Comparative Analysis of Pax-6 Sequence and Expression in the Eye Development of the Blind Cave Fish Astyanax fasciatus and its Epigean Conspecific

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The Pax-6 gene encodes a transcription factor essential for eye development in a wide range of animal phyla. In order to elucidate a possible role of Pax-6 in the eye regression of a blind cave form of the freshwater fish Astyanax fasciatus (Characidae, Teleostei) we investigated the expression of Pax-6 in eyes and brains of different larval stages by in situ hybridization. Pattern, strength, and time course of Pax-6 expression were not altered in the tissues of the cave form when compared to the epigean form. Pax-6 was even expressed in the highly degenerated eyes of late larval stages of the cave form. Comparative sequence analysis of Pax-6 cDNA clones of both forms of Astyanax fasciatus showed the complete integrity of cave fish Pax-6 mRNA. These results suggest that Pax 6 is not involved in the evolutionary process of eye degeneration in this model system of cave-living fishes. Comparison of the Astyanax Pax-6 cDNA with the other available fish Pax-6 sequence from zebrafish revealed putative fish-specific regions of homology. A stretch of 19 N-terminal amino acids is nearly identical on the nucleotide and amino acid levels in both fish species but not present in all other known Pax-6 sequences.

Introduction

Pax-6 is one of nine paired-box-containing genes presently found in vertebrates. The common feature of this multigene family is the DNA-binding paired box which was first described in the Drosophila segmentation gene paired (prd) (Bopp et al. 1986). Some of the Pax genes, among them Pax-6, have an additional complete DNA-binding domain, the homeodomain. Pax-6 is expressed in the eye, brain, and spinal cord during embryogenesis (Strachan and Read 1994). The sequence and expression pattern is well conserved over a wide range of animal phyla, including mammals, birds, amphibians, fishes, echnoderms, and insects (for a review see Halder, Calaerts, and Gehring 1995). Several naturally occurring mutants with defective Pax-6 expression have been described so far in vertebrates: the small eye mutation in mice (Sey) (Hill et al. 1991) and rat (rSey) (Matsuo et al. 1993) as well as the human diseases aniridia (AN) syndrome (Ton et al. 1991), Peters’ anomaly (Hanson et al. 1994), and autosomal dominant keratitis (Mizayans et al. 1995). Sey and AN mutations cause severe abnormalities, especially of the eye and nose, in the homozygous state and are lethal, whereas in heterozygous mutants the development of the nose is not affected and eye defects occur in milder form. Pax-6 is also involved in the morphogenesis of the insect eye since the eyeless mutation which was found in the Drosophila Pax-6 homolog causes absence of the complex eyes (Quiring et al. 1994).

The analysis of Pax-6 mutants has clearly established that this transcription factor plays a central role in eye development (Macdonald and Wilson 1996). Disturbances in the morphogenesis of the eye are not only known from pathological cases, but can also result from nonpathological evolutionary processes. Many deep-sea organisms as well as cave-dwelling animals evolve degenerate eyes in response to the continuous darkness of their environment. Cave animals exhibit a convergent reduction of eyes and melanin pigmentation, whereas nonvisual sensory organs may show a hypertrophic development (Wilkens 1988).

To test the hypothesis that alterations of Pax-6 expression are involved in the eye regression of a cave-living freshwater fish, we have used in this study Astyanax fasciatus (Cuvier 1819), a small characid from Mexico. Sometime during pleistocene the epigean form split off a series of independent cave populations (Mitchell, Russel, and Elliot 1977). All adult fishes of the “Piedras” cave population have largely degenerated eyes with completely reduced lenses. The Astyanax system allowed classical genetic approaches to the analysis of the nature of eye defects, because the cave fishes are still interfertile with their surface form. It could be shown that the F1 generation has intermediate but still functional eyes. The F2 generation exhibits a great variability of eye sizes ranging from nearly normal to almost completely reduced (Wilkens 1988). These studies led to the assumption that at least six genetic factors are involved. The loss of function of a developmental control gene was considered to be more likely than defects exclusively in structural genes because during early ontogeny the cave fish eye develops almost all structural components of that of the epigean form (Wilkens 1988; Langecker, Schmale, and Wilkens 1993). Similarities in the morphology of the reduced cave fish eye with heterozygous small-eye mice, namely microphthalmia and rudimentary or absent lenses (Hogan et al. 1986, 1988), led us to compare Pax-6 gene expression and structure between epigean and cave forms.

Here we report that time course and expression pattern of Pax-6 mRNA in the developing eye and in dis-
crebrate brain regions is not altered in cave forms of Astyanax fasciatus. Comparison of Pax-6 cDNA clones from cave and epigean forms indicates that there are no mutations which could give rise to changes in the amino acid sequence of the predicted Pax-6 protein.

Materials and Methods
Polymerase Chain Reaction

Degenerated primers were designed using conserved regions in mouse and zebrafish Pax-6 sequences. The 5' primer 5'-GTGTCACGGCTCGTGAGTAA(G,A)AT-3' corresponds to positions 346–371 (mouse Pax-6) (Walther and Gruss 1991) and 729–754 (zebrafish Pax-6) (Püschel, Gruss, and Westerfield 1992), respectively, the 3' primer 5'-CTGGGTATTAT-TATCGTTTGT(G,A)CAIAC-3' to positions 535–560 (mouse) and 918–943 (zebrafish). The PCR reaction (50 µl) contained 500 ng genomic DNA of the epigean fish, 2 µg of each primer, 25 µM dNTPs, 4.2 U Taq polymerase (Promega, Madison, Wis.), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, and 2 mM MgCl2. The amplification was performed for 40 cycles (1 min at 94°C, 2 min at 55°C, 3 min at 72°C) in a Hybid thermocycler. Because the amplification led to a single but slightly diffuse band of approximately 230 bp, the cloned PCR-products were analyzed by dot-blot hybridization at low stringency with a heterologous mouse Pax 6 probe. Sequencing of positive clones confirmed the identity of the Pax-6 fragment.

Animals

Epigean fishes from Rio Teapa and cave fishes from the Sotano de las Piedras (San Luis Potosí) were originally caught in Mexico and bred in the laboratory. After spawning, the fertilized eggs of both epigean and cave specimens were transferred into shallow breeding containers and kept under a light-dark cycle of 12 h at 25 ± 1°C. Hatching of the larvae occurred after 18 h (Langecker, Schmalle, and Wilkens 1993; Langecker, Wilkens, and Schmalle 1995).

Preparation and Screening of cDNA Libraries

Poly(A)+ RNA was prepared from whole 40-h old larval fish with the MicroFastTrack mRNA Isolation kit (Invitrogen, San Diego, Calif.). Then, 3.5 µg of the epigean form and 2.5 µg of the cave form RNA were denatured by glyoxylation, separated on a 1% agarose gel and blotted onto Genescreen plus (Dupont) using standard procedures (Sambrook, Fritsch, and Maniatis 1989). After UV-crosslinkage with an energy of 120 mJ/cm2, the membrane-bound RNA was deglyoxylated by incubation for 10 min in 20 mM Tris-HCl (pH 8.0) at 65°C. A 900-bp Pst I/Apa I fragment from the 3' end of one Pax-6 cDNA clone was random labeled with 32P-α-dCTP to a specific activity of 9 × 108 cpm/µg DNA. Prehybridization was performed for 4 h at 50°C in 50% deionized formamide, 4 × SSPE, 1% SDS, 5 × Denhardt's reagent and 100 µg/ml of heat-denatured calf-thymus DNA followed by hybridization overnight under the same conditions in hybridization buffer containing 1 × 107 cpm/ml of the labeled 900-bp fragment. After hybridization the membranes were washed two times for 15 min in 0.1 × SSC; 0.1% SDS at room temperature (RT), and 30 min at 50°C.

Genomic Southern Blotting

Genomic DNA of the epigean and the cave forms was isolated according to a standard procedure (Herrmann and Frischauf 1987) and digested with 100 Units EcoRI and HindIII, respectively. The transfer of the alkali-denatured DNA to Genescreen plus and the prehybridization and hybridization with the cloned Pax-6-specific PCR fragment (random labeled to a specific activity of 3 × 109 cpm/µg DNA) were performed exactly as described for the RNA blot. The membrane was washed two times for 10 min at room temperature in 2 × SSC, 0.1% SDS.

In situ Hybridization
Probe Labeling

The radioactively labeled antisense and sense RNA probes were generated by in vitro transcription from the linearized plasmid containing the 230-bp Pax-6 specific PCR fragment using 50 µCi 35P-α-UTP and SP6 or T7 RNA polymerases, respectively. The incorporation rate was determined by TCA precipitation. After two subsequent ethanol precipitations, the probes were resus-
Developing and Mounting

Slides were pretreated with 3-aminopropyltriethoxysilane (Sigma, Deisenhofen, Germany). Cryostat cross sections of 10 μm thickness were cut from whole fish larvae embedded into Tissue Tek (Miles Scientific, Elkhart, Ind.) at −20°C and transferred onto the slides.

Fixation of Tissue

Sections were fixed with 4% paraformaldehyde in 1 × PBS, 1 mM MgCl₂ (5 min, RT). After washing the slides in 1 × PBS (1 min, RT) a UV-crosslinking step with an energy of 120 mJ/cm² was performed, followed by a second wash in 1 × PBS (1 min, RT). After incubation in 0.2 M HCl (10 min, RT) and 1% Triton X-100 in 1 × PBS (2 min, RT) and rinsing in 1 × PBS (1 min, RT), the slides were dehydrated in 60%, 80%, 95%, and 100% ethanol (5 min, RT each) and air-dried (RT).

Hybridization

Before hybridization, the slides were prehybridized in a buffer containing 50% formamide, 750 mM NaCl, 25 mM PIPES, 25 mM EDTA (pH 7.5), 5 × Denhardt’s reagent, 0.2% SDS; 250 μg/ml yeast tRNA, and 250 μg/ml calf-thymus DNA. About 1.5 ml prehybridization buffer was applied per slide, followed by incubation in a humid chamber for 5 h at 50°C. Then, 70 μl of hybridization buffer containing 4 ng of radioactive probe was applied per slide and covered with a coverslip. Hybridization was carried out overnight at 50°C in a humid chamber.

Washing and Autoradiography

Coverslips were removed in 4 × SSC at RT. After rinsing the slides three times for 5 min in 2 × SSC at RT they were incubated in 0.5 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 7.5), and 40 μg/ml RNase A at 37°C for 30 min, followed by an incubation in the same buffer without RNase. After incubation in 0.2 × SSC at RT for 15 min the slides were washed two times in 0.2 × SSC at 50°C for 15 min each and than dehydrated in the graded ethanol series. After drying at RT, the slides were heated to 42°C on a slide warmer and dipped in NTB-3 photo emulsion (Kodak, Rochester, N.Y.) at 42°C. After drying for 3 h at RT the slides were exposed for 13 days in light-tight boxes at 4°C.

Results

Blot Hybridizations and Isolation of Pax-6 cDNA Clones

We compared the overall genomic structure of the Pax-6 gene in epigean A. fasciatus and the Piedras cave form by genomic Southern blot analysis. In both fish forms the Pax-6-specific probe derived from the paired box hybridized to a single band of approximately 12 kb after EcoRI and 0.9 kb after HindIII digests, respectively (fig. 1A). This result suggested that at least the part of the Pax-6 gene covered by the probe was not rearranged in the cave form. To determine the length of Astyanax fasciatus Pax-6 mRNA we performed a Northern blot analysis with poly(A)+ RNA of 40-h-old epigean and cave fish larvae and found in both cases a single transcript of approximately 2.7 kb (fig. 1B).

Since the DNA and RNA blot hybridization experiments do not allow the detection of more subtle changes of the genes, we analyzed Pax-6 cDNA clones derived from epigean A. fasciatus and the cave form. We chose to prepare cDNA libraries from whole 40-h-old fish larvae because at this stage, when the degenerative events in the cave fish eye begin, the proportional amount of developing eye-derived tissue and Pax-6 expression are optimal.

Comparison of Pax-6 cDNA Sequences from Epigean A. fasciatus and the Piedras Cave Form

Comparative sequence analysis of two Pax-6 cDNA clones of the epigean fish and three of the cave fish revealed a total number of seven polymorphic sites. Four of these nucleotide differences are present in the two representative sequences shown in figure 2. The deduced amino acid sequences of both fish forms are identical. The Astyanax fasciatus gene, when compared with the Pax-6 sequences of other species, showed the highest homology to the zebrafish, Brachydanio rerio, Pax-6. The nucleotide sequence identity between A. fasciatus and zebrafish Pax-6 cDNAs in the coding region amounts to 88%. On the protein level, the sequences are 98% identical. Both A. fasciatus and B. rerio Pax-6 proteins exhibit 19 N-terminal amino acids which are not found in all other known Pax-6 homologues. The predicted protein sequences in this part differ by only one amino acid (fig. 3).

While the homeodomain of the A. fasciatus Pax-6 protein is 100% identical to the zebrafish and mouse homologues, the paired domain shows some conservative exchanges. The tyrosine residue in position 123 of the homeodomain of the epigean A. fasciatus Pax-6 is replaced by phenylalanine, and the valine at position 144 is changed to isoleucine. Glycine at position 118 is used in both fish species instead of serine in the mouse sequence (fig. 3).

One of the isolated A. fasciatus Pax-6 cDNA clones of the cave fish contained an additional exon of 42 bp in the sequence encoding the paired domain which also has been found at the identical position in zebrafish and other vertebrate Pax-6 homologues (Püschel, Gruss, and Westerfield 1992; Walther and Gruss 1991; Glaser, Walton, and Maas 1992). It has already been shown that this
exon has an influence on the DNA-binding properties of the paired domain (Epstein et al. 1994). The protein sequence of this alternative exon is highly conserved; in A. fasciatus only the second amino acid is altered from histidine to glutamine (fig. 3).

None of the cDNA clones analyzed contained the complete 3' untranslated region (UTR). Comparison with the length of the mRNA as determined by Northern blot analysis and with the 3' UTR of the zebrafish Pax-6 cDNA indicated that approximately 300 bases and the poly(A) tail are missing, most probably due to internal oligo(dT)-priming at an A-rich sequence around 2.1 kb downstream from the 5' end as already observed in zebrafish (Püschel, Gruss, and Westerfield 1992).

Some A. fasciatus Pax-6 cDNA clones revealed the presence of two insertions at the 5' end but not in other regions of the mRNA (data not shown). The significance of these insertions, which occur at positions where introns have been found in the Pax-6 gene of the quail (Martin et al. 1992), remains to be determined.

Expression of Pax-6 mRNA in the Eye

We analyzed expression of the A. fasciatus Pax-6 gene between 30 h and 150 h of development by in situ hybridization of 10 μm cryostat sections using 33P-labeled RNA probes.

At 30 h of development, the size and morphology of the cave fish eye is still very similar to the eye of the epigean counterpart (fig. 4A and C). The only differences are found in the pigment epithelium and the lens. The first pigmentation appears in the pigment epithelium and the lens fibers start to develop in the epigean form, although none of these developments are visible in the cave fish eye. However, the retina consists in both forms of 5–6 rows of germinal cells. The Pax-6 hybridization signal is equally distributed over the neuroretina. The lens has, if any signal at all, a significantly lower signal than the retina (fig. 4B and D).

In 51-h-old fish larvae the differences between epigean and cave fish eye become strikingly significant (fig. 4E and G). The pigment epithelium in the epigean eye is clearly more developed than in the earlier stage. The lens size has increased and lens fiber cells are producing crystallins visible by refraction of light in the darkfield image. In the cave fish eye hardly any pigmentation develops and the lens seems to remain in the same developmental stage as before. The neuroretina of both forms has developed further. Now, two zones without nuclei become visible: the thinner outer plexiform layer between photoreceptor cells and the inner nuclear layer and the wider inner plexiform layer between the inner nuclear layer and the ganglion cell layer. The diameter of the eyeball of the cave fish is now significantly smaller than in the epigean form. The Pax-6 expression is at its peak (fig. 4F and H). The signal marks the inner part of the inner nuclear layer (in) as well as the ganglion cell layer (gl). A slight signal in the lens epithelium (le) of the epigean form is also visible. No significant differences in strength and expression pattern between both fish forms could be detected.
Comparative Analysis of Pax-6 in Astyanax

**FIG. 2.**—Comparison of the nucleotide sequences and the deduced amino acid sequence of the Pax-6 cDNA clones "epigean 2-4" (epig.) and "cave 2-3" (cave). Identical nucleotides are indicated by dots. Gaps introduced are represented by dashes. The stop codon at the end of the coding region is marked by an asterisk. The boxed regions outline the N-terminal paired box and the central homeobox. The location of the alternative exon is indicated by the arrowhead. The nucleotide sequence data represented in this paper will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence databases under the accession numbers Y07548 (epigean 2-4) and Y07546 (cave 2-3).
In 150-h-old fish larvae, the Pax-6 signal is restricted to the same cell layers as in the 51-h-old larva, but the expression level is reduced (fig. 4I and J). Although the cave fish eye is already heavily degenerated, Pax-6 mRNA could be detected in the remaining cells of the neuroretina at a level that is comparable to that of the epigean form (fig. 4K and L). We have compared the pattern of Pax-6 mRNA expression in the developing brain and eye is not indicated in the schematic overview of the larval head on figure 5.

The most rostral section (fig. 5a) passed through the anterior and posterior parts of the ventricle and included the prosencephalon with the olfactory bulbs and the prospective diencephalon, which surrounds the eyes. Pax-6 mRNA is expressed in the pair of olfactory bulbs (ub) and the prosencephalic site of the prosencephalon/mesencephalon boundary (dm). The next plane of section (fig. 5b) is located about 40 \( \mu m \) more caudally. Expression in the olfactory bulbs has decreased and the hybridization signal at the prosencephalon/mesencephalon boundary is no longer visible. Additional Pax-6 mRNA expression appears in the prospective diencephalon (de). This zone of expression extends for more than 100 \( \mu m \) caudally and can still be detected on the next plane of section (fig. 5c). A faint hybridization signal in the rhombencephalon is visible there and in the following plane of section (fig. 5d). The expression of Pax-6 mRNA in the developing eye (e) can be detected in all sections and is low compared to that in most of the other brain regions. There were no significant differences in the pattern and strength of Pax-6 mRNA expression in the larval brain at 30 h of epigean and cave-living A. fasciatus.

**Discussion**

Recent studies on the eye regression of cave-dwelling A. fasciatus suggest that structural eye-specific genes may have been inactivated during evolution (Langecker, Schmale, and Wilkens 1993; Yokoyama et al. 1995) but that, primarily, defects in developmental control genes are responsible for the cave phenotype (Wilkens 1988). We have thus studied Pax-6 sequence and expression in the developing eye of epigean and cave specimens. It has been shown that Pax-6 performs discrete functions in different cell types during all stages of eye differentiation and that this transcription factor may well be a master control gene for eye development (Macdonald and Wilson 1996). At early stages of development, Pax-6 may have a general role in controlling the growth of the optic vesicle; later it is required to specify the identity of retina and lens cell types. Even in the mature eye tissue, Pax-6 is still expressed and may affect the function of structural genes, such as crystallin genes which have been shown to contain Pax-6 binding sites in their promoter regions (Cvekl et al. 1995; Richardson, Cvekl, and Wistow 1995). Analysis of Sey mutant mice has indicated that reduced levels of Pax-6 protein do not affect specification of cells, but influence the size of the induced structure (Hogan et al. 1988). Inappropriate Pax-6 function could well explain the observations that in the cave fish (1) the growth rate and developmental timing of cytodifferentiation of the presumptive eye are altered in the cave fish and is in accordance with those reported for other vertebrates (Strachan and Read 1994).
It cannot be completely ruled out that Pax-6 is not involved in the process of eye regression. Although the levels of Pax-6 mRNA expression in epigean and cave-living A. fasciatus are identical within the limits of the in situ hybridization method, the amount of mRNA does not necessarily reflect the amount of protein synthesized. Since the effects of Pax-6 are concentration-dependent (Macdonald and Wilson 1996; Schedl et al. 1996), only quantification of Pax-6 protein would provide ultimate proof of the amount present in the eyes of both fish forms.

The binding capacity of Pax-6 protein to its target genes can also be influenced by alternative splicing. Pax-6 including the alternative exon in the paired box has a unique spatiotemporal expression and different DNA-binding properties. In humans, an alteration of the
Fig. 5.—*Pax-6* expression pattern in a series of brain cross sections of 30-h-old epigean and cave fish larvae as determined by in situ hybridization using a $^{33}$P-labeled *Pax-6* specific antisense RNA probe. Planes of sections a–d are indicated within the central schematic drawing of a sagittal section of the head region of the larva. For each plane, a pair of bright- and dark-field photomicrographs of the epigean form and the cave form, respectively, is shown. Numbers and corresponding patterns specify relevant head regions. Arrows in microphotographs point to hybridization signals in: de, diencephalon; dm, diencephalon/mesencephalon boundary; e, eye; ob, olfactory bulb; re, rhombencephalon. Signals within the pigment epithelium (pe) and the adhesion gland (ag) and at the outer margins of the sections were also obtained with a sense RNA probe and therefore do not represent specific hybridizations. Scale bar is 100 µm for the microphotographs; the schematic is not drawn to scale.
ratio of the short and long forms due to a splice site mutation leads to a defined disease phenotype of eye-related structures (Epstein et al. 1994). The ratio of the two splice variants involving the paired-box alternative exon in the developing eye of epigean and cave-living *A. fasciatus* is not known, and the characterization of the splice sites has to await cloning of the chromosomal genes.

During the long-term process of evolution of the vertebrate eye, many genes were recruited and fine-tuned to fulfill their specific functions. Although regressive evolution of the eye took place on a shorter time scale, it most likely involves the alteration of several gene functions. Further investigations are necessary to elucidate the types of mutations which have occurred during regressive evolution and to identify the target genes.

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