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Original Article

Retinal homeobox genes and the role of cell proliferation in cavefish eye degeneration

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ABSTRACT The teleost *Astyanax mexicanus* exhibits eyed surface dwelling (surface fish) and blind cave dwelling (cavefish) forms. Despite lacking functional eyes as adults, cavefish embryos form eye primordia, which later arrest in development, degenerate and sink into the orbit. We are comparing the expression patterns of various eye regulatory genes during surface fish and cavefish development to determine the cause of eye degeneration. Here we examine *Rx* and *Chx/Vsx* family homeobox genes, which have a major role in cell proliferation in the vertebrate retina. We isolated and sequenced a full-length *Rx* cDNA clone (*As-Rx1*) and part of a *Chx/Vsx* (*As-Vsx2*) gene, which appear to be most closely related to the zebrafish *Rx1* and *Alx/Vsx2* genes respectively. *In situ* hybridization shows that these genes have similar but non-identical expression patterns during *Astyanax* eye development. Expression is first detected in the optic vesicle, then throughout the presumptive retina of the optic cup, and finally in the ciliary marginal zone (CMZ), the region of the growing retina where most new retinoblasts are formed. In addition, *As-Rx1* is expressed in the outer nuclear layer (ONL) of the retina, which contains the photoreceptor cells, and *As-Vsx2* is expressed in the inner nuclear layer, probably in the bipolar cells. With the exception of reduced *As-Rx-1* expression in the ONL, the *As-Rx1* and *As-Vsx2* expression patterns were unchanged in the developing retina of two different cavefish populations, suggesting that cell proliferation is not inhibited. These results were confirmed by using PCNA and BrdU markers for retinal cell division. We conclude that the CMZ is active in cell proliferation long after eye growth is diminished and is therefore not the major cause of eye degeneration.

KEY WORDS: *Rx1 homeobox gene, Vsx2 homeobox gene, cavefish, eye degeneration, cell proliferation*

Introduction

During the last decade, regulatory genes have been identified encoding transcription factors and signaling molecules that coordinate eye development and evolution (Tomarev, 1997; Gehring and Ikeo, 1999; Lupo *et al.,* 2000; Mathers and Jamrich, 2000). These genes are structurally and functionally conserved across wide phylogenetic distances and can be responsible in mutant form for degenerative human eye diseases (Jordan *et al.,* 1992; Hanson *et al.,* 1994: Burmeister *et al.,* 1996; Percen *et al.,* 2000). Despite progress in understanding the molecular basis of eye development, little is known about the control of eye degeneration, particularly in animals that have lost their vision during adaptation to specialized habitats (Nevo, 1999; Jeffery, 2001).

We study the regulation of eye degeneration in the cavefish *Astyanax mexicanus* as a model system in evolutionary developmental biology (Jeffery, 2001). This species consists of two forms: an eyed surface dwelling form (surface fish) and an eyeless and depigmented cave dwelling form (cavefish). *Astyanax* cavefish populations are present in at least 29 different caves in northeastern Mexico (Mitchell *et al.,* 1977). Some of these cavefish populations may be genetically unique and have lost their eyes and pigmentation independently (Dowling *et al.,* 2002). Morphological studies indicate that cavefish embryos develop a small optic

*Abbreviations used in this paper: Alx/*Alx, Aristaless-like homeobox gene and encoded protein; *As-Rx1/*As-Rx-1, *Astyanax* retinal homeobox gene 1 and its encoded protein; *As-Vsx2/*Vsx2, *Astyanax* visual system homeobox gene 2 and its encoded protein; bp, base pair; cDNA, DNA complementary to RNA; BrdU, bromodeoxyuridine; *Chx/*Chx, *C. elegans*-like homeobox genes and their encoded proteins; kb, kilobases or 100 bases; CMZ, ciliary marginal zone of the retina; INL, inner nuclear layer of the retina; NJ, neighbor joining method; ONL, outer nuclear layer of the retina; pf, post-fertilization; OP domain, octapeptide domain; OAR domain, Opt, aristaless and rax domain; ORF, open reading frame; PCNA, Proliferating Cell Nuclear Antigen; *Prox1*, prospero-like homeobox gene 1; RT-PCR, reverse transcription mediated PCR; RPE, retinal pigment epithelium; *Rx/*Rx, retinal homeobox genes and their encoded proteins; SSC, 0.1M NaCl/0.015 M sodium citrate, pH 7.0; SDS, sodium dodecyl sulfate; UTR, untranslated region.

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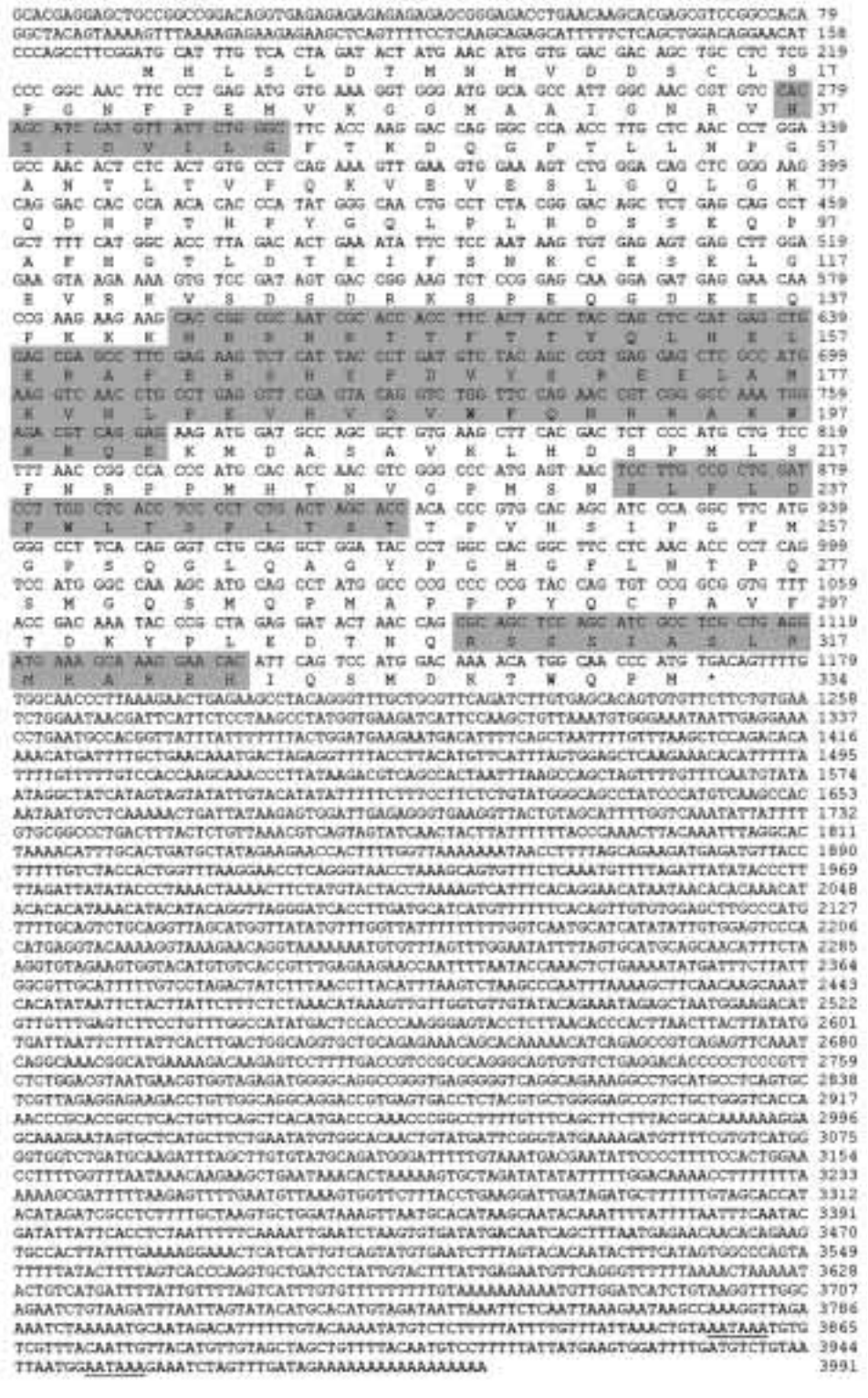

Fig. 1. The nucleotide and deduced amino acid sequences of the *As-Rx1* cDNA. *The nucleotide and amino acid positions are numbered on the right. The N-terminal OP domain, the central* paired *class homeodomain, the trailing Rx domain, and the C-terminal OAR domain are shaded in respective order from 5′ to 3′ in the sequence. The poly (A) addition sites in the 3′UTR are underlined.*

primordium containing a lens vesicle and an optic cup (Cahn, 1958; Wilkens, 1988). The cavefish eye primordium begins to grow and differentiate but later arrests in development, degenerates, and sinks into the orbit. The first sign of eye degeneration is programmed cell death in the lens (Jeffery and Martasian, 1998). The lens appears to play a central role in eye development because transplantation of a surface fish lens into a cavefish optic cup prevents optic degeneration, restoring an eye to adult cavefish (Yamamoto and Jeffery, 2000).

Genetic crosses suggest that multiple genes are responsible for cavefish eye degeneration (Wilkens, 1988). Thus far, however, most genes examined in cavefish embryos exhibit the same expression patterns as surface fish. The opsin gene is expressed early during cavefish retinal development, but its expression disappears as degeneration begins (Langecker *et al.,* 1993). Despite concurrent programmed cell death in the lens, beta and gamma crystallin genes are expressed during eye development in the Pachón cavefish population (Jeffery *et al.,* 2000; A. G. Strickler, unpublished results). In contrast, expression of an alphaA crystallin gene has not been detected in another cavefish population (Behrens *et al.,* 1998). The *Prox1* gene, which is important in lens and retina development (Tomarev, 1997; Wigle *et al.,* 1999), is also expressed normally in the developing cavefish lens and retina (Jeffery *et al.,* 2000). At present, *Pax6* is the only regulatory gene whose expression pattern is known to change during cavefish eye development (Strickler *et al.,* 2001). In surface fish, *Pax6* is expressed in two bilateral domains in the region of anterior neural epithelium corresponding to the presumptive optic vesicles. In cavefish embryos, however, the *Pax6* expression domains are reduced in size and withdrawn from the midline, suggesting a role for midline signaling in cavefish eye degeneration (Strickler *et al.,* 2001). The lack of major changes in the expression of eye regulatory genes may be due to additional functions in the development of non-optic tissues (Tomarev,1997).

Regulatory genes with expression patterns mainly in the optic primordia have not been investigated in cavefish. Here we describe the isolation and expression patterns of the *Rx1* and *Alx*/*Vsx2* retinal homeobox genes in surface fish and cavefish embryos. These retinal homeobox genes encode *paired* class homeodomain proteins, which function in the developing retina (Liu *et al.,* 1994; Mathers *et al.,* 1997; Mathers and Jamrich, 2000; Zhang *et al.,* 2000; Chuang and Raymond, 2001) and are excellent markers for retinal cell prolifera-

tion. We show here that the expression pattern of these genes in the ciliary marginal zone (CMZ), the region of the retina where new retinoblasts are formed, is unaltered in two different cavefish populations. The expression of *Rx1* and *Vsx2* and labeling of the CMZ with the cell division markers PCNA and BrdU strongly suggest that inhibition of retinal cell proliferation is the not the primary cause of eye degeneration during cavefish development.

Results

Isolation of Astyanax Rx1

To identify an *Astyanax Rx* gene, we used degenerate primers to amplify a 471 bp DNA fragment by RT PCR. The DNA sequence encoded part of a *paired* class homeobox typical of the *Rx* genes (Bürglin, 1994; Gehring *et al.,* 1994). The DNA fragment was used to screen a surface fish cDNA library. A single 3.9 kb cDNA clone *(As-Rx1)* was isolated and sequenced (Fig. 1). The *As-Rx1* cDNA contains a 999 bp ORF flanked by a 170 bp 5' UTR and a long 2822 bp 3' UTR, which is terminated by a 17 bp poly (A) tract (Fig. 1). Two putative poly (A) addition signals (AATAAA) are present immediately upstream of the poly (A) tract. The deduced As-Rx1 protein contains an N-terminal octapeptide (OP) (Noll, 1993), a *paired* class homeodomain (Bopp *et al.,* 1986), an Rx domain, and a C-terminal *paired* tail or OAR domain (Furukawa *et al.,* 1997), indicating that it encodes an *Rx* homeobox gene.

The As-Rx1 amino acid sequence was aligned with other Rx proteins (Fig. 2). Three *Rx* genes, *Rx-1*, *Rx-2*, and *Rx-3*, have been reported in zebrafish (Mathers *et al.,* 1997; Chuang *et al.,* 1999). The *Astyanax* Rx1 homeodomain is identical to those of the zebrafish, *Xenopu*s, and chicken Rx1 and Rx2 proteins. Sequence conservation in the OP, Rx, and OAR domains suggests that As-Rx is most closely related to zebrafish Rx1 and Rx2. The complete amino acid sequence of As-Rx1 is 79% similar to zebrafish Rx-1, 69% similar to Rx-2, and 46% similar to Rx-3. A phylogenetic tree was constructed using the Rx protein sequences (Fig. 3). The results showed that As-Rx1 clusters with the zebrafish and chicken Rx1 and is more distantly related to the Rx2 and Rx3 proteins. We conclude that As-Rx1 is likely to be encoded by an *Astyanax Rx1* gene.

Isolation of Astynanx Vsx2

We used the same strategy to identify an *Astyanax Chx/Vsx* gene. A 319 bp DNA fragment (As-Vsx2) was amplified by RT PCR and sequenced. The sequence indicated that As-Vsx2 encoded part of a *paired* class homeodomain (Bürglin, 1994; Gehring *et al.,* 1994) and a downstream CVC domain (data not shown), diagnostic features of the Chx/Vsx family of homeodomain proteins (Svendsen and McGhee, 1995). Despite extensive screening with the *As-Vsx2* we were unable to obtain a corresponding cDNA clone. The deduced As-Vsx2 protein sequence was compared to the corresponding regions of other Chx/Vsx proteins in the database. The As-Vsx2 sequence is identical to the homeodomain and CVC domains of goldfish Vsx2 and mouse Chx10, and is very similar to these regions in zebrafish Alx/Vsx2, medaka Vsx2, and chick Chx10. Phylogenetic trees constructed using nucleotide (Fig, 4A) and amino acid (Fig. 4B) sequences

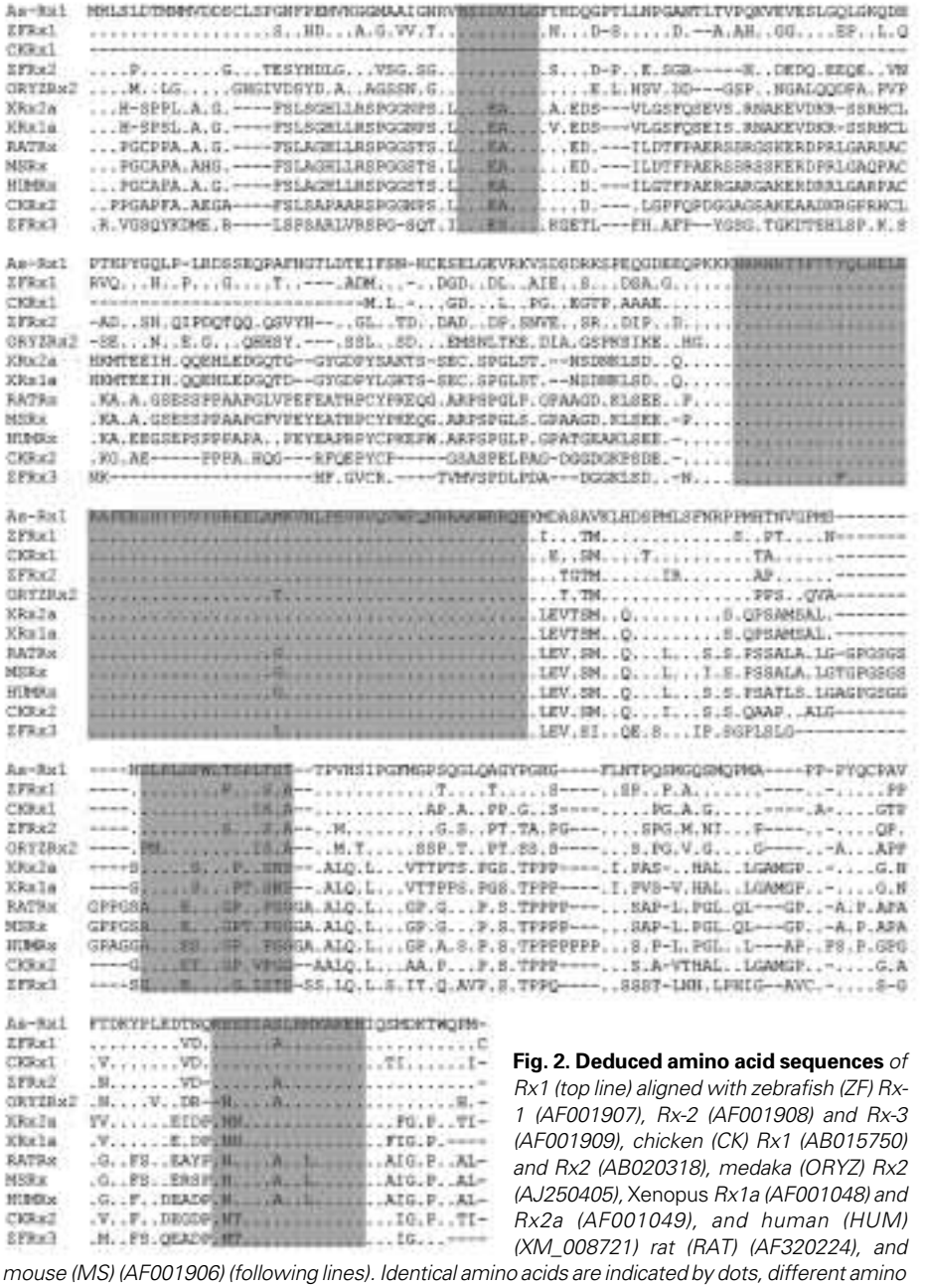

Fig. 2. Deduced amino acid sequences *of Rx1 (top line) aligned with zebrafish (ZF) Rx-1 (AF001907), Rx-2 (AF001908) and Rx-3 (AF001909), chicken (CK) Rx1 (AB015750) and Rx2 (AB020318), medaka (ORYZ) Rx2 (AJ250405),* Xenopus *Rx1a (AF001048) and Rx2a (AF001049), and human (HUM) (XM_008721) rat (RAT) (AF320224), and mouse (MS) (AF001906) (following lines). Identical amino acids are indicated by dots, different amino acids are indicated by letters, and gaps or unidentified regions (e.g. CKRx-1) by hyphens or broken lines respectively. The shaded regions are as described in Fig. 1.*

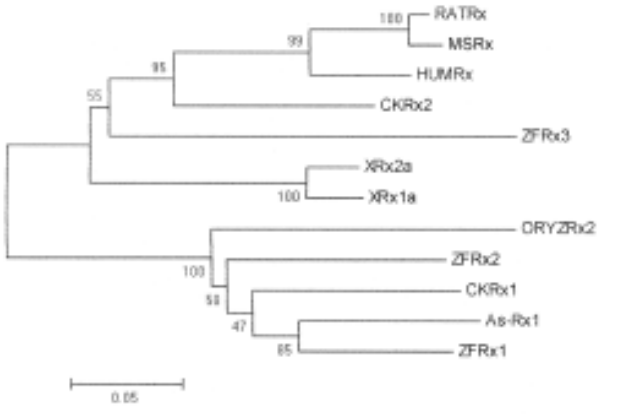

Fig. 3. A phylogenetic tree of Rx proteins constructed by the NJ method. *The length of branches is proportional to the phylogenetic distance. The accession numbers of the sequences are indicated in Fig. 2. The scale bar represents an evolutionary distance of 0.05 amino acid substitutions. The numbers at the nodes indicate the percentages of 1000 bootstrap replicates in which the same internal branch was recovered. Other details are the same as in Fig. 2.*

showed *As-Vsx2* clustered with goldfish *Vsx2*, zebrafish Alx/Vsx2, and mouse *Chx10* (Fig. 5). Thus, we conclude that *As-Vsx2* is likely to represent part of an *Astyanax Vsx2* gene.

Rx1 *Expression during Surface Fish and Pachón Cavefish Development*

Eye primordia are first apparent in *Astyanax* surface fish and cavefish embryos when the optic vesicles protrude laterally from the diencephalon at about the 5-somite stage. Later, the optic vesicles invaginate to form the bilayered optic cup, and the overlying surface ectoderm buds to form the lens vesicles. After hatching, the lens vesicle and optic cup increase in size and differentiate in concert to form the crystalline lens and neural retina/retinal pigment epithelium respectively. The retina grows primarily from the CMZ. As new retinoblasts are produced, they move from the CMZ toward the central region of the retina and differentiate into the neuronal and glial cell layers. In Pachón cavefish embryos, the lens and retina begin to develop but then arrest in growth and start degenerating by about 3 days pf (Wilkens, 1988; Langecker *et al.,* 1993; Yamamoto and Jeffery, 2000). Programmed cell death, which is the first sign of optic arrest and degeneration, is first detected in the lens at about 36 hpf (Jeffery and Martasian, 1998).

To determine whether changes in *Rx1* expression are involved in eye degeneration, we compared surface and Pachón cavefish embryos by whole mount *in situ* hybridization (Fig. 5). In zebrafish and other vertebrate embryos, *Rx1* transcripts are first detected in the neuroepithelium at the neurula stage (Mathers *et al.,* 1997; Casarosa *et al.,* 1997; Chuang *et al.,* 1999). Later in development, expression becomes restricted mainly to the optic vesicles and then to the presumptive retinal region of the optic cup. We were unable to detect *Rx1* mRNA accumulation in *Astyanax* neurula (data not shown) or tailbud stage embryos (Fig. 5 A,D), although other transcripts are easily detected at this stage of development (Strickler *et al.,* 2001). Instead, *Rx1* mRNA was first observed in the evaginating optic vesicles between the 5 and 10 somite stages in both surface fish (Fig. 5 B,C) and cavefish (Fig. 5 E,F) embryos.

At 24 hpf, *Rx1* transcripts were expressed throughout the optic cup but not in the lens vesicle, as shown in sections of whole mount *in situ* hybridized specimens (Fig. 5 G-I, H-K). Sections of later stage surface fish and cavefish embryos showed that *Rx1* expression was progressively restricted to the CMZ (Fig. 5 J-N, L-P). Significantly, *Rx1* transcripts were detected in the CMZ of cavefish larvae even at 4 or 5 days pf (Fig. 5P, data not shown), suggesting that new retinoblasts are formed despite the arrest in retinal growth. *Rx1* staining was also detected in the outer nuclear layer (ONL) of the surface fish and cavefish retina, probably in the developing cone photoreceptor cells (Fig. 5 N-P), as reported in zebrafish (Chuang *et al.,* 1999). The ONL is known to partially degenerate during cavefish retinal development (Langecker *et al.,* 1993, Yamamoto and Jeffery, 2000). Consistent with these studies, *Rx1* expression appears to be reduced and fragmented in the cavefish ONL (Fig. 5P). The results show that *Rx1* expression remains qualitatively unchanged during the first 5 days of Pachón cavefish development.

Vsx2 *Expression during Surface Fish and Pachón Cavefish Development*

The expression pattern of *Vsx2* was also determined by *in situ* hybridization. *Vsx2* expression was similar to *Rx1* throughout sur-

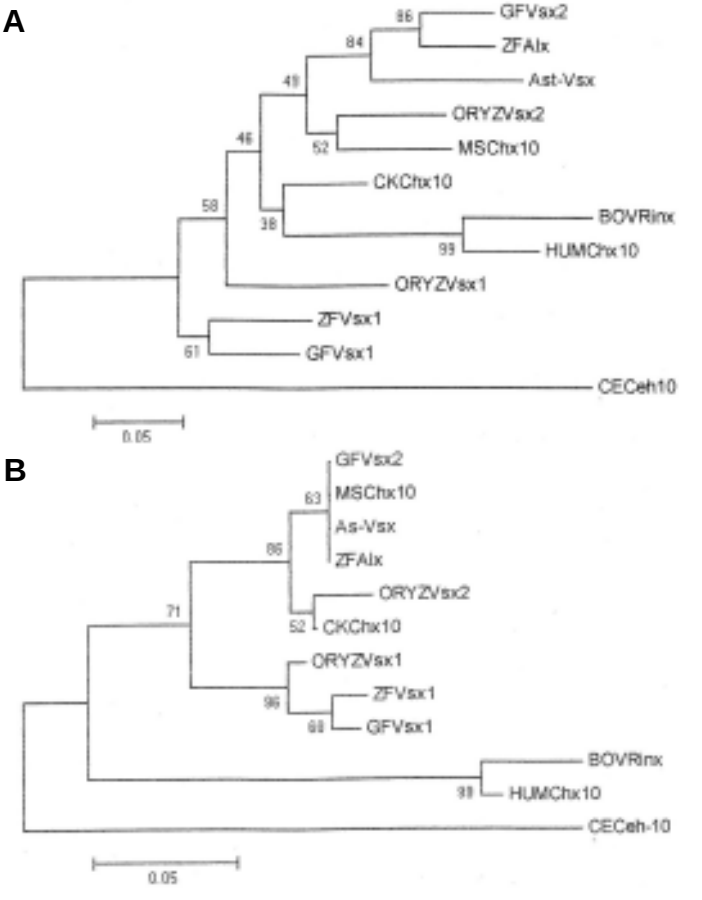

Fig. 4. Phylogenetic trees of *Vsx2* nucleotide (A) and deduced amino acid (B) sequences constructed by the NJ method. *The scale bar in (B) represents an evolutionary distance of 0.05 nucleotide substitutions. The accession numbers for various* Chx/ Vsx *family members used in the trees are indicated in Fig. 4. Other details are the same as in Fig. 3.*

face fish and Pachón cavefish development (Fig. 6). Transcripts were first detected during optic vesicle formation (Fig. 6 A-F), then became restricted to the optic cup (Fig. 6 I-K), and later predominated in the CMZ (Fig. 6 J, N, L-P). *Vsx2* expression was also detected in the inner nuclear layer (INL) of the retina (Fig. 6 M-P), probably corresponding to *Vsx2* reported in the bipolar cells (Liu *et al.,* 1994; Chen and Cepko, 2000). Again, qualitative differences in expression could not be detected between surface fish and Pachón cavefish embryos (Fig. 6). Notably, expression was still detected in the CMZ of Pachón cavefish embryos as late as 4 days pf, after eye growth had ceased (Fig. 6P). The results indicate that *Vsx2* expression is similar during surface fish and Pachón cavefish eye development.

Rx1 *and* Vsx2 *Expression during Los Sabinos Cavefish Development*

Similarly to Pachón cavefish, Los Sabinos cavefish form a small eye primordium, which then arrests and degenerates later in development (Wilkins, 1988). The Pachón and Los Sabinos cavefish are genetically distinct and may have evolved independently from an eyed surface fish ancestor (Dowling *et al.,* 2002). We conducted *in situ* hybridizations with Los Sabinos cavefish embryos to determine whether *Rx1* and *Vsx2* expression has changed during eye development. The results were similar to those obtained in Pachón cavefish. Figure 7 shows *Rx1* (Fig. 7A) and *Vsx2* (Fig. 7B) expression in the CMZ of Los Sabinos cavefish at 36 hr pf. These results suggest that *Rx1* and *Vsx2* expression occur in the CMZ during eye degeneration in Los Sabinos cavefish.

Cell Proliferation in the Cavefish Retina

The *Rx1* and *Vsx2* expression studies suggest that retinal cell proliferation continues during cavefish eye degeneration. To confirm these results, we compared retinal cell division more directly using PCNA and BrdU markers. PCNA positive cells were detected in the CMZ of surface and Pachón cavefish at 10 days and 30 days pf (Fig. 8), indicating that new retinoblasts are still being formed late in cavefish development. In addition, BrdU pulse-chase experiments were conducted to determine whether the proliferating CMZ cells are displaced into the inner parts of the

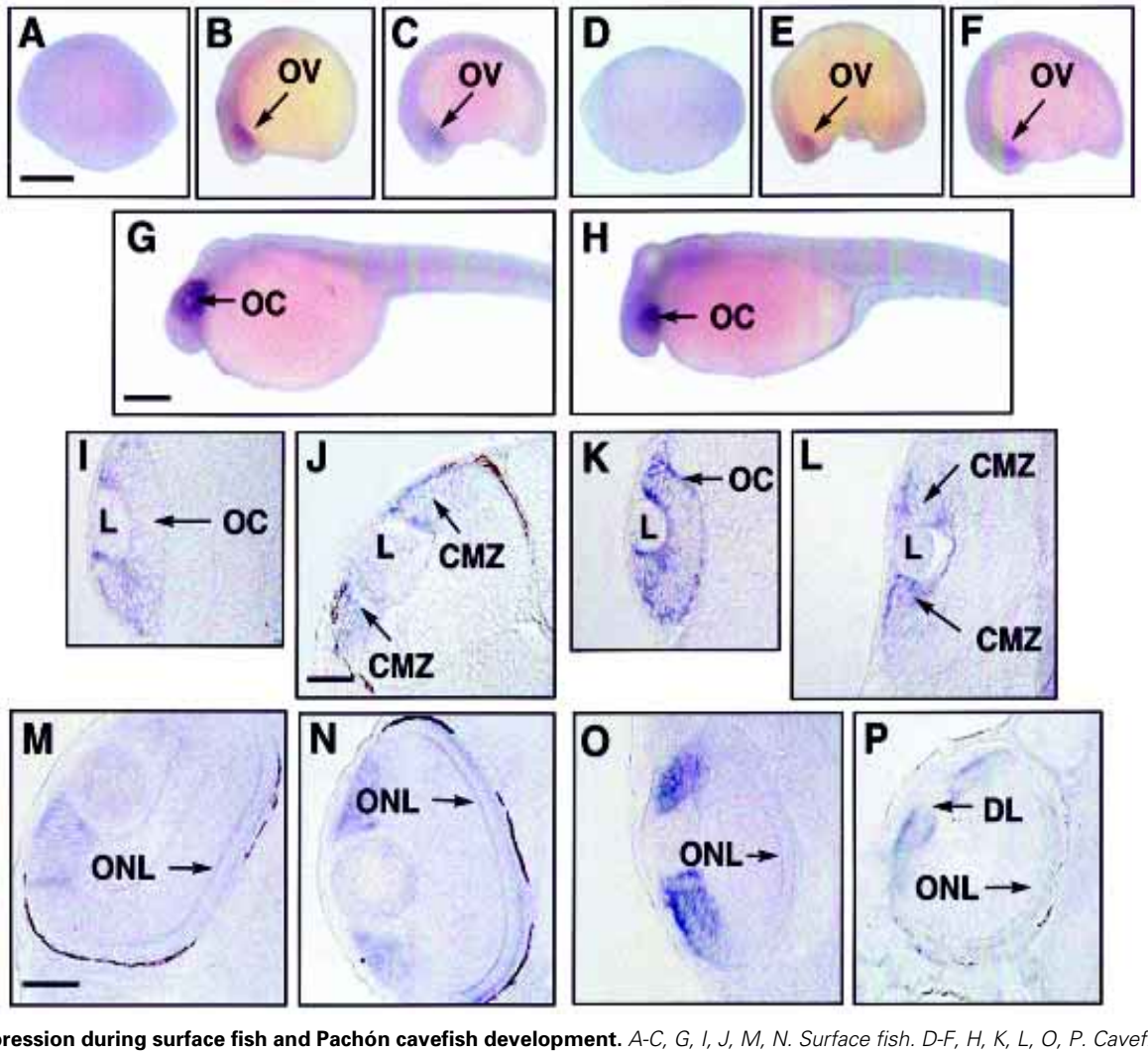

Fig. 5. *Rx1* expression during surface fish and Pachón cavefish development. *A-C, G, I, J, M, N. Surface fish. D-F, H, K, L, O, P. Cavefish. Whole mount* in situ *hybridized embryos at the tailbud* **(A,D)**, *10 somite* **(B,E)**, *18 somite* **(C,F)**, *and 24 h pf* **(G,H)** *stages. Sections through the optic area of whole mount* in situ *hybridized embryos and larvae at 24 h* **(I,K)**, *36 h* **(J,L)**, *48 h* **(M,O)**, *72 h* **(N)**, *and 96 h* **(P)** *pf. Abbreviations: OV, optic vesicle; OC, optic cup; L, lens; DL, Degenerating lens; CMZ, ciliary marginal zone; ONL, Outer nuclear layer of the retina; Scale bars in A and G, 200 μm; magnification is the same in (A-F) and (G, H). Scale bar in J, 30 μm; magnification is the same in (I-L). Scale bar in (M), 40 μm; magnification is the same in (M-P).*

retina, as occurs during normal retinal development (Johns, 1977; Harris and Perron, 1998; Perron *et al.,* 1998). As expected from PCNA staining, BrdU was incorporated into the surface fish and cavefish CMZ during the pulse (Fig. 9 A,D). During the chase, the BrdU labeled cells were displaced from the CMZ to the interior of the retina, eventually contributing cells in the inner and outer retinal layers (Fig. 9 B,C,D,E). The results demonstrate that despite the dramatic decline in retinal growth new retinoblasts are formed in the cavefish CMZ and displaced to the interior of the retina.

Discussion

We have determined the role of retinal cell proliferation in the degenerating cavefish eye by studying the expression patterns of the *Rx1* and *Vsx2* retinal homeobox genes. Although cavefish lack functional eyes as adults, eye primordia are formed during embryogenesis, which later arrest and degenerate (Wilkens *et al.,* 1988; Jeffery, 2001). One of the hallmarks of developmental arrest is the slowing and eventual cessation of retinal growth. Homeobox genes of the *Rx* and *Chx/Vsx* families were selected for study here because they are expressed early during optic development and are required for retinal cell proliferation (Mathers *et al.,* 1997; Barabino *et al.,* 1997; Zhang *et al.,* 2000).

The *Rx* retinal homeobox genes are characterized by a *paired* class homeodomain, a diagnostic *Rx* domain, and two smaller conserved regions at the N and C termini. One or two closely related *Rx* genes have been isolated in *Drosophila, Xenopus*, chicken, mouse, and human (Eggert *et al.,* 1998; Andreazzoli *et al.,* 1999; Mathers *et al.,* 1997; Cararosa *et al.,* 1997; Ohuchi *et al.,* 1999), and three *Rx* genes (*Rx1*, *Rx2*, and *Rx3*) have been reported in zebrafish (Chuang *et al.,* 1999). On the basis of sequence conservation inside and outside of the four conserved domains and clustering with other *Rx1* genes in phylogenetic trees, *Astyanax As-Rx* appears to correspond to an *Rx1* gene.

In zebrafish the *Rx1* gene is first expressed in presumptive optic primordia in the anterior neuroepithelium, then in the optic vesicles, and finally in the retina (Chuang *et al.,* 1999, Chuang and Raymond, 2001). *Rx1* expression initially occurs throughout the retina but later becomes restricted to the CMZ, the source of new retinoblasts, and to the layer of cone photoreceptors in the ONL. The expression pattern of *Astyanax Rx-1* resembles zebrafish *Rx1*, including confinement of activity to the CMZ and ONL during

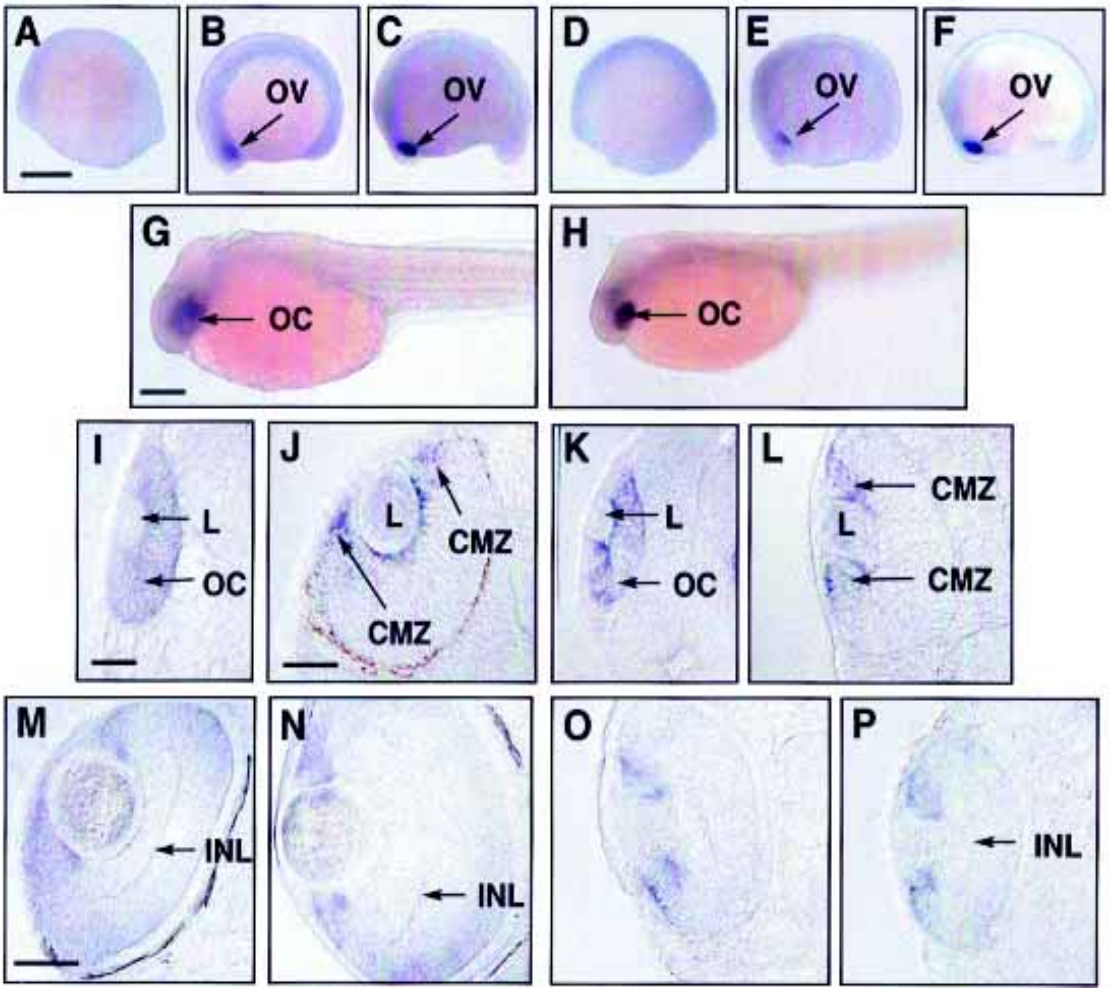

Fig. 6. *Vsx2* expression during surface fish and Pachón cavefish development. *(A-C, G, I, J, M, N) Surface fish; (D-F, H, K, L, O, P) Cavefish. Whole mount* in situ *hybridized embryos at the tailbud* **(A,D)**, *10 somite* **(B,E)**, *18 somite* **(C,F)**, *and 24 h pf* **(G,H)** *stages. Sections through the optic area of whole mount* in situ *hybridized embryos and fry at 24 h* **(I,K)**, *36 h* **(J,L)**, *48 h* **(M,O)**, *72 h* **(N)** *and 96 h* **(P)** *pf. Abbreviations: INL, Inner nuclear layer of the retina. Other details and scale bars are the same in as in Fig. 6.*

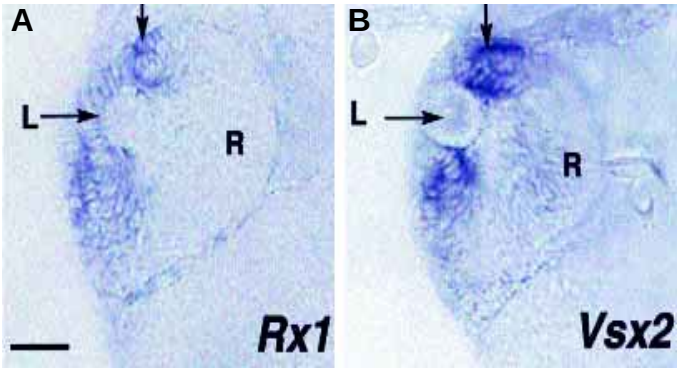

Fig. 7. *Rx1 and Vsx2* expression in Los Sabinos cavefish embryos. (A) Rx1 *expression in the optic area of a sectioned whole-mount* in situ *hybridized embryo at 36 h pf.* **(B)** Vsx2 *expression in the optic area of a sectioned, whole-mount* in situ *hybridized embryo at 36 hpf. The scale bar in A is 30 μm; magnification is the same in A and B. R, retina. Other details are the same as in Fig. 6.*

late optic development. Despite repeated attempts, however, *Rx1* expression could not be detected in *Astyanax* embryos prior to optic vesicle formation. It would be interesting to determine if other *Rx* genes (e.g. *Rx2* and *Rx3*) are expressed prior to optic vesicle formation in cavefish.

The photoreceptor cells of the ONL are among the optic tissues that degenerate during cavefish development (Langecker *et al.,* 1993). The opsin gene is expressed in the cavefish ONL but its activity is transient and declines beyond the level of detection during later optic development (Langecker *et al.,* 1993). The only difference in *Rx1* expression we have observed between in surface fish and cavefish embryos was also in the photoreceptor layer*: Rx1* expression is weak, transient, and restricted to the central zone of the ONL during cavefish eye development. Presumably, *Rx1* expression in the ONL occurs in the cone photoreceptor cells, which are known to express this gene in zebrafish (Chuang *et al.,* 1999). Similar results were obtained using anti-rhodopsin to follow differentiation of the rod photoreceptor cells in cavefish embryos (Yamamoto and Jeffery, 2000). These results suggest that a feature of the degenerating cavefish retina may be the suppression of genes involved in photoreceptor cell differentiation. The changes in *Rx1* gene expression in the ONL could be a cause or a consequence of eye degeneration.

The *Chx*/*Vsx* genes are another small family of homeobox genes characterized by a *paired* class homeodomain/CVC domain (Svendsen and McGhee, 1995). Two orthologs have been isolated from goldfish (*Vsx1* and *Vsx2*) (Levine *et al.,* 1994; 1997), zebrafish (*Vsx1* and *Alx*/*Vsx2*) (Barabino *et al.,* 1997; Passini *et al.,* 1998a, b), and chicken (*Chx10* and *Chx10-1*) (Chen and Cepko, 2000), whereas only one gene (*Chx10*) has been identified in the mouse and human (Liu *et al.,* 1994; Belecky-Adams *et al.,* 1997). We isolated As-Vsx2, a DNA fragment corresponding to a *Chx*/*Vsx* gene. The partial As-Vsx2 sequence and phylogenetic trees suggest that *As-Vsx2* is most closely related to the goldfish *Vsx2* and zebrafish *Alx*/*Vsx2* genes. The goldfish and zebrafish *Vsx1* and *Alx*/*Vsx2* genes have similar overlapping expression domains, initially in the optic cup and later in the proliferating and differentiating retinal cells (Levine *et al.,* 1997; Passini *et al.,* 1998a, b). Similar to other teleosts, *As-Vsx2* is expressed throughout the retina but is later confined to the CMZ and the INL during *Astyanax* development. Thus, expression in the INL and not the ONL distinguishes *As-Vsx2* from the related *As-Rx1* gene.

The early expression patterns in the neurula and optic vesicles and eventual confinement of *Rx1* and *Chx/Vsx* to the optic primordia earmarked these genes as candidates for regulators of cavefish eye degeneration. Our results indicating that the temporal and spatial expression patterns of *As-Rx1* and *As-Vsx2* are generally unchanged in Pachón and Los Sabinos cavefish embryos do not support this possibility. Although we have not excluded the possibility that the positive *in situ* hybridization signals represent untranslatable mRNAs arising from partially expressed pseudogenes, we believe that this possibility is unlikely. The rudimentary cavefish lens does not produce lens fibers, but it expresses both gamma crystallin mRNA and protein (Jeffery *et al.,* 2000; Strickler, unpublished results). Likewise, opsin mRNA and protein are expressed transiently in the cavefish retina (Langecker *et al.,* 1993; Yamamoto and Jeffery, 2000; A. G. Strickler, unpublished results). These structural genes operate at the termini of gene networks and might be expected to evolve into pseudogenes under conditions of relaxed selection. Better candidates for regulators of eye degeneration may be genes operating downstream and/or in parallel to the *Rx1* and *Vsx2* retinal homeobox genes. For example, *Pax6*, which is eventually expressed in the retina but whose expression domains are smaller in cavefish optic primordia (Strickler *et al.,* 2001), might be more

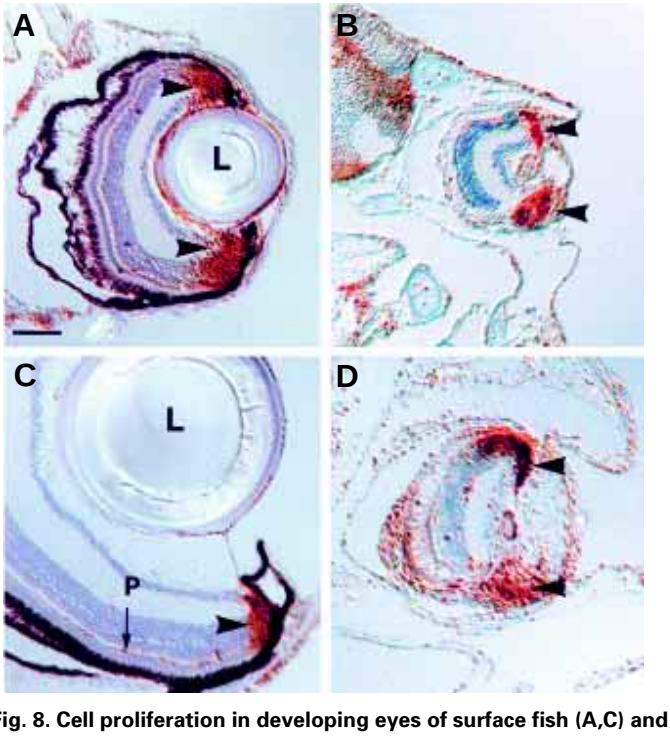

Fig. 8. Cell proliferation in developing eyes of surface fish (A,C) and Pachón cavefish (B,D) larvae determined by staining sections with PCNA antibody. *At 10* **(A,B)** *and 30 days pf* **(C,D)** *brown PCNA staining is present in the CMZ and in photoreceptor cells of the retina (P). PCNA staining is also seen in the lens epithelium of the surface fish and in the anterior portion of the cave fish lens. L, lens; Arrowheads: PCNA staining in the ciliary marginal zone. Scale bar, 30μm; magnification is the same in each frame.*

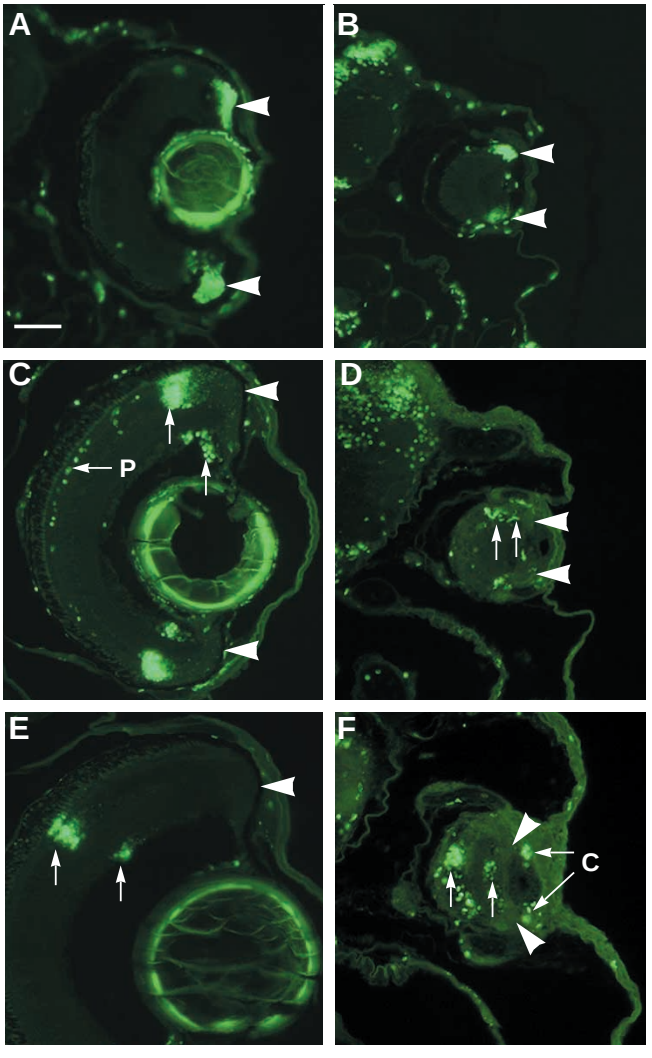

Fig. 9. Cell proliferation in surface fish (A-C) and Pachón cavefish (D-E) retinae determined by a BrdU pulse-chase labeling experiment with sections of surface and cave fish eyes stained for BRDU incorporation. *Surface fish and cavefish were exposed to BrdU from day pf 8 to 10 (pulse experiment)* **(A,D)**. *The chase started at day 10 and extended to day 12* **(B,E)** *or day 14* **(C,F)** *pf. BrdU labeling is indicated by green fluorescence. During the pulse (A,D), BrdU labeling is present in the CMZ. During the chase (B,C,E,F), BrdU labeling is present in retinal cells that have migrated away from the CMZ, as well as in proliferating photoreceptor cells e.g. (P). Arrowheads in A-F, CMZ; arrows in (B,C,E,F) BrdU labeled retinal cells during the chase. In (F), BrdU labeling is also seen in conjunctiva cells (c) that are beginning to occlude the eye structures. Scale bar, 30 μm. Magnification is the same in each frame.*

directly involved in generating the eyeless phenotype. It is also possible that retinal homeobox genes other than *Rx1, Vsx*2, and *Pax6* may have roles in eye degeneration.

The major reason for studying the *Rx1* and *Vsx2* homeobox genes is their role in cell proliferation during development of the vertebrate retina. The eyes of cold-blooded vertebrates, such as fishes and amphibians, continue to grow throughout life in proportion to the body. The constant increase in eye size is due to the proliferative activity of the optic germinal layers, including the CMZ in the retina and the lens epithelium, which produce new progenitor cells from an indelible stem cell lineage (Johns, 1977; Perron *et al.,* 1998; Harris and Perron, 1998). Thus, arrested retinal growth could be caused by inhibition of cell division in the cavefish CMZ. In contrast to this idea, we have shown that As-*Rx1* and *As-Vsx2* transcripts are still present in the cavefish CMZ for days following the decline in retinal growth. Similarly, the *Pax6* and *Prox1* genes, markers of retinal cell differentiation (Perron *et al.,* 1998), are also expressed in the CMZ during the degenerative phase of cavefish eye development (Jeffery *et al.,* 2000; Strickler *et al.,* 2001). More direct demonstration of cell proliferation using the cell division markers PCNA and BrdU also showed that the cavefish CMZ is a source of new retinoblasts, which are subsequently displaced to the interior of the retina. Our studies show that cell proliferation continues in the cavefish CMZ at least through the first 30 days of cavefish development, long after the eye has significantly reduced its rate of growth and sunken into the orbit.

Our results strongly suggest that retinal cell proliferation is not the primary cause of arrested eye growth in cavefish. An alternative hypothesis for future consideration is that persistent cell proliferation is balanced by cycles of cell death in the cavefish retina.

Materials and Methods

Biological Materials

The *A. mexicanus* (=*fasciatus*) stocks originated from surface fish collected at Balmorhea State Park, Texas and cavefish collected at La Cueva de El Pachón (Pachón cavefish) in Tamaulipas, Mexico and La Cueva de Los Sabinos (Los Sabinos cavefish) in San Luis Potosí, Mexico. Stocks were maintained as described previously (Jeffery and Martasian, 1998). Embryos were obtained by natural spawning and reared to adults at 25 C. Under these conditions, the tailbud stage occurs at 10-11 h, the 18 somite stage at 16 h, and hatching at 22 hpf.

Isolation of Rx *and* Chx/Vsx *DNA*

Total RNA was extracted from 18 somite surface fish embryos using the RNA/DNA Maxi Kit (Qiagen, Valenica, CA, U. S. A.), and cDNA was prepared for RT PCR using the First Stand cDNA Synthesis Kit (Roche Molecular Biochemicals, Indianapolis, IN, U. S. A.). RT PCR was performed using degenerate oligonucleotide primers and the PCR Master Kit (Roche) as described by Jeffery *et al.* (2000). The primers used to amplify *Rx* DNA were 5'-CGAGAAGTCACACTACCCTG-3 '(forward primer) and (5'-CATCCTCAGWGMGGCAATGC-3' (reverse primer). The primers used to amplify *Chx/Vsx* DNA were 5'-GAAGGCACAGGACAGTHTTYAC-3 '(forward primer) and (5'-ACCTAGAAGCCAGGGAGCRCA-3' (reverse primer). PCR products of the expected size were ligated into the PCR-Script vector (Stratagene, LaJolla, CA) and sequenced. Blast analysis indicated that parts of *Astyanax Rx* and *Chx/Vsx* (As-Vsx2) genes had been amplified. The DNA fragments were used to screen a cDNA library prepared from 18 somite stage *Astyanax* surface fish RNA by conventional procedures under the following conditions. The filters were hybridized in 50% formamide, 5 X SSC, 5X Denhardt's solution, 0.1 % SDS, 0.1 M Tris-HCl, pH 7.4, 5 μg/ml salmon sperm DNA at 42 C for 16 h, washed once with 2 X SSC, 0.1% SDS for 20 min at room temperature, and three times with 0.5 X SSX, 0.1% SDS at 37 C for 20 min. The screens using *Rx* DNA yielded a single cDNA clone *(As-Rx1),* which was sequenced and shown to correspond to an *Rx1* gene. The Genbank accession numbers of *As-Rx1* and *As-Vsx2* are AF264703 and AF418642 respectively.

Construction of Phylogenetic Trees

Sequences were aligned using ClustalX (Thompson *et al.,* 1997). The MEGA version 2.1 software program was used to construct phylogenetic trees (Kumar *et al.,* 2001). For phylogenetic trees constructed with nucleotide sequences, distances were calculated using the Jukes-Cantor method,

and the neighbor joining (NJ) method was used for tree construction. For phylogenetic trees constructed with amino acid sequences, distances were calculated using the p-distance method, and tree construction was by the NJ method. The degree of support for internal nodes was assessed using 1000 bootstrap replications.

In Situ *Hybridization*

Antisense riboprobes generated from the *As-Rx1* and *As-Vsx2* DNA sequences were used for *in situ* hybridization following the procedure described by Püschel *et al.* (1992). Embryos subjected to *in situ* hybridization were observed directly as whole mounts or were embedded in Paraplast, sectioned at 10 μm, and the sections were observed after mounting on subbed slides.

PCNA Immunohistochemistry

PCNA antibody staining was performed according to Yamamoto, and Jeffery (2000). Samples were embedded in paraplast and sectioned at 8 μm. The sections were incubated with a polyclonal antibody to PCNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The staining was visualized using DAB substrate. The sections were counterstained with hematoxylin and eosin prior to photography.

BrdU Pulse Chase Experiments

Surface fish and cavefish larvae were soaked in a 100 μM solution of BrdU from 8 to 10 days pf. An aliquot of larvae was removed from the BrdU solution after 2 d and fixed overnight in 4% paraformaldehyde. The remaining larvae were rinsed several times into fresh water, chased without BrdU and subsequently fixed at 12 and 14 days pf. The samples were embedded in paraplast and sectioned at 10 μm. The sections were dewaxed and an anti-BrdU antibody conjugated to fluorescein (Roche Biochemicals, Indianapolis, IN, USA) was used to detect BrdU incorporation. For BrdU detection samples were washed twice in PBS containing 0.5% BSA and 0.1% Tween 20, incubated in 0.05% trypsin and 0.05% calcium chloride in PBS at 37° C for 5 min., incubated in 10 mg/ml trypsin inhibitor for 10 min, incubated in 4 M HCl for 15 min, rinsed twice 5 min. in PBS/BSA/Tween 20, incubated with 50 ug/ml antibody in PBS/BSA/Tween 20, washed three times for 5 min with PBS/BSA/Tween 20, mounted, and viewed by fluorescence microscopy.

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