while grinding. After the liquid nitrogen had evaporated, the powder was added slowly to an extraction buffer containing 10 mM Tris pH 8.0, 100 mM EDTA pH 8.0, 0.5% SDS, and 200 µg/ml proteinase K (10 ml of buffer/g of material), and gently swirled until all the powder had submerged. The mixture was incubated at 45°C overnight, cooled to room temperature, and extracted twice with an equal volume of equilibrated phenol. The phases were separated by centrifugation at 2000 rpm for 20 min. The aqueous phase was removed carefully and extracted with an equal volume of

phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged. The aqueous phase was transferred to fresh tubes and NaCl was added to a final concentration of 200 mM. Two volumes of cold ethanol were added, and the tube was gently swirled until the solution was thoroughly mixed. DNA precipitated immediately and was removed using a sealed, U-shaped pasteur pipette, and subsequently transferred to a tube containing 70% ethanol. The pasteur pipette with the spooled DNA was removed from the ethanol, drained, and air dried for 5 min. The DNA was resuspended at 4°C o/n in 5-10 ml of 10 mM Tris pH 8.0, 5 mM EDTA pH 8.0, and 100 µg/ml (DNAse free) RNase A. Phenol:chloroform:isoamyl alcohol extraction was followed by ethanol precipitation with two volumes of ethanol and 0.1 volume of 7.5 M ammonium chloride. The DNA was spooled, washed in 70% ethanol, and suspended in 0.5-1 ml of 10 mM Tris pH 8.0 and 1 mM EDTA pH 8.0 (Sambrook et al., 1989; Westerfield, 1993).

Isolation of Total RNA

Total RNA was prepared by the guanidine isothiocyanate procedure (Ausubel et al., 1992). Staged embryos or dissected adult tissues that were quick-frozen in liquid nitrogen were lysed in two volumes of a buffer containing 4 M guanidine isothiocyanate, 100 mM Tris pH 7.5, and 1% ß-mercaptoethanol (GITC). 1.5 ml of 5.7 M CsCl in 50 mM EDTA pH 8.0 was placed in a 13 X 51 mm diethyl pyrocarbonate (DEPC) treated

centrifuge tube. 3.5 ml of the lysate was layered on top, and the tubes were centrifuged at 35,000 rpm in a SW-55 rotor (Beckman Instruments, Palo Alto, CA) for 18 h at 18°C. The supernatant was removed carefully with a pasteur pipette, and the RNA pellet resuspended in 360 µl TES (10 mM Tris pH 7.5, 5 mM EDTA pH 8.0, 1% SDS). Phenol:chloroform:isoamyl alcohol extraction was performed once. The aqueous phase was transferred to fresh tubes, 40 µl of 3 M sodium acetate pH 5.2, and 1 ml of 100% ethanol were added, the RNA precipitated at -80°C for 30 min., and centrifuged for 15 min. The pellet was washed in 70% ethanol, centrifuged and resuspended in 20 µl DEPC treated water.

Polymerase Chain Reaction Amplification of POU Domain Sequences from Zebrafish Genomic DNA and Embryonic cDNA

The polymerase chain reaction (PCR) was performed typically as follows, unless specified otherwise. The primers used for the initial amplification of POU sequences from zebrafish were designed from the deduced amino acid sequence of known POU domain genes in various organisms (He et al., 1989). 20 µM each of EcoPOU and BamPOU primers were added to 250 µM of each dNTP, 1 X Taq polymerase Buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$, and 20 ng of either zebrafish genomic DNA or a 19 h λcDNA library (a gift from Dr. Kai Zinn, University of California, Berkeley), in a 100 µl reaction volume. The reactions were overlaid with mineral

oil, incubated at 95°C for 10 min. and subsequently incubated at 80°C for 20 min. 1.25 units of Taq DNA polymerase (Promega, Madison, WI) were added prior to starting the PCR cycles. The PCR program used consisted of 35 cycles of denaturation for 1.5 min. at 95°C, annealing at 55°C for 1 min. and extension at 72°C for 3 min. A final extension of 10 min. at 72°C was included in the program. A programmable thermocycler (MJ Research Inc., Watertown, MA) was used to perform the reactions. The PCR amplification products were analyzed by agarose gel electrophoresis (Sambrook et al., 1989).

Cloning and Nucleotide Sequence Determination of the PCR Amplification Products

The major amplification product of 400 bp was purified using GENECLEAN II (Bio 101, La Jolla, CA), digested with Eco RI and Bam HI, and ligated with Eco RI and Bam HI linearized pBluescript II KS+ (Stratagene, La Jolla, CA) plasmid using T_4 DNA ligase (Promega, Madison, WI). Alternatively, the PCR products were directly ligated with the plasmid pCR II (Invitrogen, San Diego, CA). 5-10 µl of the ligation mixture was used to transform 50 µl of competent XL-1 Blue (Stratagene, La Jolla, CA) or INVF-α cells (Invitrogen, San Diego, CA). Competent cells were prepared and transformed by standard procedures (Sambrook et al., 1989).

Table 1. Sequences of Primers used in PCR Amplifications and Nucleotide Sequence Determination

Primer	Sequence
BamPOU	5′ CGGATCCTTTAAAGAICGICGIATIAAACTIGG 3′
	C GCT A A GT
EcoPOU	5′ GGAATTCTGICTICGATTACAAAACCAIACIC 3′
	G T G G G
5Brn1-1	5′ AAACCGCCCGCTAATCTTCTTCC 3′
3Brn1-1	5′ TAGGGGGTGGGGGACATTTTACA 3′
5Brn2a-1	5′ CGCTGGGGACCCTCTACGGCAAC 3′
3Brn2a-1	5′ CCCTGCGCGGCGATCTTATCCAG 3′
5Brn2c-1	5′ CCACGGGCAGTCCCACCAGTATC 3′
3Brn2c-1	5′ TGATCTCCTGCGCCGACGGTTTG 3′
5Brn2c-2	5′ ACCCATGCCGGCAACGGAGTTTT 3′
3Brn2c-2	5′ AGAGGGCAATATCCACGATTATCA 3′

5Brn2c-3	5′	CCTGGGCTGATGTGGGGAGAGTT 3′
3Brn2c-3	5′	CTGGGCTGGAGCTCCAAGGTGAG 3′
5Brn2c-4	5′	TTACGCTCAAGCTGCACTTATTT 3′
3Brn2c-4	5′	GCTCGCAGTTGTGACGTTGTGGT 3′
5Brn3-2	5′	CCAGGCGGATGTGGGATATGCTC 3′
3Brn3-2	5′	GGTCCGGTTTCCCGTTTTTCTCC 3′
5zmax-1	5′	GAAGAATGAGCGACAACGATGAT 3′
3zmax-1	5′	TTCCGTCGCATGTACTGGATGTA 3′

Recombinant plasmids were screened initially by blue-white selection. The white colonies were picked and grown for use in alkaline minipreps for isolating DNA (Sambrook et al., 1989). The plasmid DNA samples thus isolated were digested with the enzyme Eco RI alone or in combination with Bam HI. The digestion products were analyzed by electrophoresis on a 1.2 % agarose gel.

Plasmids harboring inserts of ~400 bp were used to determine the nucleotide sequence by the dideoxy chain termination method (Sanger et al., 1977) using Sequenase™ version 2.0 (United States Biochemical, Cleveland, OH). Single stranded or double stranded DNA templates were used for the reactions. 6% acrylamide/8M urea gels were used to resolve the products of the reactions, and visualized by autoradiography. The DNA sequence obtained was analyzed using DNA Strider 1.0 (Marck, 1988).

Screening cDNA Libraries using a Radioactive Probe

Conventional filter hybridization with a radioactively labeled Brn2a fragment was used as a probe to isolate the cDNA corresponding to it (Ausubel et al., 1992). Approximately 1 X 10^6 plaques of a zebrafish 33 h embryonic library in λgt11 (a gift from Dr. Kai Zinn, University of California, Berkeley) was plated with E. coli LE 392 host culture according to standard procedures (Ausubel et al., 1992), and incubated at

37°C until plaques covered the plates but were not confluent. The plates were placed at 4°C for at least 1 h. NitroPlus nitrocellulose membranes (Micron Separations Inc., Westboro, MA) were placed on the plates for 2-3 min. and their orientations were marked on the filters as well as the plates. The filters were sequentially placed face up for 2 min. each on plastic wrap covered with:

0.2 M NaOH/1.5 M NaCl

0.4 M Tris-Cl, pH 7.5/2 X SSC

2 X SSC

The filters were briefly placed face up on paper towels to blot any residual liquid, and subjected to 120,000 μJ of UV crosslinking using a UV Stratalinker™ 1800 (Stratagene, La Jolla, CA). The filters were prehybridized in a buffer containing 5 X SSPE (50 mM $NaPO_4$, 0.9 M NaCl, 5 mM EDTA), 5 X Denhardt's solution (0.1% BSA, 0.1 % Ficoll, 0.1% polyvinyl pyrrolidone), 0.2% SDS, for at least 3 h at 65°C. A random hexamer labeled Brn2a probe was added to the prehybridization buffer and incubated at 65°C overnight (o/n). The filters were washed sequentially for 30 min. each in 2 x SSC/0.2% SDS at 63°C twice, and in 0.4 X SSC/0.2% SDS twice. The filters were exposed to Kodak XAR film o/n at -80°C. Lysates from plates corresponding to positively hybridizing filters were

used for secondary screening at a lower plaque density. Filter hybridization was performed on these plates as described above. This procedure was repeated two more times until every single isolated plaque on a plate showed a signal upon autoradiography.

Large Scale Preparation of λ cDNA

A large scale liquid lysate of the plaque-purified recombinant λ phage (designated as λ c5a) was prepared according to standard procedures (Ausubel et al., 1992; Sambrook et al., 1989). 5 X PEG (207 g polyethylene glycol 6000, 49.5 g NaCl, 6 g dextran sulfate, 350 ml H_2O) was added to a final concentration of 1 X PEG, and left to precipitate o/n at 4°C. The lysate was spun at 8000 rpm for 10 min. in a SS-34 rotor (Beckman Instruments, Palo Alto, CA). The pellet was washed and resuspended in 10 ml of 10 mM Tris pH 7.5/100 mM NaCl (T/N), and spun again at 10,000 rpm for 10 min. at 4°C. The supernatant was transferred to SW 28 tubes (Beckman Instruments, Palo Alto, CA). The pellet was resuspended in 10 ml of T/N, spun for 10 min. at 10,000 rpm in a SS-34 rotor, and the supernatant pooled in the SW 28 tubes. The supernatant was spun at 25,000 rpm in a SW 28 rotor (Beckman Instruments, Palo Alto, CA) for 90 min. at 4°C. For every 500 ml of initial lysate, the phage pellet was resuspended in 5 ml T/N containing 10 mM $MgSO_4$, using a 22 g needle. A 5 ml aliquot of 65% CsCl (w/w in H_2O) was added and the mixture was

spun at 65,000 rpm o/n in a vTi 80 rotor (Beckman Instruments, Palo Alto, CA) at 20°C. The white phage band was removed and dialyzed against 10 mM Tris pH 7.5/200 mM NaCl/1 mM EDTA. The phage suspension was digested with 50 µg proteinase K/ml of suspension at 45°C for 30 min. and subsequently extracted thrice with phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was transferred to fresh tubes, precipitated with 2 volumes of ethanol and 0.1 volume of 3 M sodium acetate, and spun at 10,000 rpm for 10 min. The pellet was washed in 70% ethanol and resuspended in 500 µl TE (10 mM Tris pH 7.5, 1 mM EDTA pH 8.0). The DNA was then subjected to extraction with high salt and ethidium bromide (Stemmer, 1991) in order to remove any contaminating proteins. 30 µl of 10 mg/ml ethidium bromide and 280 µl of 7.5 M ammonium acetate were added to the phage DNA, vortexed well, and extracted with phenol:chloroform:isoamyl alcohol twice.
Ethanol precipitation was followed by resuspension of the DNA in 50 µl of TE.

Subcloning of the cDNA Insert and Nucleotide Sequence Determination

A 500 ng sample of the λc5a phage DNA was digested with the restriction enzyme Eco RI in a 20 µl reaction volume. The 2.5 kb cDNA insert was ligated with Eco RI linearized plasmid pKS+, and transformed into competent XL1-Blue™ cells (Stratagene, La Jolla, CA). Recombinants were selected by

blue-white selection. Individual white colonies were picked and grown in LB broth containing ampicillin and subjected to the alkaline lysis procedure to prepare plasmid DNA (Ausubel et al., 1987). The plasmid DNA samples were digested with Eco RI to test for the presence of a 2.5 kb insert. One plasmid sample that showed the desired size insert was further tested by PCR using the degenerate POU primers, EcoPOU and BamPOU, to determine if it indeed harbored a POU cDNA.

The nucleotide sequence of pc5a was determined by the dideoxy chain termination method (Sanger, 1977). The templates used were single or double stranded DNA templates of pc5a, and various subclones derived from it. The sequence was analyzed using DNA Strider 1.0.

Screening cDNA Libraries by PCR

Since conventional filter hybridization using a radioactive probe failed to give a cDNA clone for Brn2c, a PCR-based sib-selection strategy (Isola et al., 1991; Amaravadi and King, 1994) was employed to screen four zebrafish λ ZAP cDNA libraries (kindly provided by Dr. Dave Grunwald, University of Utah) that were prepared from the following stages: gastrula, neurula, post-somitogenesis, and adult, respectively. Aliquots of 2 µl from each library were initially amplified using Brn2c specific primers (5Brn2c-1 and 3Brn2c-1) to determine if the transcript was present in these stages. The post-somitogenesis library showed the strongest

signal and was used for subsequent screening. The primary screen was performed by plating approximately 540,000 plaques. The recombinant phage were mixed with 4 ml of an o/n culture of E. coli LE 392, and incubated at 37°C for 10 min. Top agarose (6g agarose, 10 g tryptone, 8 g NaCl /1) was melted and cooled to 42°C, added to the phage/E. coli mixture, and rapidly poured on NZCYM plates at 8 ml/plate. The plates were incubated at 37°C until plaques covered them. A 10 ml volume of suspension medium (SM) containing 5.8 g NaCl, 2 g $MgSO_4.7$ H_2O, 50 ml Tris 7.5, and 0.1% gelatin per liter was poured on each plate and incubated at 4°C for at least one hour to allow the phage to elute into the SM. The SM with phage was siphoned from the plates and mixed well with a drop of chloroform. Two µl of each of these phage lysates were used in 20 µl PCR reactions to screen for a Brn2c cDNA clone. Aliquots of positively amplifying lysates from the primary screen were replated at a lower density (~ 5000 plaques) and screened using PCR. This was repeated two more times until isolated single plaques could be cored, eluted in 100 µl of SM, and screened by PCR.

In Vivo Excision of pBluescript from the λ clone

The ExAssist™/SOLR™ system (Stratagene, La Jolla, CA) was used to excise the bluescript phagemid (containing the cDNA) from the λ ZAP vector (Stratagene, La Jolla, CA). A 100 µl aliquot of the positively amplifying phage designated as

λ932 (1 X 10^5 pfu) was mixed with 200 µl of XL1-blue cells at O.D.$_{600}$=1, and 1 µl of ExAssist helper phage (1 X 10^6 pfu/ml) in a 50 ml conical tube, and incubated at 37°C for 15 min. A 3 ml aliquot of superbroth was added to the mixture, which was then incubated with shaking at 37°C. The tube was heated at 70°C for 20 min., and spun at 2000 rpm for 20 min. The supernatant containing the plasmid packaged as filamentous phage was decanted to a fresh tube. To plate the rescued phagemid, 200 µl of SOLR cells (OD_{600} = 1.0) was added to 50 µl of the phagemid stock, and incubated at 37°C for 15 min. 100 µl aliquots of the mixture was plated on LB-ampicillin plates, and incubated o/n at 37°C. Colonies appearing on the plate were picked and subjected to PCR using 5'2c-1 and 3'2c-1 primers to ascertain if they harbored the desired cDNA.

Nucleotide Sequence Determination of the cDNA Insert

The nucleotide sequence of pSK-932 was determined by Sanger's chain termination method. Subcloning and nested unidirectional deletions (Ausubel et al., 1987; Henikoff, S., 1987; Promega Protocols and Applications Guide, 1991) were performed to generate templates for the reactions. In addition, specific primers were designed to facilitate sequence determination of the opposite strand.

Determination of Copy Number by Southern Hybridization with Genomic DNA

Zebrafish genomic DNA was isolated as described above. 10 µg of DNA was subjected to digestion with the restriction enzymes Eco RI, Bam HI, Hind III, Pst I, and Pvu II, individually or in various combinations. The digestion was performed in a 200 µl volume, o/n at 37°C. The DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol, centrifuges, and resuspended in 15 µl H_2O. The DNA was subjected to electrophoresis through a 0.7% agarose gel, and then sequentially treated in: 0.25 M HCl, 10 min., 0.5 M NaOH/1 M NaCl for 30 min., and 0.5 M Tris pH 7.5/3 M NaCl for 30 min. The DNA was capillary transferred (Ausubel et al., 1992) onto a Zetaprobe™ membrane (Bio-Rad, Hercules, CA) o/n using 10 X SSC (87.5 g sodium chloride and 44 g sodium citrate/l) as the transfer buffer. After transfer, the membrane was rinsed briefly in 2 X SSC and air-dried at room temperature for 10 min. The DNA was fixed on the membrane by baking at 80°C under vacuum for 30 min. The membrane was placed in a hybridization bottle with 15 ml of prehybridization buffer containing 5 X SSC, 5% blotting powder (Boehringer Mannheim Biochemicals, Indianapolis, IN), 50 % formamide (Sigma Chemicals, St. Louis, MO), and 0.2% SDS, for at least 3 h. at 42°C. The Brain insert to be tested for copy number was gel-purified and used as the template to make a radioactive DNA probe by random hexamer priming (Sambrook et

al., 1989). The probe was heated at 65°C for 5 min., and added to the prehybridization buffer. Hybridization was performed o/n at 42°C. Washes were performed as follows: 1 wash at 42°C in 2 X SSC/0.2% SDS for 20 min., 1 wash for 20 min. in 2 X SSC/0.2% SDS at 63°C., 2 washes for 20 min. each in 0.2 X SSC/0.2% SDS at 63°C. The blot was exposed to XAR-5 or Biomax (Eastman Kodak company, Rochester, NY) film at -80°C for 3 days.

Determination of Developmental Expression by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA from embryos at various stages was prepared by the guanidine isothiocyanate method as described earlier (Ausubel et al., 1992). 2.5 µg of each RNA sample was used as the template in reverse transcription reactions. Each RNA sample was suspended in 15 µl of DEPC-treated H_2O, denatured at 95°C for 3 min., and snap chilled on ice. To this were added 6 µl of 5 X RT first strand buffer (BRL, Bethesda, MD), 6 µl of deoxynucleotide triphosphates at 2.5 mM each (Pharmacia Biotech Inc., Piscataway, NJ), 1.5 µl of 25 mM $MgCl_2$, 100 pM of random hexamer primers, and 0.5 µl RNasin ribonuclease inhibitor (Promega, Madison, WI) in 30 µl reactions. cDNA synthesis was performed using 200 units of Superscript Reverse Transcriptase (BRL, Bethesda, MD) at 37°C for 1 h (RT+). No enzyme was added to the negative controls (RT-) for each RNA sample in the reverse transcription

reactions. 2 µl aliquots of the RT reactions were used in separate 20 µl PCR reactions with 20 µM of 5' and 3' primers for each Brain gene. The reaction mixture included 2 µl of 10 X Taq polymerase buffer (Promega, Madison, WI), 2 µl of 25 mM $MgCl_2$, 3 µl of 1.25 mM deoxynucleotide triphosphates, and 1 unit of Taq DNA polymerase. The polymerase was added after a 20 min. hot-start at 80°C. PCR with primers specific for zebrafish Max was used to determine the quality of the RT reactions. The reactions were trace labeled with 0.5 µCi α^{32}P-dCTP. Negative controls for the PCR reactions used included no template and aliquots of RT- for each RNA sample. The PCR conditions were as follows: 25 cycles of 1.5 min. denaturation at 95°C, 1 min. annealing at 58°C, and extension for 3 min. at 72°C. A final extension for 10 min. at 72°C was included. 5 µl volumes of each PCR reaction were electrophoresed on a 6 % polyacrylamide gel. The gel was dried and exposed to Biomax film (Eastman Kodak company, Rochester, NY) o/n at -80°C.

Whole Mount In Situ Hybridization to Determine the Spatial Expression Pattern in Embryos

Staged zebrafish embryos were dechorionated manually (for embryos younger than 10 h) or with Pronase (20 µg/ml), washed and fixed overnight in 4 % paraformaldehyde (PFA) in 1X Phosphate buffered saline (PBS). The embryos were stored in methanol at -20°C for at least 2 h. The embryos were

rehydrated in a methanol/PBST series (100% methanol, 75% methanol/25% PBST, 50% methanol/50% PBST, 25% methanol/75% PBST, 100% PBST) for 15 min. each, followed by 2 washes in PBST (1 X PBS, 0.1% Tween-20). Embryos older than 10-12 h were treated in 10 µg/ml proteinase K in PBST for 5-10 min., postfixed in PFA for 20 min., and washed 5 times in PBST. Embryos were then prehybridized in hybridization buffer (50% formamide, 5 X SSC, 0.1% Tween-20, 50 µg/ml heparin, 500 µg/ml tRNA) for at least 2 h at 65°C. Digoxigenin labeled sense and antisense RNA probes were synthesized by in vitro transcription using 1 µg of linearized plasmids as template (Boehringer Mannheim Biochemicals), and hydrolyzed to 200-300 bases using sodium bicarbonate/sodium carbonate (Oxtoby and Jowett, 1993). The prehybridized embryos were incubated with fresh hybridization buffer containing digoxigenin labeled RNA probe at 50-100 ng/200 µl buffer and incubated o/n at 65°C. Rehydrated 24-48 h embryos were used to preadsorb a 1:200 dilution of the anti-digoxigenin antibody conjugate (Boehringer Mannheim Biochemicals, Indianapolis, IN) in PBST containing 2% sheep serum and 2% goat serum (Sigma Chemical Company, St. Louis, MO)for 4-5 h at room temperature. The preadsorbed antibody dilution was stored at 4°C. Following hybridization with the RNA probe, the embryos were washed at 65°C for 20 min. each in 100% hybridization buffer, 50% hybridization buffer/50% 2 X SSC, and 100% 2 X SSC. This was followed by 2 washes in 0.2 X SSC for 30 min. each at 60°C.

The embryos were washed for 5 min. each in 75% 0.2 X SSC/25% PBST, 50% 0.2 X SSC/50% PBST, 25% 0.2 X SSC/75% PBST and 100% PBST. The embryos were incubated for 1 h at room temperature with shaking in PBST containing 2% sheep serum and 2% goat serum (Sigma Chemical Company, St. Louis, MO) and then with a 1:2000 dilution of the preadsorbed antibody for 4 h. The embryos were washed in PBST 5 times for 15 min. each, followed by 3 washes for 5 min. each in staining solution (100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween-20) and stained in staining solution with 4.5 µl/ml of a 50 mg/ml stock of Nitroblue tetrazolium (NBT) and 3.5 µl/ml of a 50 mg/ml stock of BCIP (X-phosphate) (Boehringer Mannheim Biochemicals, Indianapolis, IN), until sufficient signal was seen. The reaction was stopped by washing in PBST and the embryos were cleared in methyl salicylate or glycerol (Oxtoby and Jowett, 1993; Westerfield, 1993).

Determination of Tissue Specific Expression Patterns in Adults

Adult male and female zebrafish were dissected and various organs or tissues were quick-frozen in liquid nitrogen. RNA was extracted from the individual tissues by the guanidium isothiocyanate procedure as described (Ausubel et al., 1992). Reverse transcription of 2.5 µg of RNA from each tissue was carried out essentially as described for the developmental series. PCR with specific 5' and 3' primers for each individual Brain gene in reactions trace-labeled with 0.5

μCi of α^{32}P-dCTP was performed using 2 μl aliquots of each RT sample. Negative controls for the PCR reactions included no template, an aliquot of a RT reaction performed with no RNA, and individual RT(-) reactions for each RNA sample. The products of the PCR reactions were electrophoresed through a 6% polyacrylamide gel and visualized by autoradiography.

RESULTS

Polymerase Chain Reaction Amplification of POU Sequences from Zebrafish

Based on the amino acid sequence of known POU domain proteins from other organisms, degenerate oligonucleotide primers were designed that spanned the POU domain. The 5' primer (BamPOU) was based on the sequence F/LKQ/VRRIK, and the 3' primer (EcoPOU) corresponded to VVRVWFCN. Using these two primers on genomic or 19 h cDNA templates at an annealing temperature of 55°C, a major amplification product of 400 bp was obtained (Fig. 1). Several higher molecular weight bands could also be observed in the amplification performed with genomic DNA as the template, and these disappeared when higher annealing temperatures were used (not shown).

Cloning and Nucleotide Sequence Determination of the Amplification Product

The 400 bp major product was cloned in the plasmids pKS+ or pCR II. The nucleotide sequence of the 400 bp inserts was determined by Sanger's dideoxy chain termination method (Fig. 2). The obtained sequences were compared with those of known POU domain genes from other organisms (He *et al.*, 1989). The amplification product from the 19 h cDNA template showed homology to the mammalian Brn2 gene. When the deduced amino acid sequences were compared, there were only 5 differences

Figure 1. Agarose Gel Electrophoretic Analysis of Fragments Amplified by PCR with the Degenerate POU Primers. Lane 1, lambda Hind III marker; lane 2, PCR amplification products from genomic DNA template.

between the fish and rat Brn2 POU domains (Fig. 3). The amplification product from genomic DNA revealed two additional POU domain genes, one of which also showed homology to the mammalian Brn2 gene, but was distinct from the cDNA PCR product (Brn2a). The second was similar to the mouse Brn3.1 gene. The Brn2 homologue from genomic DNA was designated as Brn2c. At least two independent PCR generated clones each of Brn2c and Brn3 were isolated and sequenced. The Brn2c sequence differed from the cDNA product (Brn2a) by 6 amino acids (Fig. 3). Nucleotide comparison between the Brn2 clones showed 86% homology (Fig. 2). The Brn3 PCR product showed 94% identity to the rat Brn 3 POU domain sequence at the amino acid level (Fig. 3).

Screening of a 33 hour cDNA Library

Radioactively labeled Brn2a was used as a probe to screen the 19 h cDNA library to isolate a full-length clone. The 19 h cDNA library failed to give any positively hybridizing plaques. Screening of a 33 h λ cDNA library (a gift from Dr. Kai Zinn, University of California, Berkeley) was performed. Approximately 1 X 10^6 plaques of the 33 h library were plated with *E. coli* LE 392 host culture, and screened by the conventional radioactive plaque hybridization method. The positively hybridizing plaque (λc5a) was purified and used to subclone the cDNA insert.

Figure 2. Nucleotide sequence of the PCR products.

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Brn2a  GGATCCTTCAAGCAGCGGAGGATGAAGCTGGGCTTCACGCAGGCGGACG
Brn2c  GGATCCTTTAAACTGCGGCGGATGAAACTGGGCTTTACTCAGGCCGACG
Brn3   GGATCCTTTAAGCAGCGGAGGATGAAGTGGGGAGTGACCCAGGCGGATG

Brn2a  TGGGGCTCGCGCTGGGGACCCTCTACGGCAACGTCTTC.........TC
Brn2c  TCGGGTTAGCGCTGGGAACTCTTTACGGAAATGTTTTC.........TC
Brn3   TGGGCTCTGCTCTCGCCAACCTGAAGATACCGGGGGTCGGCTCGCTGAG

Brn2a  CCAGACCACCATCTGCAGGTTCGAGGCGCTCCAGCTGAGCTTCAAGAAC
Brn2c  TCAGACCACCATTTGCAGGTTCGAGGCTCTCCAGCTGAGCTTCAAGAAC
Brn3   CCAAAGCACCATCTGCAGGTTCGAGTCCTTAACACTTTCACACAACAAC

Brn2a  ATGTGCAAACTCAAGCCGCTGCTGAACAAGTGGCTGGAGGAGGCGGACT
Brn2c  ATGTGCAAACTTAAGCCACTGCTAAACAAGTGGCTGGAGGAGGCCGACT
Brn3   ATGATCGCCCTCAAACCCGTCCTCCAAGCTTGGCTGGAGGAAGCTGAAG

Brn2a  CCACTTCGGGAAGCCCGACCAGCCTGGATAAGATCGCCGGCAGGGCAGG
Brn2c  CGACCACGGGCAGTCCCACCAGTATCGACAAAATAGCAGCTCAAGCAGG
Brn3   CTGCTTACCGGGAGAAAAACTGGGAAACCGGACCTTTCAATGGGAACGA

Brn2a .AAAAGGAAAAAGCGCACCTCCATCGAGGTGAGTGTCAAGGGGGCCCTGG
Brn2c .AAACGAAAGAAGCGCACTTCCATTGAGGTGAGCGTGAAGGGAGCCTTGG
Brn3  GAGGAAACGAAAGCGTACGTCCATCGCAGCTCCGGAGAAGCGATCGCTCG

Brn2a  AGAGCCACTTCCTCAAGTGTCCCGAAACCAGGCGCTCCGAGATCAACTC
Brn2c  AGAGCCATTTTCTGAAATGCCCCAAACCGTCGGCGCAGGAGATCACCTC
Brn3   AGGATATTTTGCAATCCAGCCGCACCGTCGTCGGAAAAAATCGCGGCTA

Brn2a  GCTGGCGGACAGCCTGCAGCTGGAGAAGGAGGTGGTGCGCGTCTGGTTC
Brn2c  TCTGGCCGACAACCTGCAGCTGGAAAAAGAGGTGGTCCGCGTCTGGTTC
Brn3   TCGCGGAGAAGTTGGATCTAAAG.AAGACGTGGTTCGCGTCTGGTTCTG
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Subcloning the cDNA Insert and Nucleotide Sequence Determination

The DNA from λc5a was isolated and the 2.5 kb cDNA insert was cloned into Eco RI digested pKS+ (Fig. 4). Various subclones as well as exonuclease III deletion clones were generated to facilitate sequence determination. The nucleotide sequence of double as well as single stranded templates was determined and the amino acid sequence was deduced. Sequence comparison with mammalian POU domain proteins showed that c5a is a homologue of Brain 1, showing 75 % identity to the mouse Brn1 (Hara et al., 1992) amino acid sequence (Fig. 5).

The zebrafish Brn1 contains stretches of the amino acids alanine, proline, serine and histidine near the amino terminus. The zebrafish gene shows remarkable homology to the mouse Brain 1 sequence even in regions outside the conserved POU domain (Fig. 5). As this work was in progress, the cloning and characterization of a zebrafish POU domain gene, ZFPOU1, was described by Matsuzaki and others (1992). The sequence of Brn1 is identical to that of ZFPOU1.

Expression of Zebrafish Brn1 During Development

The expression pattern of Brn1 was determined by RT-PCR using primers specific for Brn1. Brn1 can be detected at very low levels during gastrulation (6-8 h). The expression is

Figure 3. Putative Amino Acid Sequence of the Cloned PCR Fragments, Compared to the Homologous Sequences from the Rat. Brn 2a is the PCR fragment cloned from a 19 h cDNA library. Brn2c and Brn3 are the products obtained from amplification of genomic DNA.

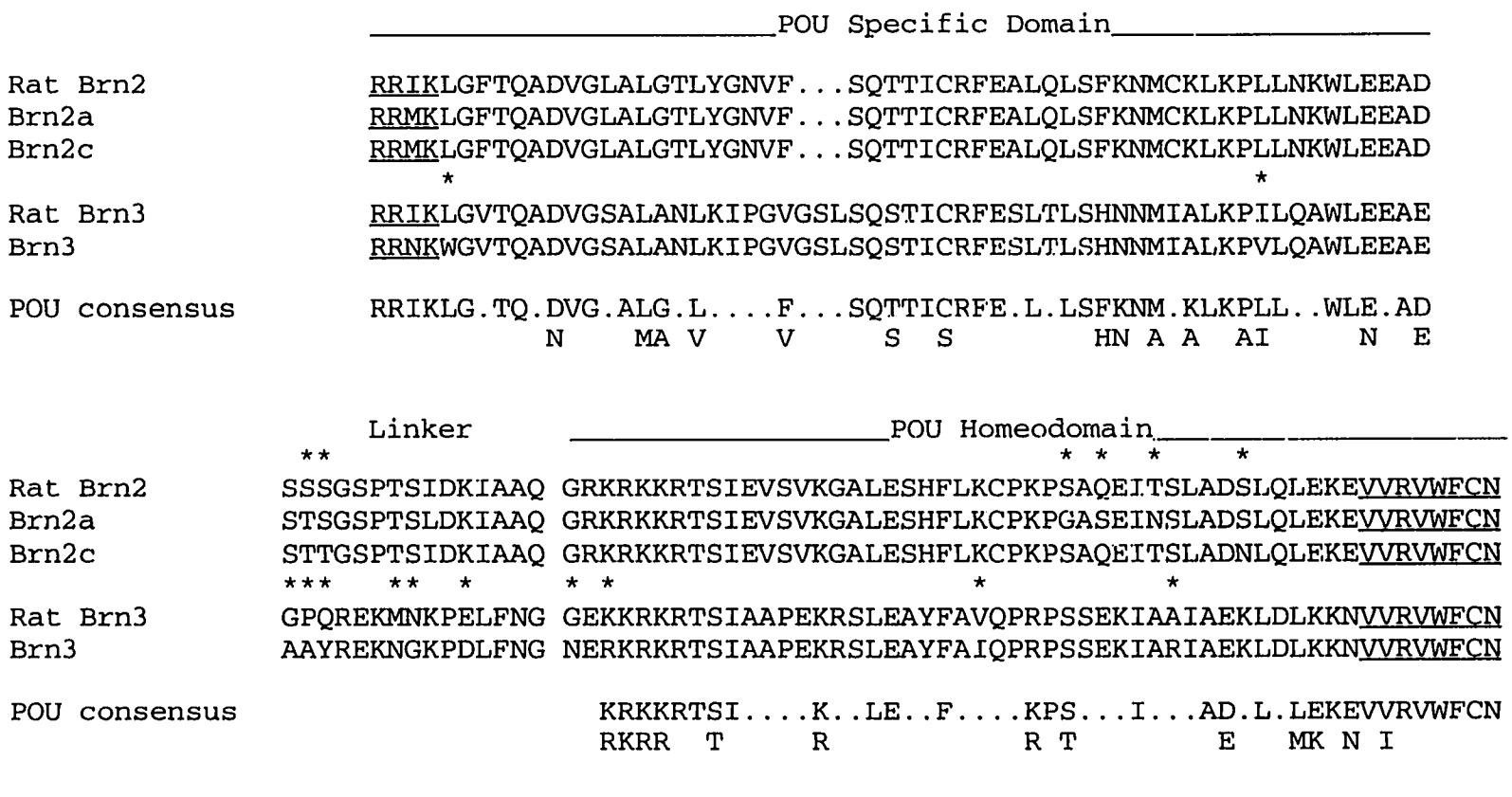


Figure 4. Agarose Gel Electrophoretic Analysis of a Plasmid Containing the Insert from the c5a cDNA Clone. Lane 1, Eco RI digested plasmid with the 2.2 kb insert shown with the arrow; lane 2, Lambda Hind III marker.

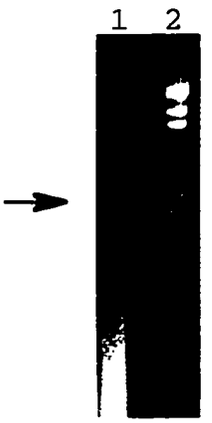
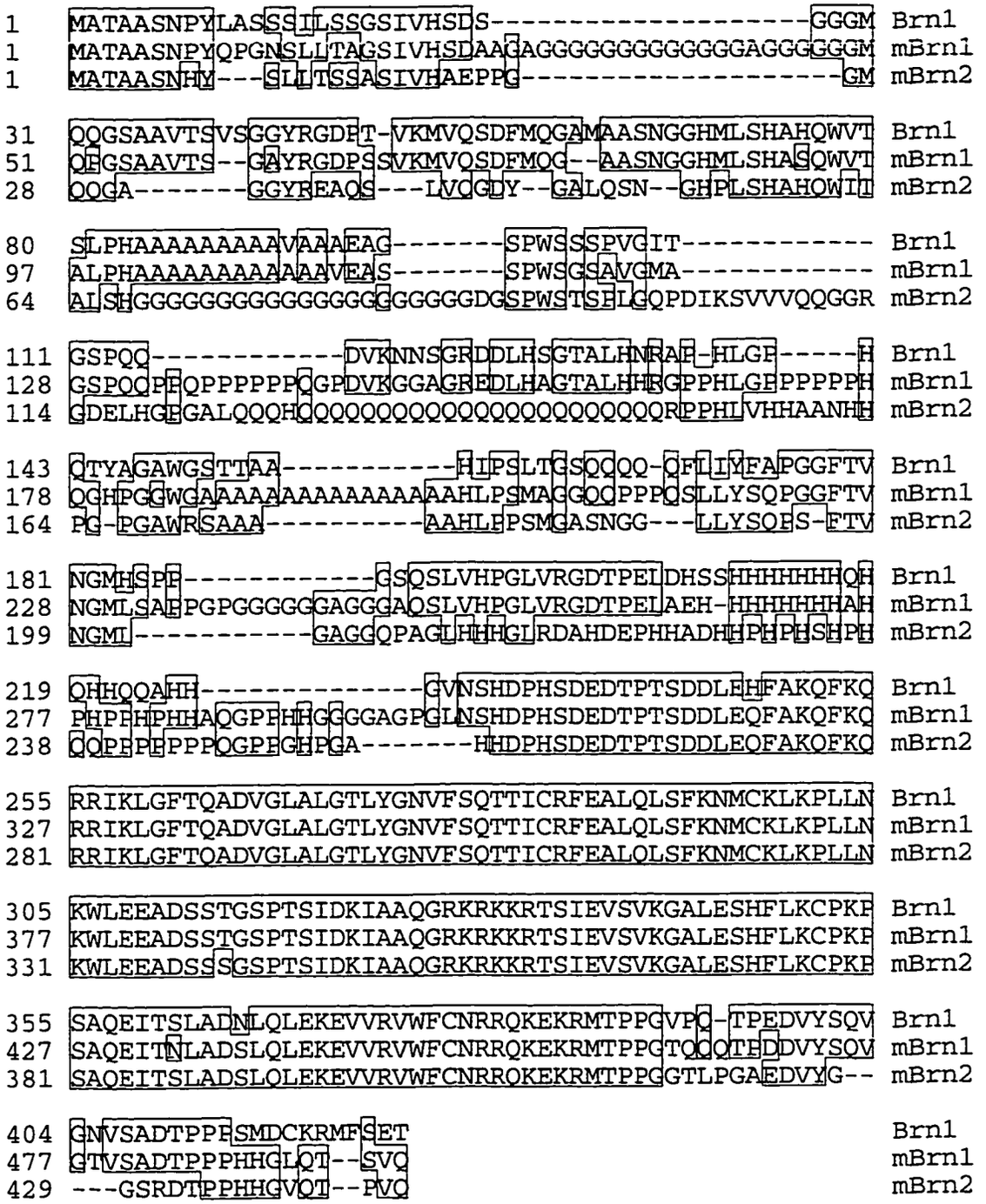


Figure 5. Comparison of the Zebrafish and Mouse Brn1 and Brn2 Amino Acid Sequences. The dots indicate gaps included to facilitate alignment of the sequences. The translation termination codons are indicated by asterisks (*).

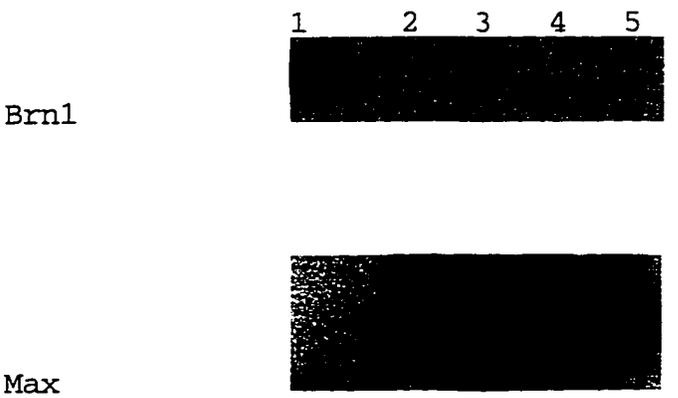


stronger during neurulation (10-14h) (Fig. 6). Although not evident in this figure, independent experiments suggest that the Brn1 transcript is still present after 24 h. The rat Brn1 gene can be detected as early as day 8 in the embryonic nervous system (He et al., 1989). Primers specific for zebrafish Max were used for PCR on the same templates to serve as controls. The same level of Max products were obtained from all the reverse transcription templates (Fig. 6, lower panel).

Spatial Localization of the Brn1 Transcript in Developing Embryos

Whole mount in situ hybridization with a digoxigenin labeled Brn1 antisense RNA probe was used to detect the presence of the Brn1 transcript in embryos at various stages of development. An anti-digoxigenin antibody conjugated to alkaline phosphatase was used which gave a purple colored precipitate wherever the probe bound in the embryo. A probe corresponding to the sense strand was used as a negative control. The Brn1 transcript could be detected in 10 h embryos, all along the neural keel. The staining was particularly intense in the anterior region, where the brain would form (Fig. 7a). By the 15 somite stage (15 h), regions of intense and faint staining can be discerned in the brain, giving a metameric or segmented appearance to the stained

Figure 6. Developmental Expression of the Zebrafish Brn1 Transcript Detected by RT-PCR. 2.5µg of total RNA from the following stages was converted to first strand cDNA and used in PCR reactions with Brn1 specific primers. 25 cycles were performed and a 600 bp product was expected. Lane 1, genomic DNA control; lane 2, 1 cell embryos; lane 3, gastrula; lane 4, neurula; lane 5, post-somitogenesis.

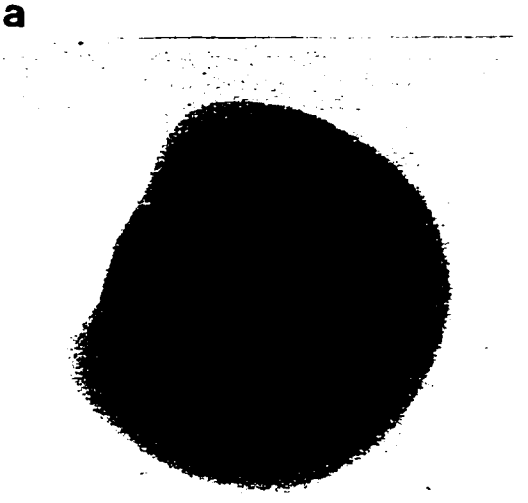


regions (Fig 7b). In 18 h embryos, staining is intense in the posterior forebrain and anterior mid-brain, and in regions of the hindbrain. Staining can also be detected along the spinal cord. The anterior forebrain, posterior mid-brain, and the eyes do not show any staining (Fig. 7c). The MyoD transcript (a gift from Dr. Eric Weinberg, University of Pennsylvania) was used as a positive control for the staining reaction (not shown).

Tissue-Specific Expression of Brn1 in Adult Zebrafish

RNA from various adult tissues was isolated for use in RT-PCR reactions with the Brn1 specific primers, 5Brn1-1 and 3Brn1-1. The 900 bp products were analyzed by acrylamide gel electrophoresis and autoradiography as described in the methods. Low levels of the Brn1 transcript could be detected in the eye. The brain showed high levels of the transcript, and in other tissues tested, such as gills, ovary and liver, the transcript could not be detected (Fig. 8). Primers for the zebrafish Max gene were used in PCR reactions with the same cDNA preparations to evaluate their quality and to ensure that all the samples had equivalent amounts of cDNA. The amplification with the Max primers yielded several products of varying intensities, consistent with the identification of more than one Max homologue in zebrafish. The levels of the products in various tissues were also consistent with the observed expression patterns (Schreiber-Agus et al., 1993).

Figure 7. Whole Mount In Situ Hybridization with Brn1. Digoxigenin labeled RNA probes synthesized from the entire Brn1 cDNA were used. a. A 10 h embryo showing hybridization in the neural keel (arrowhead) in the anterior region of the embryo. b. A 15 h embryo with staining in the midbrain and hindbrain. c. A 18 h embryo with staining in the posterior forebrain, anterior midbrain (m), regions of the hindbrain (h), and the spinal cord (s). The yolk is indicated by 'y'. Magnification 50 x.



Screening for a Brn2c cDNA

A PCR based sib-selection scheme was employed to isolate a cDNA corresponding to Brn2c. Initial amplification of four cDNA libraries suggested that Brn2c was represented best in the post-somitogenesis stage library. 540,000 plaques were screened and finally, two individual plaques that amplified the desired size fragment were isolated (Fig. 9). One of these, λ93 could be excised to give a phagemid harboring the desired cDNA. The rescued phagemid was plated on LB agar plates with ampicillin. The colonies that amplified the desired 141 bp product with 5Brn2c-1 and 3Brn2c-1 were grown for plasmid minipreps. Restriction digestion with Eco RI and Xho I showed that the cDNA insert was 1.8 kb (Fig. 10).

Nucleotide Sequence Determination of the Brn2c cDNA

The nucleotide sequence was determined by Sanger's method. Double stranded templates of the pSK-932 plasmid as well as exonuclease III digested derivatives were used as templates for the reactions. The cDNA contained one large open reading frame of 1.2 kb (Fig. 11). The remaining 600 bp were 3' untranslated sequences. Comparison of the deduced amino acid sequence of the large open reading frame with known POU domain proteins shows that it is related to the mouse, rat and human Brn1 gene, showing 73 % identity to mouse Brn1 at

Figure 8. Adult Tissue-Specific Expression of Brn1. 2.5 µg of total RNA from various dissected tissues was converted to first strand cDNA, and subjected to 25 cycles of PCR with Brn1-specific primers. The lower panel shows amplification with primers specific for Zebrafish Max. Lane 1, genomic DNA; lane 2, eyes; lane 3, gills; lane 4, ovary; lane 5, brain; lane 6, testes; lane 7, liver.

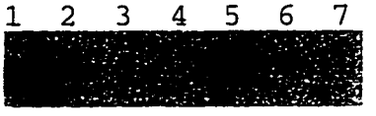
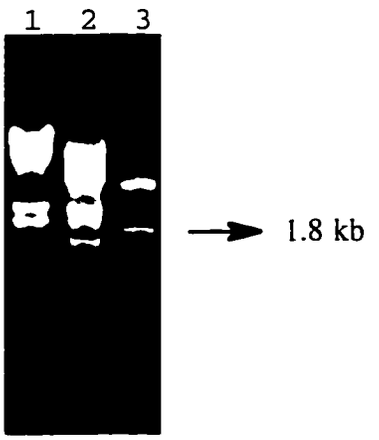


Figure 9. Positively Amplifying Purified Plaques. PCR based sib-selection was usd to screen a library with Brain 2c specific primers. At the 4^{o} level of screening, isolated single plaques were picked and amplified. Lane 1, SM buffer control; lane 2, positively amplifying plaque 93; lane 3, positively amplifying plaque 96; lane 4, lambda Hind III/Pvu II marker.

Figure 10. Agarose Gel Electrophoretic Analysis Showing a 1.8 kb cDNA Insert. The recombinant plasmid was digested with the restriction enzymes Eco RI and Xho I to release the insert. Lane 1, Lambda Hind III marker; lane 2, lambda Hind III/Pvu II marker; lane 3, Eco RI and Xho I digested plasmid with a 1.8 kb insert.



the amino acid level. However, Brn2c is distinct from the previously described zebrafish Brn1 gene. Brn2c shows 73 % homology at the amino acid level to the mouse Brn1 (Fig. 12), and will be referred to as Brn1.1. The Brn1.1 cDNA encodes a putative protein of about 410 amino acids. This clone represents a partial cDNA, with the first 4 amino acids of the coding region missing, shown by alignment with known class III POU domain proteins. Towards the amino terminus of the deduced amino acid sequence of Brn1.1, there are several stretches of the amino acids glutamine, histidine, alanine and serine, similar to other members of Class III POU domain proteins.

Southern Analyses of Brn1.1

The entire 1.8 kb cDNA insert was used to generate a labeled Brn1.1 probe for southern blot analysis. Genomic DNA was digested with various restriction enzymes that included Eco RI, which has a restriction site near the 3' end of the cDNA. There were two bands seen in the Eco RI digested lane in the southern blot, as would be expected. Similarly, with one internal Pst I site, two fragments were expected, and were detected (Fig. 13). There are no internal Bam HI or Hind III sites in the cDNA, and only one fragment hybridized in each of these lanes. Under the conditions used, no cross-hybridization was seen with Brn1 or any other POU domain gene. Hence, Brn1.1 is a single copy gene

Figure 11. Nucleotide Sequence of the Brn1.1 cDNA Clone and the Putative Amino Acid Sequence. The nucleotide and amino acid numbers are given at the begining and end of the coding sequence.

1
g gct tca aac cct tac ctg gcc agc agc acc att ctc tca tca gcc
  ala ser asn pro tyr leu ala ser ser thr ile leu ser ser ala

tcg ctc gtg cac tcg gag tcc gga gtg gtg gca atg cag ccg gga
ser leu val his ser glu ser gly val val ala met gln pro gly

agt ggt gca gtt aca tcg gtg tcg ggg ggt tac aga gga gac ccc
ser gly ala val thr ser val ser gly gly tyr arg gly asp pro

aca gtc aag atg gtg cag agc gac ttc atg caa gga gcc atg gcc
thr val lys met val gln ser asp phe met gln gly ala met ala

gcc agt aac ggg ggt cac atg ctg agc cat gcg cat cag tgg gtc
ala ser asn gly gly his met leu ser his ala his gln trp val

acc tca ctg ccc cac gcg gct gcc gca gcc gcc gca gcc gcc gca
thr ser leu pro his ala ala ala ala ala ala ala ala ala ala

gca gca ggc gaa gcc ggc tca cct tgg agc tcc agc cca gtt ggt
ala ala gly glu ala gly ser pro trp ser ser ser pro val gly

atg gcc ggg agc cca cag caa caa gac gtc aag agc agt tca aac
met ala gly ser pro gln gln gln asp val lys ser ser ser asn

agg gaa gac cta cac tcg ggc acc gcc ttg cac cac aga ccc acg
arg glu asp leu his ser gly thr ala leu his his arg pro thr

cat tta ggg gct cac cag tcg cac caa agc gca tgg ggt ggc acc
his leu gly ala his gln ser his gln ser ala trp gly gly thr

aca gcc tcc cac atc tcc acc atc acc gga cag cag cag tcc cag
thr ala ser his ile ser thr ile thr gly gln gln gln ser gln

cag tct ctc atc tat tcc cag cct ggt ggt ttc aca gtc aac ggc
gln ser leu ile tyr ser gln pro gly gly phe thr val asn gly

atg tta aac cct ccc gga ggt ttt gtg cac cct ggg ctg atg tgg
met leu asn pro pro gly gly phe val his pro gly leu met trp

gga gag tct cca gaa atg gat cac cat cat cat cac cac cac cac
gly glu ser pro glu met asp his his his his his his his his

cag cac cac gcg gga gtg aac agc cac gac tcg cac tca gac gag
gln his his ala gly val asn ser his asp ser his ser asp glu

gac acg ccg acc tcc gac gac ctg gag cag ttc gcc aaa caa ttt
asp thr pro thr ser asp asp leu glu gln phe ala lys gln phe

aag cag cgt cgg atc aaa ctg ggc ttt act cag gcc gac gtc ggg
lys gln arg arg ile lys leu gly phe thr gln ala asp val gly

tta gcg ctg gga act ctt tac gga aat gtt ttc tct cag acc acc
leu ala leu gly thr leu tyr gly asn val phe ser gln thr thr

att tgc agg ttc gag gct ctc cag ctg agc ttc aag aac atg tgc
ile cys arg phe glu ala leu gln leu ser phe lys asn met cys

aaa ctt aag cca ctg cta aac aag tgg ctg gag gag gcc gac tcg
lys leu lys pro leu leu asn lys trp leu glu glu ala asp ser

acc acg ggc agc ccc acc agt atc gac aaa ata gca gct caa ggc
thr thr gly ser pro thr ser ile asp lys ile ala ala gln gly

agg aaa cga aag aag cgc act tcc att gag gtg agc gtg aag gga
arg lys arg lys lys arg thr ser ile glu val ser val lys gly

gcc ttg gag agc cat ttt ctg aaa tgc ccc aaa ccg tcg gcg cag
ala leu glu ser his phe leu lys cys pro lys pro ser ala gln

gag atc acc tct ctg gcc gac aac ctg cag ctg gaa aaa gag gtg
glu ile thr ser leu ala asp asn leu gln leu glu lys glu val

gtc cgg gtc tgg ttc tgc aac cga agg cag aag gag aag agg atg
val arg val trp phe cys asn arg arg gln lys glu lys arg met

acg ccg ccc ggg gta ccc cag acg ccc gag gac gtg tac acc cat
thr pro pro gly val pro gln thr pro glu asp val tyr thr his

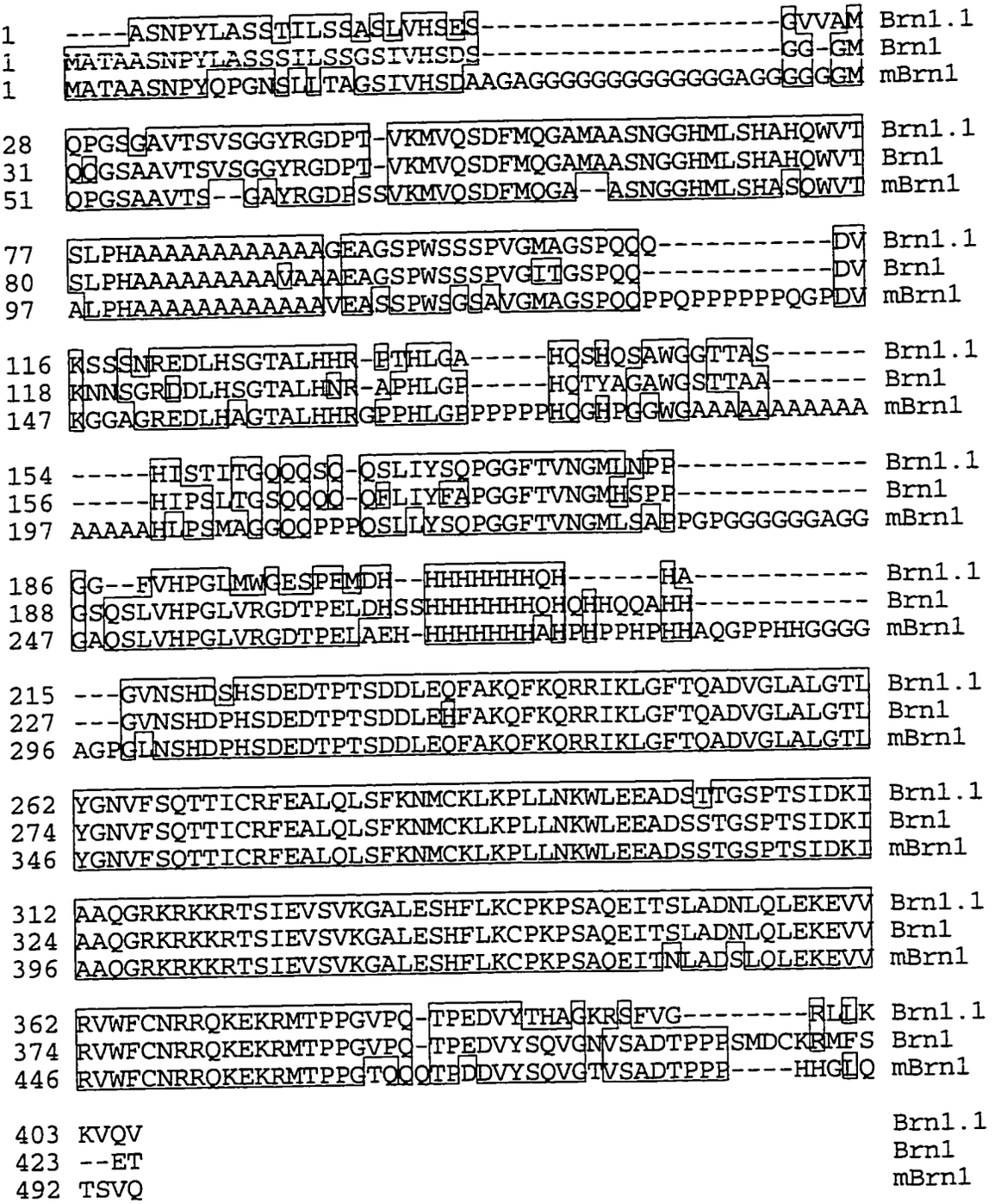
gcc ggc aaa cgg agt ttt gtt ggt aga tta ctt aaa aag gtg caa
ala gly lys arg ser phe val gly arg leu leu lys lys val gln

1218/406
gtt tga aagatgaaccggacagcaaccacaacgtcacaactgcgagctcctatggcc
val OPA

aggagattctggtgcactgacaccgaaaacataaccagcccaaagccaacataagactttat
ggattgaaattgcaaaagaactcccaaggacatggatcaatgcagacaaacattaaatatta
aacagtggaagaatttaccccaggacacgtatcaattttacgcacaggaattatattac
tcattttaacaaaaacggaattgtcttgtctactcttggagtattatgtgcaaacaaactac
acctttctatttcatagtatttttgtggacaacacttctgtaaatagctttacacagagaa
actctcaagaaaaagagaaaagatactctcaagctgcactatttccaattcagccgacc
ccttgtaaaaaccttttttttgttgttgtttggctgataatcgtggatattgccctctcttca
ctgctttttccaggcagaatttttggtgggttttgataagtctgtgtaaatttggcaatat
gaattctctgtgttgagttgatttaaatgatttgctttatatttttcactaaaatcaatttc
agataaaaaaaaaaaaaaaaa

1847

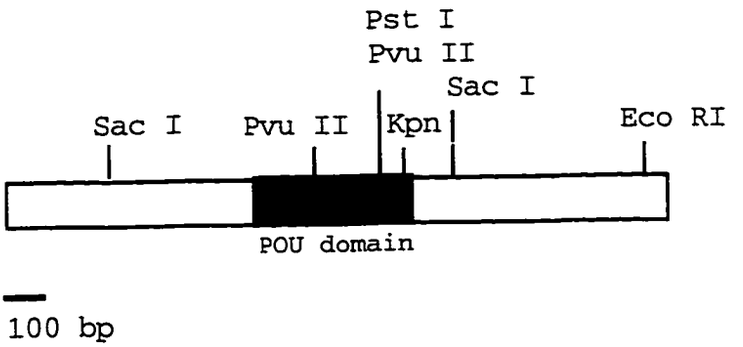
Figure 12. Comparison of the Putative Amino Acid Sequence of Brn1.1 with the Zebrafish Brn1 and Mouse Brn1 Sequences. Dots indicate gaps included to facilitate alignment of the sequences. -- indicate missing sequence. * indicate the translation termination codon.



Developmental Expression of Brn1.1 Detected by RT-PCR

Total RNA from embryos at various stages was isolated and used in RT-PCR reactions with Brn1.1 specific primers. Low levels of the Brn1.1 transcript can be detected in the reactions performed with the 1 and 2 cell embryo cDNA sample. The transcript was not detected in the cDNA synthesized from 6-8 h embryos, which were at mid- to late gastrulation. An upregulation of the transcript is seen during neurulation (10-16 h), and in the post-somitogenesis (24-28 h) stages (Fig. 14). The same cDNA preparations were subjected to amplification with primers specific for zebrafish Max, and showed equivalent amounts of cDNA for each stage analyzed. Genomic DNA was used a control for the PCR reactions and a larger size amplification product can be detected in the lane corresponding to this sample, suggesting the presence of a small (~200 bp) intron in this region of genomic DNA. Low levels of this larger product can be detected in the neurula and post-somitogenesis RT-PCR lanes as well. The RT(-) negative controls for the neurula and post-somitogenesis samples did not yield either of the two products in the PCR reactions, showing the lack of any contaminating genomic DNA or cDNA. The larger product seen in the RT-PCR with these samples very likely represents unspliced RNA.

Figure 13. Southern Blot Analysis with Brn1.1.
Zebrafish genomic DNA was digested with several restriction enzymes and subjected to southern blot analysis with a Brn1.1 full-length probe. The panel on the top of the page shows a schematic representation of a partial restriction map of Brn1.1. The lower panel shows the southern blot. Lane M, Lambda Hind III marker; lane 1, uncut genomic DNA; lane 2, Eco RI digested DNA; lane 3, Bam HI; lane 4, Hind III; lane 5, Eco RI and Bam HI;lane 6, Eco RI and Hind III; lane 7, Bam HI and Hind III; lane 8, Pst I; lane 9, Pvu II; lane 10, Pst I and Pvu II.



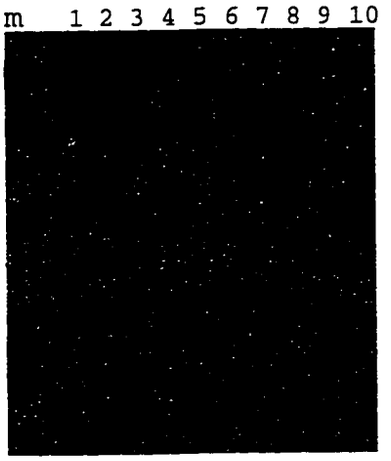
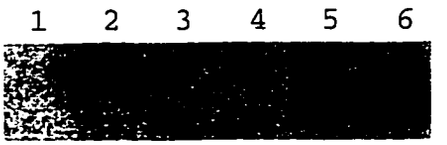


Figure 14. Developmental Expression of Brn1.1. Total RNA from the following stages was converted to first strand cDNA and used in a 25 cycle PCR reaction with primers specific for Brn1.1. Lane 1, negative control; lane 2, genomic DNA positive control; lane 3, 1-2 cell; lane 4, 6-8 h gastrula; lane 5, 10-16 h neurula; lane 6, 24-28 h post-p somitogenesis.



Tissue-Specific Expression of Brn1.1 in Adult Zebrafish

RT-PCR was performed on RNA from various adult tissues. The Brn1.1 transcript is present in low levels in the eye ovary, and testes. The highest levels appeared to be in the brain. The transcript could not be detected in the liver or gills (Fig. 15). The larger size product could not be detected in any of the samples and even the genomic DNA positive control failed to yield the larger product in the reaction shown.

Expression of Brn1.1 in Embryos Detected by Whole Mount In Situ Hybridization

Expression was detected in early cleavage embryos. Expression was not detected in gastrula stage embryos. By 15 h post-fertilization, distinct staining was seen in the entire midbrain. Staining was also detected in the optic tract. By 18 h, expression was detected in the optic tract, mid brain, and hindbrain. Krox-20 expression in rhombomeres 3 and 5 of the hindbrain was used as a reference for staining in the hindbrain. In 42 h pharyngula stage embryos, there was faint staining observed in the optic nerve and midbrain regions, with intense staining in the gills (Figures 16, 17, and 18).

Figure 15. Adult tissue expression of Brn1.1. Total RNA from various adult tissues was used to synthesize first strand cDNA for use in 25 cycle PCR reactions with Brn1.1 primers. Lane 1, genomic DNA; lane 2, eyes; lane 3, gills; lane 4, ovary; lane 5, brain; lane 6, testes; lane 7, liver.

Figure 16. Whole Mount In Situ Hybridization with Brn1.1. The entire 1.8 kb Brn1.1 insert or a 500 bp 3' untranslated region fragment were used to generate digoxigenin labeled Brn1.1 RNA probes. a. An 8 cell embryo with staining in all blastomeres at magnification 50 X. b. Anterior region of a 16 h embryo double labeled with Krox-20 and Brn1.1 at 200 X magnification. Krox-20 staining is seen in rhombomeres 3 and 5 in the hindbrain (r3 and r5). Brn1.1 staining is seen in the midbrain (m). c. Anterior region of a 18 h embryo with intense staining in the midbrain (m) at 200 X magnification. Krox-20 expression in r3 is marked as a reference.

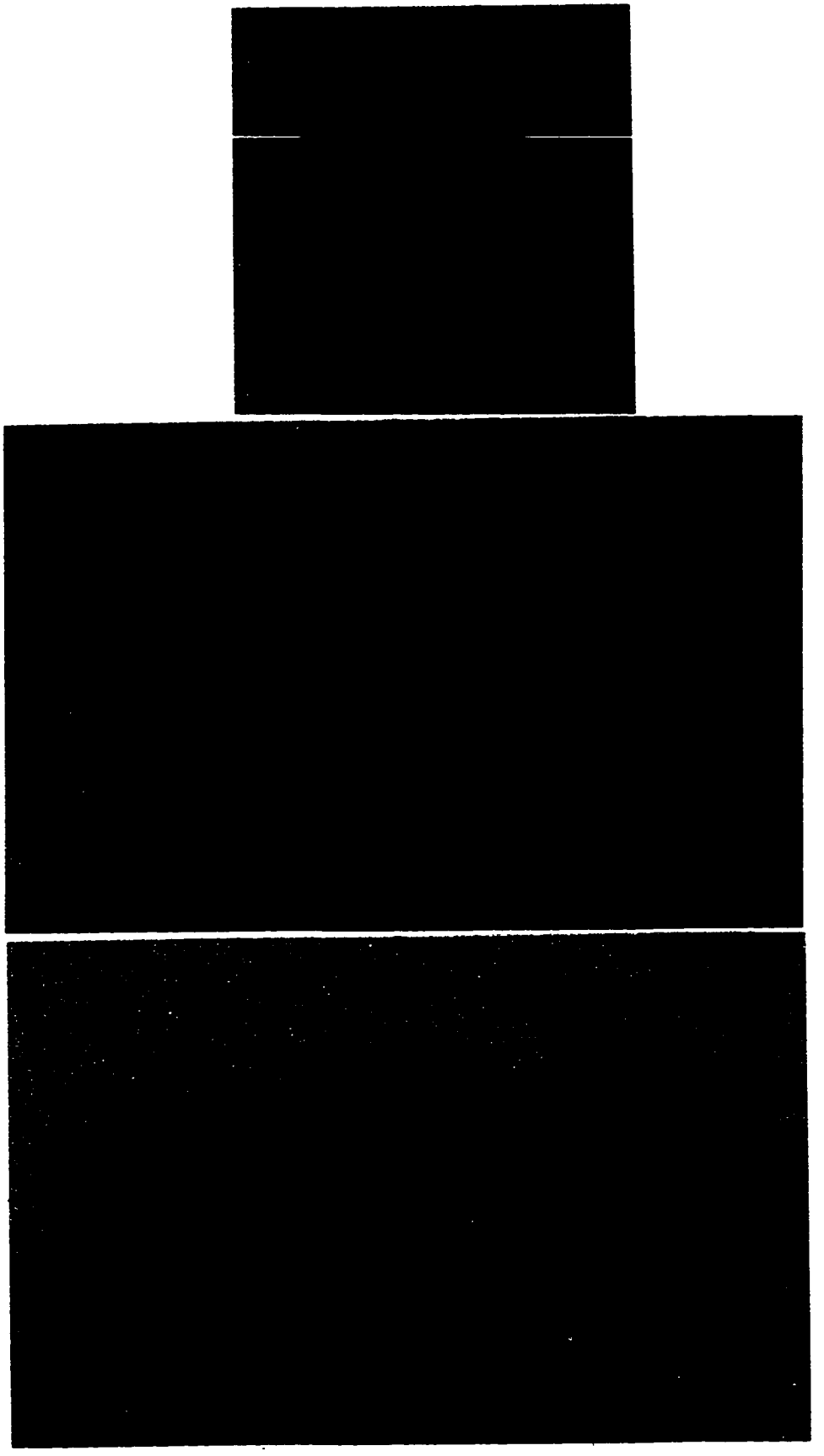
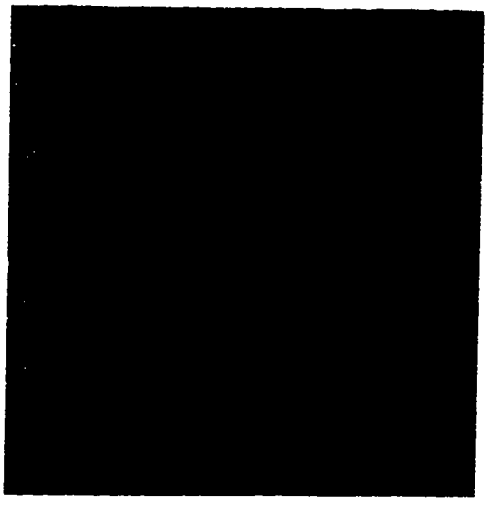


Figure 17. Whole Mount In Situ Hybridization with Brn1.1.
a. An 18 h embryo with Brn1.1 expression in the midbrain and hindbrain at 50 X magnification.
b. 200 X magnification of the same embryo showing intense staining in the midbrain (m), and hindbrain (h).



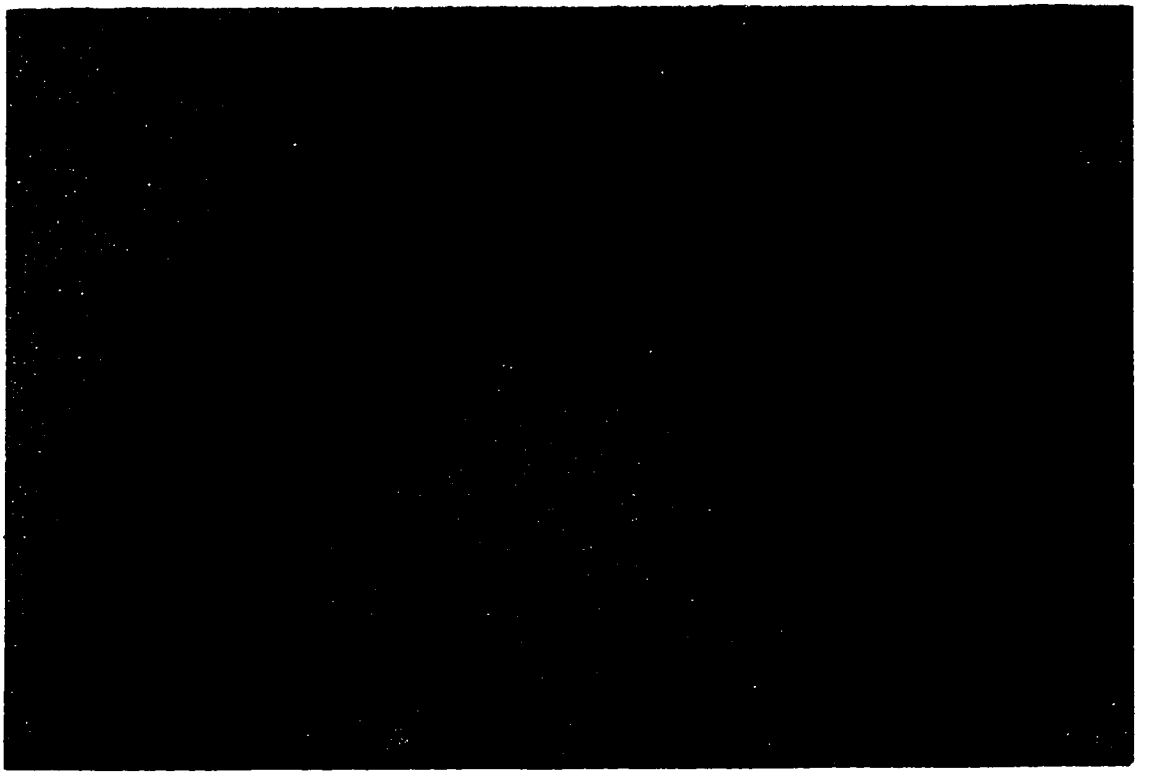
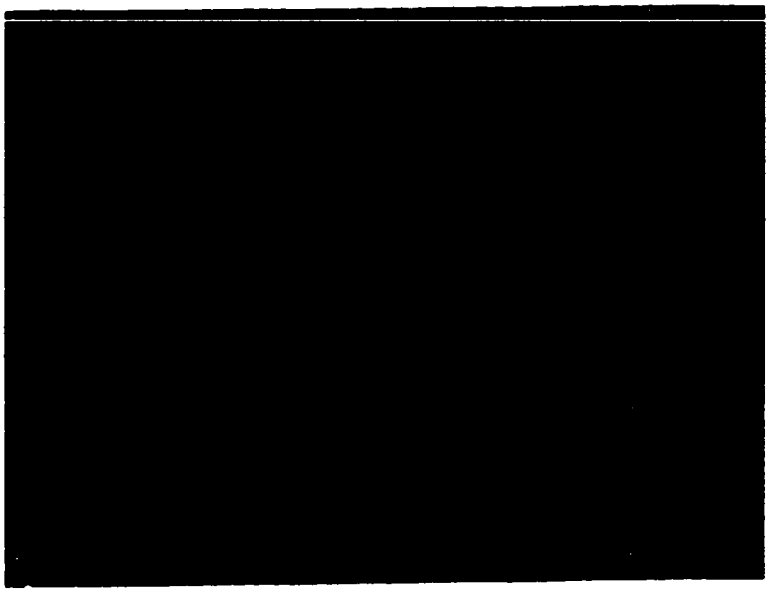
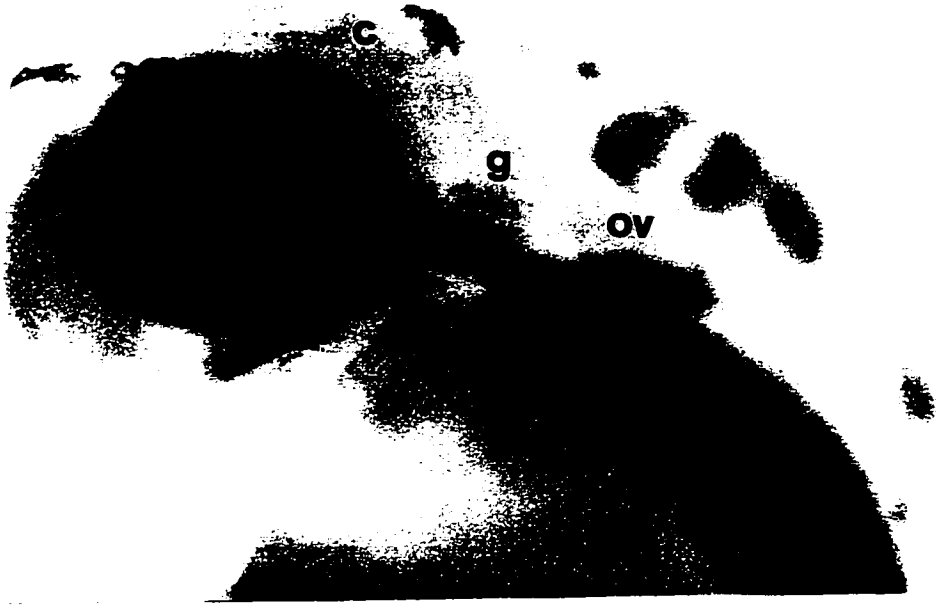


Figure 18. Whole Mount In Situ Hybridization with Brn1.1. a. Dorsolateral view of an 18 h embryo at 200 X showing Brn1.1 expression in the optic stalk (os), and the midbrain (m). The eye (e) shows very little staining. b. Lateral view of the anterior region of a 42 h pharyngula embryo at 400 X. Staining is seen in the cerebellar fold (c) and the midbrain anterior to it (not marked). Staining is also seen in the gill apparatus (g). The eyes do not show staining. The clear area adjacent to the gills is the otic vesicle (ov). Dark areas seen in the eye, along the dorsal surface and around the otic vesicle are melanocytes. Some background staining can be seen in the yolk.





b

Developmental Expression of Brn2a

RT-PCR was performed on RNA from various developmental stages using primers specific for Brn2a. The Brn2a transcript can be detected at low levels during gastrulation (6-8 h). The level increases during neurulation (10-16 h), and the highest intensity was detected in the post-somitogenesis stages (24-28 h) (Fig. 19).

Tissue-Specific Expression of Brn2a in Adults

RT-PCR was performed on various adult tissues as described. The Brn2a transcript can be detected at low levels in the eye and testes samples. The brain shows the strongest signal. The transcript was not detected in any of the other tissues tested, such as liver, ovary, and gills (Fig. 20).

Southern Analysis Using the Brn2a Insert

The 400 bp Brn2a PCR fragment was used as a probe in southern hybridization. Several fragments of varying intensity were detected in the autoradiograph (Fig. 21). For instance, with no internal Eco RI site in the fragment used to generate the Brn2a probe, a single hybridizing fragment would be expected. However, two strongly hybridizing fragments, and one weakly hybridizing fragment can be detected (Fig. 21). This indicates cross-hybridization with other closely-related POU sequences.

Figure 19. Developmental Expression of Brn2a. Total RNA from the following stages was used to generate first strand cDNA and useed in 25 cycles of PCR with primers speecific for Brn2a. Lane 1, genomic DNA; lane 2, 1-2 cell; lane 3, 6-8 h gastrula; lane 4, 10-16 h neurula; lane 5, 24-28 h post-somitogenesis.

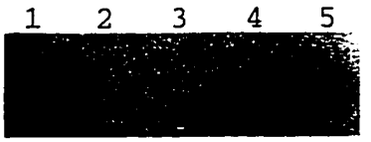


Figure 20. Adult Tissue Expression of Brn2a. Total RNA from various adult tissues was used to generate first strand cDNA, and subjected to 25 cycles of PCR with primers psecific for Brn2a. Lane 1, genomic DNA; lane 2, eyes; lane 3, gills; lane 4, ovary; lane 5, brain; lane 6, testes; lane 7, liver.

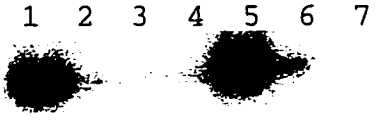
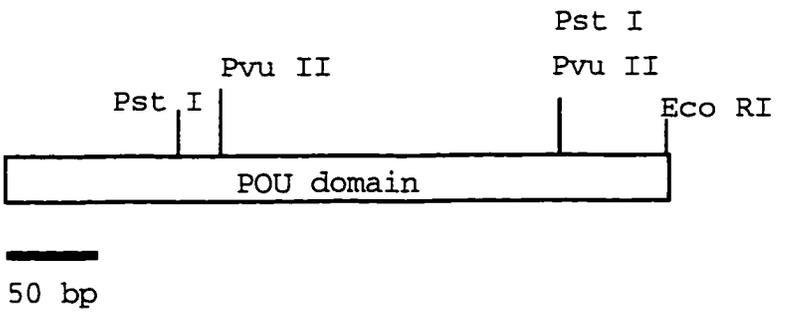
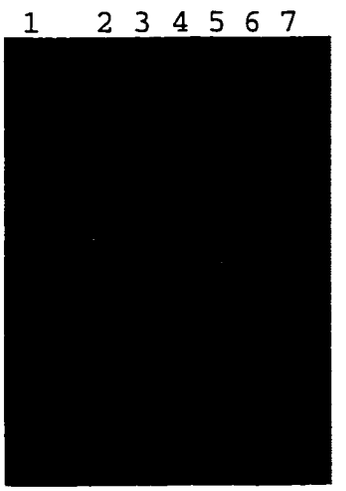


Figure 21. Southern Analysis using a Brn2a Probe. Zebrafish genomic DNA digested with several restriction enzymes and subjected to southern hybridization with a Brn2a probe. The upper panel shows a schematic representation of a partial restriction map of the 400 bp Brn2a. The lower panel shows the southern blot. Lane 1, genomic DNA uncut;lane 2, Eco RI; lane 3, Hind III; lane 4, Eco RI and Hind III; lane 5, Pst I; lane 6, Pvu II; lane 7, Pst I and Pvu II.





Whole Mount In Situ Hybridization with Brn2a

The Brn2a probe detected expression in all blastomeres of early blastula stage embryos. Expression was seen everywhere in 15 h embryos, with intense staining in the mid and hind brain regions (Fig. 22). By 18 h, expression was ubiquitous, with intense staining localized to the midbrain, hindbrain, and spinal cord.

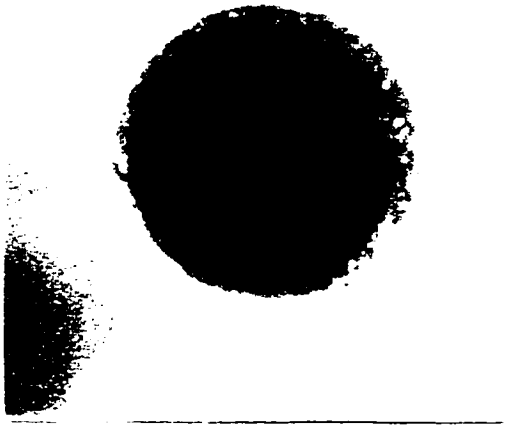
Developmental Expression of Brn3

The expression of Brn3 in embryos was detected by RT-PCR with primers specific for Brn3. The expected 141 bp fragment could not be detected until 72 h, by which time the embryos hatch from their chorions. However, a larger 250 bp fragment could be detected in the 1-2 cell embryo samples. This transcript could be detected at trace levels in the late gastrula stage sample as well (Fig. 23).

Adult Tissue-Specific Expression of Brn3

The expected 141 bp RT-PCR product was detected in low levels in the brain, gills and at moderate levels in the testes. The strongest expression was seen in the eyes. A higher molecular weight product of about 250 bp was detected in the ovary sample (Fig. 24). Neither transcript was detected in the liver.

Figure 22. Whole Mount In Situ Hybridization with Brn2a. a. Early blastula embryo at 65 X showing intense staining in all blastomeres. b. A 12 h embryo at 65 X with staining in the prospective anterior brain and optic placode. c. An 18 h embryo (without the yolk sac) at 65 X showing intense staining in the midbrain (m), hind brain (h), and spinal cord (s). Less intense staining can also be seen in the eyes (e).



b

c

Southern Analysis of Brn3

Southern analysis was performed with the Brn3 POU domain fragment as a probe. Several bands of strong and weak intensity were seen, suggesting cross-hybridization with other related POU sequences (Fig. 25).

Whole Mount In Situ Hybridization with Brn3

The expression pattern of Brn3 sequences in embryos was determined by whole mount in situ hybridization with the 400 bp fragment as a probe. Staining was seen at all stages tested. All blastomeres in 8-16 cell early cleavage embryos showed intense staining (Fig 26). In 18 h embryos, staining was ubiquitous, with intense staining in the eyes and anterior regions of the developing brain.

Figure 23. Developmental Expression of Brn3. 2.5 µg of total RNA from embryos at the following stages was used to generate first strand cDNA. 25 cycles of PCR was performed with primers specific for Brn3. Lane 1, negative control; lane 2, genomic DNA control; lane 3, 1-2 cell; lane 4, 6-8 h gastrula; lane 5, 10-16 h neurula; lane 6, 24-28 h post-somitogenesis; lane 7, 30-48 h; lane 8, 72 h larvae. The arrow indicates the expected product of 141 bp. The larger product seen in lanes 3 and 4 is 250 bp.

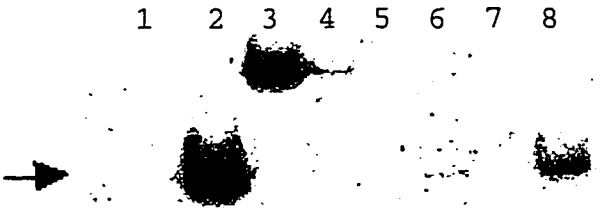


Figure 24. Adult Tissue Expression of Brn3. Total RNA from various adult tissues was used to synthesize first cDNA for use in 25 cycles of PCR with Brn3-specific primers. Lane 1, negative control; lane 2, genomic DNA; lane 3, eyes; lane 4, gills; lane 5, ovary; lane 6, brain; lane 7, testes; lane 8, liver.

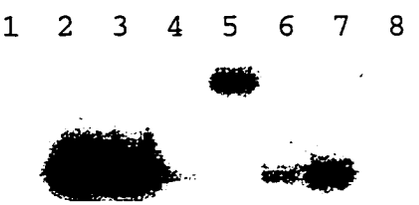
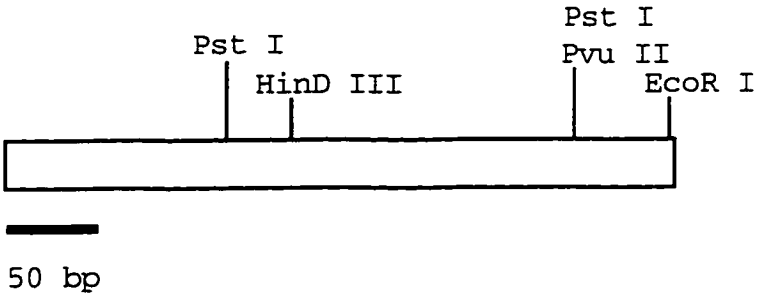


Figure 25. Southern Blot Analysis using a Brn3 Probe. Genomic DNA was digested with several restriction enzymes, electrophoresed, capillary-transferred onto a nylon membrane, and hybridized with a radioactive Brn3 probe. The upper panel shows a partial restriction map of the Brn3 PCR fragment used as template to generate the probe. The lower panel shows the southern blot. Lane 1, uncut genomic DNA; lane 2, Eco RI digested DNA; lane 3, Hind III; lane 4, Eco RI and Hind III; lane 5, Pst I; lane 6, Pvu II; lane 7, Pst I and Pvu II.



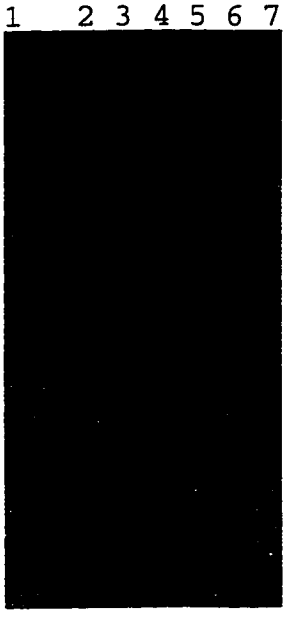
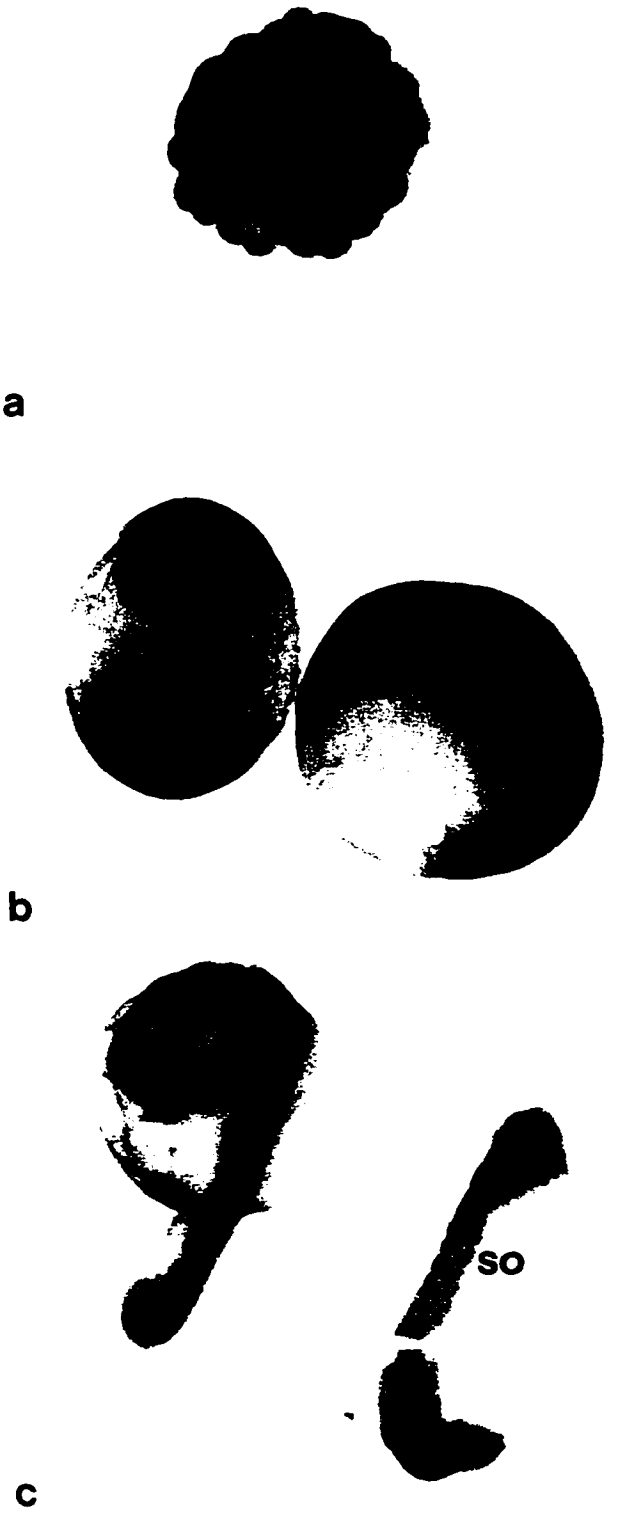


Figure 26. Whole Mount In Situ Hybridization with Brn3. a. A 64 cell embryo at 65 X magnification showing staining with the Brn3 probe in all blastomeres. b. 10 h embryos at 65 X magnification with staining all along the axis. c. A 20 h embryo at 65 X magnification showing ubiquitous staining all over the embryo. 'e' indicates the eyes and 'so' indicates somites.



DISCUSSION

Isolation of POU-Domain Containing Fragments from Zebrafish DNA using PCR

Fragments showing similarity to two major classes of POU domains (He et al., 1989) were amplified by PCR from zebrafish genomic DNA or 19 h cDNA templates using primers corresponding to the ends of the conserved 150-160 amino acid POU domain. Two distinct Class III fragments were isolated both of which showed homology to the mouse, rat and human Brn2 POU domains (He et al., 1989; Hara et al., 1992). In addition, a Class IV fragment was isolated that showed homology to the rat and mouse Brn3 POU domains (Gerrero et al., 1993).

Failure to isolate PCR fragments showing homology to other classes of POU domains could be due to the choice of the primers designed for the PCR amplifications. Although the primers corresponded to consensus sequences compiled from the POU domains of the Unc-86 gene of the nematode worm C. elegans to the human Brn1 and Brn2 genes, these sequences may not have been representative of all POU domains. At least two other POU domain genes have been isolated from zebrafish, pou2 (Takeda et al., 1994), and POU(c) (Johansen et al., 1993), both which were not found in this search. POU2 shows similarity to both Class II as well as Class V genes, and might represent a new intermediate class of POU domain genes

(Takeda et al., 1994). POU(c) also shows a divergent POU domain sequence and defines a separate class by itself, Class VI (Johansen et al., 1993). He and others (1989) suggested the presence of a large family of POU domain genes in mammals, and to date, over 60 members have been identified in a variety of organisms (reviewed in Rosenfeld, 1991; Wegner et al., 1993). It is likely that zebrafish have several additional POU domain genes, which remain to be isolated.

Cloning and Characterization of a Brn1 Homologue in Zebrafish

A 2.5 kb cDNA clone encompassing the entire coding sequence of a zebrafish POU domain gene was isolated by screening a 33 h library with the PCR clone Brain 2a. The cDNA encodes a putative protein of 425 amino acids, and shows homology to the mouse, rat and human Brn1 genes. The amino acid identity to the mouse Brn1 gene is 75% (Hara et al., 1992). This places the zebrafish Brn1 gene in POU domain class III.

The expression pattern of Brn1 was examined in developing zebrafish embryos and various adult tissues. Similar to the mouse, rat and human Brn1 genes (He et al., 1989; Hara et al., 1992), the zebrafish Brn1 is expressed in the embryonic nervous system, as it develops. Expression can be detected first with the onset of neurogenesis at 10 h. At this stage, the entire neural keel, which will form the brain later on, shows the presence of the transcript in whole mount in situ

hybridization. The amount of the transcript increases by 15 h, showing higher levels of the transcript in the prospective mid-brain and hind-brain regions. At 18 h, the transcript is present in the posterior forebrain, anterior midbrain, and several regions in the hindbrain. Staining in the hindbrain appeared strongest in the anterior regions, perhaps corresponding to the first three rhombomeres. Intense staining was detected in the posterior regions of the hindbrain, leading into the spinal cord, which also expresses the transcript in high levels. The transcript could not be detected in the embryonic eye. However, the adult eyes do show low levels of the transcript. Amongst various adult tissues tested, the brain shows the highest levels of the transcript.

As this work was in progress, the cloning and expression pattern of a neurally expressed zebrafish POU domain gene, ZFPOU1 was described (Matsuzaki et al., 1992). The zebrafish Brn1 gene is identical in sequence and expression pattern to ZFPOU1. However, some details were not described by Matsuzaki and others. Our use of the RT-PCR technique has allowed the detection of the Brn1/ZFPOU1 transcript in the adult eyes. In the developing embryos at 18 h, the hind brain shows an apparent segmental pattern of expression of the Brn1/ZFPOU1 transcript, with regions of high and low/no expression. Another homeobox gene isolated from the zebrafish, hlx-1

(Fjose et al., 1994) shows restricted transversal stripes in the hindbrain, which correspond with the segmental pattern of rhombomeres in the hindbrain. Similarly, in the midbrain, the homeobox genes eng-1, eng-2, and eng-3 are expressed (Ekker et al., 1993). The paired-box containing genes pax-6 and pax-2 (previously known as pax[zf-a] and pax[zf-b]) are also expressed in restricted regions of the nervous system (Krauss et al., 1991a, 1991b; Puschel et al., 1992). Some of these genes have overlapping and/or complementary domains of expression. These transcriptional regulators are likely to play important roles in the regionalization of the brain as it develops. Similarly, Brn1/ZFPOU1 could be involved in the specification of subsets of neurons, by directly or indirectly regulating other genes during differentiation of neuronal structures.

Zebrafish Have More Than One Brn1 Homologue

Using a PCR-based strategy, another Brn1-like gene, Brn1.1 was isolated from a zebrafish post-somitogenesis library. 1.8 kb of most of the coding region of a putative protein of 410 amino acids was isolated. At the amino acid level, Brn1.1 shows 73 % identity to the mouse Brain 1 gene (compared to 75% between zebrafish Brn1/ZFPOU1 and mouse Brn1). Brn1.1 is also a member of POU class III. Although the POU domain of Brn1.1 is similar to the mammalian Brn2 genes, the sequences outside the POU domain are more

homologous to the mammalian Brn1 genes. Attempts to isolate a strict Brn2 homologue in zebrafish have failed thus far. It is possible that zebrafish have several Brn1-like genes, some of which might be intermediate in sequence, expression patterns, and function, between the mammalian Brn1 and Brn2 genes. In Xenopus, two genes with >90% homology to class III genes have been described, XLPOU1 and XLPOU2, both of which produce transcripts that are localized to neural tissues in developing embryos and adults (Agarwal and Sato, 1991). The zebrafish Brn1-like genes perhaps represent an ancestral group of Class III genes, predating the divergence of mammals from other vertebrates.

The expression pattern of Brn1.1 was examined in detail in embryos as well as adult tissues by RT-PCR, and whole mount in situ hybridization in embryos. The Brn1.1 transcript can be detected at low levels in 1-2 cell embryos. The adult ovary also shows the presence of a transcript, indicating that Brn1.1 produces a maternally expressed transcript. The transcript is not detectable during gastrulation, and reappears during neurulation, with the highest levels of expression detected 24 h after fertilization. At 15 h, the transcript can be detected in a wide band of cells in the mid-brain, extending posteriorly to the junction between the mid-brain and hind-brain. This expression is clearly anterior to the expression of Krox-20 in rhombomeres 3 and 5 in the hindbrain.

By 18 h, expression spreads to the hind brain and spinal cord. Expression can also be seen in a small semicircle of cells in the eyes, in the optic stalk, just as it enters the eye. This group of cells expands to a wider region in 20 h embryos, seen in the entire optic stalk, and in cells in the outer rim of the optic cup, which will give rise to the pigmented retina (Gilbert, 1994). This corresponds to the expression domain of the zebrafish pax[zf-b]/pax-2 (Krauss et al., 1991b). Transcripts of pax[zf-b] are first seen around 12 h of development in the region of the optic stalk, with stronger expression in the anterior portion of the optic stalk. The pax[zf-b] transcripts can also be detected in the lateral parts of the optic vesicle, and by 18 h, expression becomes more homogenous and intense, as the stalk constricts. The Brn1.1 transcript shows a similar expression domain in the optic stalk. Similarly, in the midbrain, Brain 1b and pax[zf-b] are both expressed. However, the anterior boundary of Brn1.1 is much more rostral than that of pax[zf-b] in the mid-brain, extending to the anterior mid-brain. Brn1.1 was not detected in the forebrain. The posterior boundary of both pax[zf-b] and Brn1.1 extends to the junction of the mid-brain and hindbrain, where the engrailed class homeobox genes are expressed (Ekker et al., 1992). By 18 h, expression of Brn1.1 was detected in more posterior regions of the brain, with strong expression in the hindbrain. Expression in the optic stalk, mid brain and hindbrain persists at the 24 h stages as

well. In 42 h pharyngula, staining was very faint in the optic and cerebellar regions. Intense staining was seen in 3 patches surrounding the gill arches. Neural crest cells from the forebrain and midbrain migrate to form the first pharyngeal pouch, which in fishes forms part of the gill apparatus (Gilbert, 1994). The expression of Brn1.1 in neural crest cells that arise from the forebrain and midbrain and migrate to the gills could account for the staining pattern seen in the midbrain in early stages of neurogenesis, and the gill arches at later stages.

In adult zebrafish, the highest levels of expression of the Brn1.1 transcript is seen in the brain, and low levels are detected in the eye and ovary. The transcript was not detected in the gills, liver or testes. High levels of expression in the brain and in the eyes is known for several class III genes, such as the rat Brn1 and Brn2 genes (He et al., 1989), and XLPOU1 and XLPOU2 in Xenopus (Agarwal and Sato, 1991). The neural expression of Brn1.1 is similar to these genes.

However, Brn1.1 is a novel gene in its class since to date, no other maternally expressed class III gene has been described. The Brn1.1 transcript is present at very low levels in 1-2 cell embryos compared to an equivalent amount of RNA from the ovary, so it is possible that Brn1.1 is actually involved in the ontogeny of oocytes in the ovary and the low levels seen in the 1-2 cell stage is residual message. A

detailed analysis of expression in oocytes as they mature in the ovary will need to be performed to determine if Brn1.1 indeed has such a role.

Several maternally expressed POU domain genes described to date belong to class V rather than class III (Okamoto et al., 1990; Rosner et al., 1990; Scholer et al., 1990; Whitfield et al., 1993; Takeda et al., 1994). The murine Oct-3/4 transcript is present in oocytes and early embryos, but is down regulated later in development, restricted to the primordial germ cells by day 10.5. The Xenopus XLPOU-60 transcript is specifically expressed in oocytes, and is inherited maternally by the embryos, where expression declines during gastrulation, but remains detectable throughout the larval stages (Whitfield et al., 1993). The zebrafish pou2 is maternally expressed, and present till the gastrula stage in embryos (Takeda et al., 1994). A C. elegans POU gene ceh-18, which is most similar to the vertebrate class II genes Oct-1 and Oct-2, is expressed in gonadal sheath cells that signal the oocytes, but not in oocytes. Targeted mutations in ceh-18 have shown that it is required for gonad migration, maintenance of diakinesis arrest of oocytes, and epidermal differentiation (Greenstein et al., 1994). It is possible that Brn1.1 also has at least two different roles in embryogenesis: one early in oocyte differentiation and/or maturation, and a second later on in the specification of the

nervous system, in conjunction with other neurally expressed factors.

Expression Pattern of Brn2a

The Brn2a POU domain fragment was isolated by PCR from a 19 h cDNA library. The amino acid sequence of the Brn2a POU domain appears most similar to the mammalian Brn2 genes (He et al., 1989; Hara et al., 1992). The expression pattern of Brn2a as determined by RT-PCR and whole mount in situ hybridization shows that it is also a neurally restricted class III gene. Expression can be detected first during gastrulation, with increasing levels during neurulation. The transcript persists in larval stages, and in the adult, it can be seen expressed strongly in the brain. Some expression can be detected in the adult eyes as well. Whole mount in situ hybridization with the Brn2a fragment shows expression in early blastula stages. Since RT-PCR with primers specific for Brn2a failed to detect the transcript until gastrulation, it is likely that the signal seen in the whole mounts is because of cross-hybridization of the Brn2a probe with the transcripts of other related POU domain genes which could include Brn1.1. In 12 and 18 h embryos, there appears to be ubiquitous expression in the embryo, with the strongest signal seen in the developing neural tissues. It is likely that this pattern is a result of the Brn2a probe binding throughout the embryo

to several different transcripts, which include the authentic Brn2a transcript. All of these cross-hybridizing transcripts would be expected to be similar in sequence to the Brn2a POU domain. The most intense staining seen in the nervous system, particularly in the mid-brain, the hind-brain, and the spinal cord, suggests that these other transcripts are also expressed at high levels in the nervous system. Ubiquitous expression during embryogenesis, with the highest levels in the nervous system, is known for a member of the POU domain family in zebrafish: the POU(c) gene (Johansen et al., 1993), which is a class VI gene. POU(c) is highly divergent in its sequence from all other POU domain genes, and does not appear until 3 h post-fertilization, during embryogenesis. The pou(c) transcript is present throughout development, without any marked tissue-specificity, but with strongest expression in the CNS (Johansen et al., 1993). Another ubiquitously expressed POU domain gene is Oct-1 in mammals, required for lymphoid specific gene expression (Sturm et al., 1988). It is likely that there are several ubiquitously expressed POU domain genes during embryonic development of vertebrates.

A Class IV POU Domain Gene

A POU domain fragment related to the rat and mouse Brn3 genes was isolated from zebrafish genomic DNA using PCR. The expression pattern as determined by RT-PCR with Brn3-specific

primers indicate that the Brn3 transcript of the expected size is expressed late in development, during the hatching stage. In adults, the transcript was detected at low levels in the brain, gills and testes. The eyes showed the strongest expression amongst various adult tissues tested, which correlates well with the expression of the mammalian Brn3 genes in the sensory and nervous systems (Gerrero et al., 1993; Xiang et al., 1993; Turner et al., 1994). A larger transcript was also detected in the adult ovary, 1-2 cells stage embryos, and gastrulating embryos. It is possible that this larger transcript represents another Brn3 gene, or some other transcript whose sequence bears similarities to the primers used in the PCR reactions. There are at least three different Brn3-related genes in the mouse (He et al., 1989; Gerrero et al., 1993; Xiang et al., 1993). It is possible that zebrafish also have several Brn3-related genes, and the primers used in the RT-PCR reactions may be capable of amplifying these related transcripts as well. Whole mount in situ hybridization performed on embryos using the PCR amplified Brn3 fragment as a probe showed ubiquitous expression at all stages tested. Since two transcripts of different sizes were detected by RT-PCR during development, it is possible that the Brn3 probe used in whole mount embryos is also detecting more than one Brn3-related transcript. This possibility is strengthened by the results of southern hybridization with the Brn3 probe. Several cross-hybridizing

bands were detected, suggesting the presence of other POU-domain containing sequences in the genome which bear sequence similarity to the Brn3 clone. A longer cDNA clone for Brn3 would be required to define the precise expression pattern of this gene.

During development, several transcription factors exert their effects on various target genes, leading to the specification of various cell types. The target genes for several POU domain proteins remain largely unidentified, but targets for the mammalian Pit-1, Oct-1 and Oct-2, and the Drosophila drifter/cf1a gene products have been identified. Pit-1, in addition to regulating the expression of growth hormone and prolactin genes in the anterior pituitary, is autoregulatory (reviewed in Wegner et al., 1993). The Drosophila drifter/cf1a protein regulates the expression of the enzyme dopa decarboxylase in dopaminergic neurons (Johnson and Hirsch, 1990). Mutant analyses has revealed that mice with missense or nonsense mutations in the Pit-1 gene lack somatotrophs, lactotrophs, and thyrotrophs in the anterior pituitary (Li et al., 1990). Mice mutant for Pit-1 are dwarf in stature. Mutations in the human Pit-1 gene also lead to reduced stature and pituitary dysfunction (Rhodes et al., 1994). This indicates that functional Pit-1 is required for the differentiation, proliferation, as well as maintenance of these three cell types, in addition to transcriptional activation of the trophic hormones produced in these cell

types. The targets for the zebrafish Brain genes are not known. Identification of these genes, and analyses of their expression patterns could be of paramount value in understanding the function of the Brain genes.

The Brain genes are expressed in spatially and temporally restricted patterns during embryogenesis. The cis- and trans-acting regulatory elements that define the precise expression of the Brain genes are not known. It is possible that these genes are regulated by other transcriptional regulators which could include HOX, PAX and engrailed genes. The concerted expression of several homeobox genes, belonging to the PAX, HOX, engrailed, and POU domain families, in various domains of the developing nervous system of vertebrates suggests that there might be cross-regulatory interactions between the products of these genes. The restricted expression of these genes in various regions could be important for the differentiation of the nervous system.

The presence of several Brain-like genes in the developing nervous system, some of which have overlapping domains of expression also suggests combinatorial interactions between the products of these genes. For instance, different combinations of POU proteins could form homodimers or heterodimers which could positively or negatively regulate the expression of target genes. This kind of regulation is known for the Drosophila I-POU gene, which can inhibit cf1a/drifter

gene function by forming heterodimers that fail to bind DNA (Treacy et al., 1991).

The expression pattern of these Brain genes during zebrafish embryogenesis suggests that they play roles in specifying various developmental fates. The known functions of POU domain genes in other organisms suggests this as well. However, direct evidence of the functions of these genes is lacking at this point. Recently, large scale mutagenesis to identify genes affecting development in zebrafish has been performed by several laboratories (Mullins et al., 1994; Solnica-Krezel et al., 1994). These mutants are being used to generate a genetic map of zebrafish (John Postlethwaithe, pers.commun.). Also a part of this effort is to place cloned genes on the map. The position of the zebrafish Brn1, Brn1.1, Brn2a, and Brn3 genes, relative to known mutations on the map, would give a starting point to analyze the functions of these genes.

In conclusion, four distinct POU domain-containing genes were isolated from zebrafish cDNA or genomic DNA. The expression patterns of these genes shows that these genes are largely expressed in the nervous system in developing and adult zebrafish. These genes are likely to be involved in early patterning during neurogenesis, and possibly have some specific functions in the adult brain and eyes.

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