Homebox genes are expressed in the retina and brain of adult goldfish

(visual pathway/homebox sequence comparison/polymerase chain reaction)

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Communicated by John E. Dowling, January 4, 1993

ABSTRACT The goldfish (Carassius auratus) visual pathway displays continuous growth and plasticity throughout life. Since homeobox genes are important transcriptional regulators in development, we searched for homeobox genes in the adult goldfish retina and brain. Using the PCR, we discovered a repertoire of homeobox sequences expressed in these tissues. In addition to isolating homeobox sequences found in the vertebrate Hox gene clusters, a sequence identical to the chicken CHox7 homeodomain was characterized. Furthermore, a sequence with significant homologies to the Xenopus XHbox8 and leech Htr-A2 homeodomains was identified, and these sequences may define an additional class of homeodomain. Finally, a sequence belonging to the paired class (prd) of homeodomains is reported. Homeobox gene expression in the adult goldfish retina and brain may be associated with the persistent developmental features of these tissues.

The goldfish visual pathway is embryonic in nature, displaying continuous neuronal differentiation, axonal growth, and synaptic plasticity throughout life (1, 2). Cells within the retina differentiate from a limited number of precursor cell populations in a spatially restricted manner. In the outer nuclear layer, rod precursor cells divide and differentiate to form mature rods (3, 4). At the peripheral margin of the retina, primitive neuroepithelial cells divide and differentiate to form retinal ganglion cells (2). The growing retinal ganglion cell axons target tectal cells whose numbers also continuously increase. The growth pattern of retinal ganglion cells is symmetric, whereas the growth pattern of tectal cells is asymmetric. To maintain the retinotopic map, a persistent shifting of synaptic terminals between retinal ganglion cell axons and tectal cells results in continued synaptic plasticity (5). Furthermore, the goldfish visual pathway is regenerative. Following optic nerve injury, retinal ganglion cell axons regenerate and grow through the optic nerve and make appropriate functional synaptic connections (6). Injury to the retina also results in an ordered regeneration of the retinal cell types from the rod precursor cells (7, 8). These developmental and regenerative properties are not observed in the adult visual pathways of higher vertebrates. Thus, the goldfish visual pathway has emerged as an important model system to study molecular events associated with axonal growth and neurogenesis (9–12).

Neuronal development is a coordinately regulated process. Cellular differentiation, axonal outgrowth, growth termination, and eventual synapse formation and maturation can be organized into growth stages along a time course of regulated molecular events. These stages can be biochemically characterized in terms of a differential expression of structural as well as regulatory macromolecules that meet the physiological requirements of the developing neurons at each stage of growth.

Homeodomain proteins are important regulators of embryogenesis and development. The homeodomain encodes a helix–turn–helix DNA binding domain (reviewed in ref. 13) ～60 amino acids long. It was initially discovered as a conserved DNA sequence, termed the homeobox, in genes controlling segmentation and segment identity during Drosophila development (14, 15). Subsequently, homeobox genes were identified in genomes from yeast to man (ref. 16 and references therein). The expression of homeobox genes is linked to pattern formation, regional specification, and cellular differentiation during embryogenesis (reviewed in refs. 17 and 18), in limb regeneration in urodèle amphibians (19, 20) and in continuously developing adult mammalian tissues (21–23).

Given their importance as developmental regulators, we searched for homeobox genes in the adult goldfish visual system as a first step to determine if their expression can be linked to the developmental properties of this system. We used the PCR to survey the RNA populations of the adult goldfish retina and brain for homeobox gene expression. Homeodomain-specific PCR primers were designed from two highly conserved amino acid sequences within the homeodomain so that a large proportion of the homeobox gene family could be targeted for amplification. This approach was recently described as a powerful technique to detect homeobox gene expression in developing tissues and to trace homeobox genes through evolution (24, 25). Finally, we performed Northern hybridizations to assess the comparative expression levels of the identified homeobox sequences in the retina and brain RNA populations.

MATERIALS AND METHODS

Tissue and RNA Isolation. Common goldfish (Carassius auratus, 8–12 cm) were anesthetized in 0.05% tricaine methane sulfonate and placed in ice water prior to sacrifice. Brain tissue was removed and immediately frozen in liquid nitrogen. Retinal RNA was isolated and washed in 1× phosphate-buffered saline and immediately frozen in liquid nitrogen.

Total cellular RNA from brain and retina was isolated with RNA Sol B according to the manufacturer’s instructions (Bio-tec Laboratories, Houston) with the following modifications. After lysis, samples were spun at 6000 × g for 10 min at 4°C. In addition, prior to poly(A)* RNA isolation, total

Abbreviation: Antp, Antennapedia.

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†The sequences reported in this paper have been deposited in the GenBank data base [accession nos. L09685 (G1-1), L09686 (G1-2), L09687 (G2-1), L09688 (G3-1), L09689 (G3-2), L09690 (G5-1), L09691 (G5-2), L09692 (G6-1), L09693 (G7-1), L09694 (G8-1), L09695 (G9-1), L09696 (G10-1), L09697 (G11-4), and L09698 (G4-1)].
retina RNA was precipitated in 2 M LiCl overnight at 4°C and subsequently precipitated again with 0.3 M sodium acetate and 2.5 volumes of 100% EtOH. We found this step necessary to remove vitreous carryover that clogged oligo(dT)-cellulose columns. Poly(A)⁺ RNA was obtained from total RNA by two passages over a gravity column or spun column of oligo(dT)-cellulose type 7 (Pharmacia).

Homeobox Primer Design. To target a large proportion of the homeobox gene family by PCR, we designed a set of homeobox-specific primers that correspond to the most conserved peptide sequences in a compilation of homeodomain sequences (ref. 16; Fig. 1). The oligonucleotide primers have nucleotide degeneracies in specific positions to compensate for the wobble in the genetic code. Primer ANTP-L contains the nucleotide sequences corresponding to amino acids 18–20 of the Drosophila Antennapedia gene (Antp) homeodomain (refs. 14 and 15; Fig. 1). Primer HBI-R contains the complement of the nucleotide sequences corresponding to amino acids 38–53 of the Antp homeodomain (Fig. 1). Restriction endonuclease sites were engineered into the 5′ ends of the primers to facilitate cloning of the PCR products and do not represent homeobox sequence.

Detection of Homeobox Sequences from RNA by PCR. Reverse transcription. To make a first strand cDNA pool from RNA, 1–10 μg total RNA, 250 ng of oligo(dT)12–22, 0.5 mM each dNTP, 20 units of RNAsin (Promega), and 4 μl of 5× reverse transcription buffer (BRL, supplied with enzyme) in a total volume of 19 μl were heated at 70°C for 2 min, and immediately placed on ice. Two hundred units of Moloney murine leukemia virus reverse transcriptase (BRL) was added and the mixture was incubated at 37°C for 1 hr.

PCR amplification. One to 10 μl of the cDNA pool was added to 1.4 μg each of ANTP-L and HBI-R amplification primers, 200 μM each dNTP, 1.25 mM MgCl₂, 50 mM KCl, 10 mM Tris (pH 8.4), and 2.5 units of Taq DNA polymerase (Stratagene) in a total volume of 100 μl and overlaid with light mineral oil. The reaction was carried out in a DNA thermal cycler for 40 cycles with the following profile: 95°C, 1 min; 55°C, 1 min; 72°C, 3 min.

Cloning and Sequencing of cDNAs. The PCR was extracted with equal volumes of chloroform, phenol/chloroform, and chloroform again. The PCR cDNA products were resolved on 10% native polyacrylamide gel by electrophoresis (26). The band of expected size (129 bp) was gel purified and digested with Xba I and Sal I restriction endonucleases. The restriction endonucleases were removed from the cDNA by extraction with phenol/chloroform and chloroform and ethanol precipitation. The 129-bp cDNAs were subcloned into the pBluescript vector (pBS, KS polylinker; Stratagene) according to standard procedures (26). Individual clones were sequenced using the Sequenase enzyme according to the manufacturer’s instructions (United States Biochemical).

Sequence Analysis. The amino acid sequences of the PCR clones were deduced from their nucleotide sequences and were compared to a compilation of homeodomain sequences (16) and to the EMBL and GenBank data bases with the Genetics Computer Group program (27). Since the size of the homeodomain and the positions of the primer sequences in the homeodomain are conserved through evolution, the alignment of the goldfish sequences with other homeodomain sequences corresponds to amino acid positions 21–47 of the homeodomain (Fig. 1).

Northern Hybridization Analysis. Aliquots (2.5 μg) of poly(A)⁺ RNA from adult goldfish retina and brain were resolved on 1.3% agarose/2.2 M formaldehyde gels by electrophoresis (26). The RNAs were transferred by capillary blot onto Nytran (Schleicher & Schuell) according to the manufacturer’s instructions. The blots were prehybridized for 1 hr and hybridized overnight at 42°C in 1× PE [50 mM Tris·HCl (pH 7.5), 0.1% sodium pyrophosphate, 1% SDS, 1× Denhardt’s reagent, and 1 mM EDTA], 5× SSPE (26), 50% formamide, 0.1 mg of calf thymus DNA per ml, and 10 μg of yeast tRNA per ml. [32P]UTP-labeled antisense RNA probes (2 × 10⁶ cpm) were added to the hybridization mixtures. Templates for probe generation were the homeobox PCR clones in the pBS vector representing each of the homeobox groups (see Results and Discussion). The plasmids were prepared by restriction endonuclease digestion with Xba I. High specific activity antisense RNA probes were prepared by run-off transcription with the phage T3 promoter in the pBS vector and T3 RNA polymerase. After hybridization, the blots were washed at high stringency (final wash; ≥73°C in 0.1× SSPE/0.1% SDS). Filters were exposed to autoradiography using Kodak XAR-5 film with an intensifying screen at −80°C.

RESULTS AND DISCUSSION

Identification of Homeobox Sequences by PCR. To identify homeobox sequences in the adult retina and brain RNA populations, we utilized an RNA-based PCR amplification protocol similar to the approach described by Murtha et al. (25). A first strand cDNA pool was synthesized from total RNA by reverse transcription using Moloney murine leukemia virus reverse transcriptase and oligo(dT) primer. Homeobox sequences were amplified from the cDNA pool by PCR with homeobox-specific primers that correspond to two highly conserved peptide sequences within the homeodomain (Fig. 1). The expected band was 129 bp in length since the distance between primers (82 bp) is conserved throughout the homeobox gene family. The band was cloned into the pBS vector. Thirty-six independent clones were randomly selected and sequenced from two PCR experiments using retina RNA and 16 independent clones were randomly selected and sequenced from one PCR experiment using brain RNA. A multitude of nucleotide sequences was obtained from both tissues (discussed below).

The amino acid sequences of the PCR clones were deduced from the nucleotide sequences. The clones were then organized into groups on the basis of their amino acid sequences (Fig. 2). Clones within a group had the identical amino acid sequence in the predicted homeobox reading frame. Eleven

![Fig. 1.](image-url) The sequence of the Drosophila Antp homeodomain is depicted with a structural schematic. The homeobox-specific PCR primers ANTP-L and HBI-R are aligned with the peptide sequences they represent. The portion of the primers that correspond to the homeobox sequence are bracketed and degeneracies are shown in a vertical orientation; 5′ flanking sequences contain restriction endonuclease sites (underlined) to facilitate cloning of the PCR products. Primer HBI-R is the complement of the corresponding peptide sequence. All published sequences that contain the ANTP-L amino acid sequence also contain the HBI-R amino acid sequence.
Fig. 2. Thirty-six independent retina PCR clones were organized into 10 groups on the basis of their derived amino acid sequences (G1–G10). Sixteen independent PCR brain clones were organized into 5 groups (G1, G4, G6, G9, and G11). Each group is compared to the sequence of the Antp homeodomain in the region between the ANTP-L and HB1-R oligonucleotide primers (Fig. 1). The numbers above the sequences are oriented with position 1 of the Antp homeodomain and the goldfish amino acid sequences are numbered according to this convention. Dashes indicate the identical amino acid as in the Antp protein at that position. The number of clones identified for each group in each tissue is listed under INCIDENCE. Published sequences with the closest amino acid homology to each group are listed with the source of the gene (ZP-21, ref. 28; Ghox2.2 ref. 29; Xhox1b ref. 30; Hox-1.6, ref. 31; Hox-2H and HOX-2G, ref. 32; XHbox8, ref. 33; HOX-4D, ref. 34; HOX-1H, ref. 35; CHox7, ref. 36; ceb-10, ref. 37). Superscript a indicates that other published sequences exist that have the same % amino acid homology to the group clone as the group listed but, for simplicity, only one is shown.

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Error. Nucleotide variation is most notable in the G3 group (Fig. 3). The nucleotide sequences of G3-1 and G3-2 are different in 22 of 82 positions. Nucleotide variation was also observed in the G1, G4, G5, G9, and G11 groups. In all cases, when these sequences are translated, the deduced amino acid sequences are identical among the sequences in each group. However, two independent PCR clones have sequences that differ by 1 nt from other amplified sequences, and the changes resulted in single amino acid substitutions in the homeobox reading frames. These clones were grouped with the clones to which they were most similar (G1 and G7; data not shown).

To identify the types of homeodomain sequences that we isolated from the adult goldfish retina and brain, the amino acid sequences of the PCR clones were compared to a compilation of homeodomain sequences (16) and to the EMBL and GenBank data bases using the Genetics Computer Group program (27). The homeobox genes with the highest homologies to the goldfish sequences are listed in Fig. 2. The G1–G5, G7, G8, and G11 sequences are 96–100% homologous to the corresponding portions of homeodomain sequences in genes from the vertebrate Hox clusters (see references in the legend to Fig. 2).

![Fig. 3. The nucleotide sequences for the PCR clones in each group are shown. The sequences span the amplified region between the two primers. The nucleotides at position 2 correspond to the first codon position of the first amino acid for each group in Fig. 2. Dashes signify the same nucleotide at that position as that of the first sequence for each group. Nucleotide differences between sequences within a group do not change the amino acid at those positions. Individual sequences are identified by the name of the assigned group (Fig. 2) followed by a dash and number. Asterisks indicate which sequences were used as probes for the Northern hybridization experiments.](image-url)
The G6 sequence is ~85% homologous to the corresponding region of the Xenopus \textit{XHbox} 8 homeodomain (ref. 33; Fig. 4A). In addition to the high sequence similarity with \textit{XHbox} 8, the G6 sequence encodes a histidine at position 44 of the homeodomain. This histidine lies within the DNA recognition helix of the homeodomain and may confer unique DNA sequence recognition properties to \textit{XHbox} 8 (33). The leech gene \textit{Htr-A2} (ref. 38; Fig. 4A) is the only other homeobox gene found thus far to contain a histidine at position 44. These genes may define an additional class of homeodomains.

The G9 sequence has 100% homology to the corresponding region of the chicken \textit{CHox7} homeodomain (ref. 36; Fig. 2). The \textit{CHox7} homeodomain is considerably divergent from other homeodomain sequences, but may be a distant relative of the \textit{msh} type homeodomains (ref. 43 and references therein). Interestingly, the murine \textit{Msh} genes, \textit{Hox} 7.1 (44, 45) and \textit{Hox} 8.1 (46), are expressed in cells of the mouse embryo that give rise to eye structures (46). Although very little is known about \textit{CHox7} expression, identical sequences were isolated from 13.5-day mouse telencephalon cDNA by PCR (25), suggesting a role in vertebrate forebrain development.

The G10 amino acid sequence is the most divergent of all of the goldfish sequences identified. The best match for the G10 sequence was to the nematode, \textit{C. elegans}, \textit{ceh-10} homeodomain (37) at 74% homology (Fig. 4B). Among vertebrate homeobox genes, the highest homology for the G10 sequence is 67% with the homeodomains of the murine \textit{Pax-6} (39), zebrafish \textit{Pax-6} (40), and zebrafish \textit{Pax[zf-a]} (41, 42) genes (Fig. 4B; zebrafish \textit{Pax-6} and \textit{Pax[zf-a]} cDNAs are identical). \textit{ceh-10} and the \textit{Pax-6} genes contain \textit{prd}-type homeodomains (ref. 16 and references therein). Therefore, we propose that the G10 sequence encodes part of a vertebrate homeobox gene with a \textit{prd}-class homeodomain. The gene encoding the G10 homeodomain may also harbor a paired domain (47), making this gene a member of the vertebrate \textit{Pax} gene family (48).

\textit{Pax} gene expression has been correlated with vertebrate eye development. For example, the zebrafish and murine \textit{Pax-6} genes (\textit{Pax[zf-a]}) are expressed in a portion of the neural tube that will give rise to the eye (39-42). Furthermore, in hatched zebrafish larvae, \textit{Pax-6} is expressed in the retinal ganglion cell layer and in the bipolar cell layer of the retina (40, 42). However, the G10 encoding gene is not likely to be a goldfish homologue of the \textit{Pax-6} genes for the following reasons. The amino acid sequences of the zebrafish and murine \textit{Pax-6} genes are 100% homologous in the homeodomain and 97% homologous throughout the entire protein (40). Therefore, there may be a strong selective pressure during vertebrate evolution to conserve the primary structure of the \textit{Pax-6} protein. Given the evolutionary relatedness of goldfish and zebrafish, it is unlikely that the G10 sequence is part of a \textit{Pax-6} homologue, especially when the amino acid sequence similarity between the G10 sequence and the \textit{Pax-6} homeodomain (67%) is considered. Similarly, the evolutionary distance between the nematodes and fish and the high sequence homology (74%) between the G10 sequence and \textit{ceh-10} suggests that the G10 sequence may be part of a homeodomain that is a vertebrate homologue of \textit{ceh-10}.

**Steady-State Levels of Homeobox Transcripts.** We characterized the expression patterns of the homeobox clones by Northern hybridization analysis. Radiolabeled antisense riboprobes for each of the clones marked by asterisks in Fig. 3 were hybridized to 2.5 \mu g of poly(A)\(^{+}\) RNA from the adult goldfish retina and brain. Probes from the G2, G4, G6, G7, and G8 groups did not detect transcripts in either tissue (data not shown). Reverse transcription and PCR controls exclude the possibility of amplification from a genomic DNA contaminant (data not shown). Therefore, these transcripts were detected by PCR because of its extreme sensitivity. However, they are expressed below the levels of detection by Northern hybridization.

Positive hybridizations were obtained with probes from the G1, G3, G5, G9, G10, and G11 groups (Fig. 5). The G1 probes (G1-1 and G1-2) detected transcripts of 2 kb in the brain. Similarly, the G3 probes (G3-1 and G3-2) hybridized to transcripts of ~2 kb in the brain. Only the hybridization for the G3-1 probe is shown because the G3-2 hybridization was barely detectable. The G5 probes (G5-1 and G5-2) gave different patterns of expression when compared to each other. The G5-1 probe hybridized to a rare transcript at 1.25 kb that was detected only in the brain (not shown). The G5-2 probe hybridized to multiple transcripts in the retina and brain. A 1.4-kb transcript was detected in both tissues and was more abundant in the brain. A 3.5-kb transcript was detected exclusively in the brain, and a 4.4-kb band was detected in both tissues at equal intensity. The G9-1 probe detected a 2.1-kb transcript in the retina and brain. The G10-1 probe detected a major transcript at 1.6 kb and a minor transcript at 2.7 kb in the retina, and the G11-4 probe detected a 2.5-kb transcript in the brain.

The adult vertebrate retina and brain contain a diversity of cell types. The goldfish retina and brain are no exception, but in addition, there are cell populations at various stages of differentiation. The low level of expression observed for most of the goldfish homeobox transcripts (G1–G8) in the retina and brain raises the possibility that these transcripts are restricted to a subset of cells at a specific stage of differentiation or to small populations of differentiated cells. This type of expression is expected for a gene that specifies cell fate and/or maintains the differentiated state of specific cells.
These roles were proposed for the homeodomain LIM protein, Isl-1, which is expressed in specific subsets of cells in the adult rat central nervous system and endocrine system (49). Interestingly, Isl-1 is expressed in the inner nuclear layer and in a subset of retinal ganglion cells of the adult rat retina (49).

We also detected expression of homeobox genes that were relatively abundant in the retina and brain. For example, the G9-1 probe detected transcripts of similar abundance in both tissues. In contrast, the G10-1 probe detected transcripts that were specific to the retina and the G11-4 probe detected a transcript that was specific to the brain. The G10 and G11 encoding genes may be expressed in cell populations that are abundant, yet specific to their respective tissues. The retina and brain are neural tissues that develop in a layered array with the cells of each layer performing unique and specific functions. The abundant G10 and G11 transcripts may be expressed in a layer-specific manner. This is consistent with a gene product whose role is to provide positional cues for cells in constantly developing tissues such as the adult goldfish retina and brain. These proposed functions for the goldfish homebox genes have been demonstrated for the homeobox gene family during embryogenesis in other species but have yet to be shown for adult vertebrate central nervous system tissues. The expression of the genes encoding these homeobox sequences in the adult goldfish retina and brain may be associated with the retained and persistent developmental characteristics of these tissues in the goldfish or may be a general feature of the adult vertebrate retina and brain.

We thank Drs. Andrew Francis, Eric Glasgow, and Robert Druger for reading this manuscript and for helpful discussions. This work was supported by a grant from the National Institutes of Health (EY 05212) to N.S.