Isolation and developmental expression of the amphioxus Pax-6 gene
(Amphipax-6): insights into eye and photoreceptor evolution

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SUMMARY

Pax-6 genes have been identified from a broad range of invertebrate and vertebrate animals and shown to be always involved in early eye development. Therefore, it has been proposed that the various types of eyes evolved from a single eye prototype, by a Pax-6-dependent mechanism. Here we describe the characterization of a cephalochordate Pax-6 gene. The single amphioxus Pax-6 gene (AmphiPax-6) can produce several alternatively spliced transcripts, resulting in proteins with markedly different amino and carboxy termini. The amphioxus Pax-6 proteins are 92% identical to mammalian Pax-6 proteins in the paired domain and 100% identical in the homeodomain. Expression of AmphiPax-6 in the anterior epidermis of embryos may be related to development of an olfactory epithelium. Expression is also detectable in Hatschek’s left diverticulum as it forms the preoral ciliated pit, part of which gives rise to the homolog of the vertebrate anterior pituitary. A zone of expression in the anterior neural plate of early embryos is carried into the cerebral vesicle (a probable diencephalic homolog) during neurulation. This zone includes cells that will differentiate into the lamellar body, a presumed homolog of the vertebrate pineal eye. In neurulae, AmphiPax-6 is also expressed in ventral cells at the anterior tip of the nerve cord; these cells are precursors of the photoreceptive neurons of the frontal eye, the presumed homolog of the vertebrate paired eyes. However, AmphiPax-6 expression was not detected in two additional types of photoreceptors, the Joseph cells or the organs of Hesse, which are evidently relatively recent adaptations (ganglionic photoreceptors) and appear to be rare exceptions to the general rule that animal photoreceptors develop from a genetic program triggered by Pax-6.

Key words: Amphioxus, Cephalochordate, Pax-6, AmphiPax-6, Homeobox, Paired box, Eye, Photoreceptor, Pituitary

INTRODUCTION

Pax-6 genes, which encode transcription factors containing a paired domain and a homeodomain, play an important role in a variety of developmental processes ranging from regionalization to cell type specification (Pituello, 1997; Stoykova and Gruss, 1994). Recent studies of Pax-6 genes have emphasized their function as master genes high up in the genetic hierarchy directing eye development and led to the proposal of a single evolutionary origin for all animal photoreceptors (Gehring, 1996a,b; Halder et al., 1995a,b; Quiring et al., 1994). This hypothesis was further supported by the isolation of additional conserved genes like sparkling/Pax2, eyes absent/Eya and sine oculis/Six3 (Fu and Noll, 1997; Oliver and Gruss, 1997) that are involved in eye development, both in Drosophila and vertebrates.

Pax-6 homologs are expressed not only in photoreceptors, but also in olfactory receptors and parts of the central nervous system in most of the animal groups studied so far: namely, flatworms, roundworms, nemerteans, molluscs, arthropods, echinoderms, tunicates and vertebrates (reviewed by Callaerts et al., 1997). In addition, vertebrates express Pax-6 in the anterior hypophysis (pituitary) (Walther and Gruss, 1991) and in pancreatic α-cells and β-cells (Turque et al., 1994).

Here we describe the isolation of a Pax-6 gene (AmphiPax-6) of the cephalochordate amphioxus Branchiostoma floridae. Cephalochordates are thought to be the closest living invertebrate relatives of the vertebrates on the basis of anatomy and molecular biology (Presley et al., 1996; Wada and Satoh, 1994), and recent fossil evidence suggests that the cephalochordate body plan has been kept nearly unchanged since the lower Cambrian (Shu et al., 1996). Amphioxus resembles the vertebrates most conspicuously in having pharyngeal gill slits, segmented axial muscles, a notochord and a dorsal, hollow neural tube; however, amphioxus lacks an axial skeleton and definitive neural crest.

Amphioxus is unusual among animals in having four different kinds of photoreceptors: the frontal eye, the lamellae organ, the Joseph cells and the organs of Hesse (Lacalli et al., 1994). This diversity of photosensory structures might prove valuable for investigating the evolution of eyes and their...
dependence on a Pax-6-triggered genetic pathway (Whittaker, 1997). Moreover, amphioxus is the only invertebrate having structures that are probably homologous, respectively, to the vertebrate anterior hypophysis and to the vertebrate pancreatic α-cells and β-cells (Nozaki and Gorbman, 1992; Reinecke, 1981). For amphioxus, our data suggest that Pax-6 plays a role in the developing olfactory epithelium and anterior hypophysis (Hatschek’s pit), but not in the putative pancreatic cells or in the spinal cord. In the developing photoreceptors of amphioxus, Pax-6 expression was only detected in the lamellar organ and frontal eye, presumed homologs of the vertebrate pineal eye and paired eyes, respectively. In contrast, development of the Joseph cells and organs of Hesse (‘ganglionic’ photoreceptors, as defined by Salvini-Plawen and Mayr, 1977) is evidently directed by a gene cascade not including Pax-6. Therefore it is likely that almost all animal photoreceptors have a monophyletic origin and are characterized by a Pax-6-triggered gene cascade; the only exceptions to this rule probably belong to the minor class of ganglionic photoreceptors.

MATERIALS AND METHODS

Obtaining amphioxus and purification of DNA and RNA

Adults of the Florida amphioxus, Branchiostoma floridae, were collected in Tampa Bay, Florida. Gametes from electrically stimulated adults were fertilized, and the embryos and larvae were raised in laboratory culture (Holland and Holland, 1993). Genomic DNA purification and cDNA library construction are described in L. Z. Holland et al. (1996). Poly(A)+ RNA for marathon cDNA libraries was prepared from adults which were fertilized, and the embryos and larvae were raised in laboratory culture during the first 25 days of development (L. Z. Holland et al., 1996). Fertilization envelopes were removed with insect pins from prehatching stages to insure penetration of reagents. Two different riboprobes were used and both gave identical results. The first corresponds to the 585 bp fragment covering the paired box and the homeobox, and the second to the 3′ end beginning at the homeobox and including the complete 3′ UTR. After being photographed as whole mounts, embryos were counterstained pink in 1% Ponceau S in 1% aqueous acetic acid, dehydrated in ethanol, embedded in Spurr’s resin and prepared as 3.5 μm sections.

RESULTS

Isolation of AmphiPax-6

The Pax-6 homolog of the cephalochordate, Branchiostoma floridae, was isolated by a low-stringency PCR approach using degenerate primers directed against two conserved regions of paired domains corresponding to amino acids YYETG and WFSNS within the homeodomain, in combination with a Pax-6-triggered gene cascade; the only exceptions to this rule probably belong to the minor class of ganglionic photoreceptors.

Fig. 1. Molecular characterization of AmphiPax-6. (A) Nucleotide and deduced amino acid sequence of the AmphiPax-6 cDNA clone 12.1 and the alternative 5′ end of clone J2. The paired domain and the homeodomain are boxed. Arrowheads demarcate the PCR fragment used for genomic Southern blots and library screening. In frame stop codons and the putative polyadenylation signal are underlined, asterisks represent the stop codon. Numbers indicate the position of variations of the base sequences between the five sequenced clones 12.1, 4.2, 12.2, 4.1 and J2, which lead to changes in the deduced amino acid sequences: 1, alternative 5′ end for clone 4.2; 2, alternative 5′ end for clone 12.2, 4.1 and J2 as shown above, leading to the amino acids MG upstream of paired domain; 3, instead of the base G there is an A in clone 4.2, 12.2, 4.1 and J2 leading to Q in the deduced amino acid sequence; 5, deletion of the base sequence encoding amino acids CVSKILGR in clone 12.2, 4.1 and J2 leading to Q in the deduced amino acid sequence; 6, deletion of the base sequence encoding amino acids CVSKILGR in clone J2; 6, instead of the base C there is a G in clone 4.2, 12.2, 4.1 and J2 leading to R in the amino acid sequence, clone 12.1 misses at this position the base sequence encoding for amino acids WEN; 7, insertion of 17 bases in clone 4.2, 4.1 and J2 leading to LFFQA* termination in the amino acid sequence; 8, 3′ end of clone 12.2 (no poly(A)* tail); 9, 3′ end of clone 4.2 (no poly(A)* tail); 10, 3′ end of clone 4.1 (no poly(A)* tail); 11, 3′ end of clone 12.1 and J2 (polyadenylation signal and poly(A)* tail are present). (B) Schematic structure of the five sequenced AmphiPax-6 clones 12.1, 4.2, 12.2, 4.1 and J2. 5′ and 3′ UTR are indicated as lines, ORF is boxed, paired domain and homeodomain are in gray. Putative protein length is indicated above each ORF. Numbers refer to variations in the five clones as indicated in A. (GenBank accession numbers: AmphiPax-6 12.1, AJ223440; AmphiPax-6 4.2, AJ223443; AmphiPax-6 12.2, AJ223444; AmphiPax-6 4.1, AJ223442; AmphiPax-6 J2, AJ223441).
Fig. 2. Comparison of the amino acid sequence of the paired domains in yellow (A) the homeodomains in green (B) and a conserved motif at the C terminus of some Pax-6 genes. Asterisks represent the stop codon.

(A) Sequence comparison for the paired domain (yellow) and the conserved motif in the linker region. The open arrowhead highlights the location of the splice site, where via alternative splicing a 42 bp fragment is inserted into the backbone contacts.

(B) A comparison of the homeodomains in green (B) and a conserved motif at the C terminus of some Pax-6 genes. Asterisks represent the stop codon.

(C) Sequence comparison of the conserved motif found at the C terminus of some Pax-6 genes. Asterisks represent the stop codon.
genes, as for example in transcripts of up to 32 bp within the 3′ UTR. Furthermore, there are deletions and insertions that cause splicing and not due to the presence of two or more splice sites. AmphiPax-6 is quite normal for amphioxus genes like Branchiostoma floridae. Genomic Southern blot analysis of DNA pooled from 20000 embryos, derived from AmphiEn, AmphiDll, AmphiPax-5 and AmphiPax-6 clones. Sequencing of five clones named AmphiPax-6 12.2, 4.1, J2, 12.1 and 4.2 (Fig. 1) shows variation in the base sequences, leading to some differences in the deduced amino acid sequences, as well as alternative spliced forms. Clone J2 (Fig. 1) is 4557 bp long with an open reading frame (ORF) containing a putative translation initiation site, preceded by one in-frame stop codon, at position 470 and terminating at position 1921. After a long 3′ UTR, there is a polyadenylation signal followed by a poly(A)⁺ tail. AmphiPax-6 12.2, 4.1 and J2 have an alternative 5′ end (Fig. 1, Number 2) at a highly conserved splice site within codon one of the paired box of known Pax-6 genes (Fig. 2), causing an alternative N terminus as well as a change from G to R for the first position of the paired domain. AmphiPax-6 J2 misses the CVSKILGR motif within the paired domain and therefore nearly the complete α-helix3 of the N-terminal globular part of the bipartite paired domain (Fig. 1, Number 5; Fig. 2A) (Czerny et al., 1993; Xu et al., 1995). A 17 bp insert at position 1861 in AmphiPax-6 4.2, 4.1 and J2 causes an alternative termination (Fig. 1, Number 7), namely LFFQA*, instead of the relatively conserved C terminus (Fig. 2). All other variations between these five clones, causing deduced amino acid differences, as well as their accession numbers, are indicated in the caption of Fig. 1. There are base sequence differences that are not causing any changes within the deduced amino acid sequence (approximately one out of 100 positions in the coding region and one out of 40 positions in the 5′ UTR and 3′ UTR). Furthermore, there are deletions and insertions of up to 32 bp within the 3′ UTR. This level of polymorphism is quite normal for amphioxus genes like AmphiEn, AmphiDll or AmphiPax-1 (L. Z. Holland et al., 1996; N. D. Holland et al., 1997). Furthermore, the screened cDNA library was generated from approximately 20000 embryos, derived from 150 adults, and not from a single individual.

The open reading frame of AmphiPax-6 12.1 (Fig. 1) encodes a protein of 483 amino acids containing a N-terminal paired domain, a linker region of 96 amino acids containing a conserved 11 amino acid motif and a paired type homeodomain. The paired domain reveals 92% and the homeodomain 100% amino acid identity to the respective domains of the human and mouse Pax-6 proteins and they exhibit all Pax-6-specific amino acids, whereas there are significant differences towards the evolutionary closely related Pax-2, Pax-5 and Pax-8 genes (Fig. 2A,B; Balczerek et al., 1997). The C-terminal region is 174 amino acids long, proline-, serine- and threonine-rich, and contains a conserved termination motif (Fig. 2C). Therefore, the cloned Branchiostoma floridae gene is a homolog of the known Pax-6 genes of vertebrates and invertebrates.

**Genomic Southern blot analysis**

To confirm that the diversity found in the five sequenced AmphiPax-6 clones is due to polymorphism and alternative splicing and not due to the presence of two or more Pax-6 genes, as for example in Drosophila melanogaster, where two Pax-6 homologs (ey and toy) are present (Callaerts et al., 1997), Southern blot analysis was performed. A single hybridization band resulted from digestion by six out of seven restriction enzymes in genomic Southern blots probed at moderate stringency, with a fragment of AmphiPax-6 comprising the linker region flanked by parts of the paired box and homeobox (Fig. 3). This pattern is consistent with the presence of only a single Pax-6 gene in the genome of Branchiostoma floridae. The two bands after digestion with BstXI, presumably resulted from polymorphism and/or cutting within introns included in the cloned gene. The preponderance of single hybridization bands also indicate that AmphiPax-6 is a single copy gene.

**Developmental expression of AmphiPax-6**

No transcripts of AmphiPax-6 were detected by whole-mount in situ hybridization during cleavage, blastula and most of the gastrula stage of Branchiostoma floridae. At very late gastrula, there was weak transient expression in the dorsal hypoblast (Fig. 4A, arrow) and more conspicuous expression throughout the anterior third of the neural plate (Fig. 4A, arrowhead; Fig. 4B). At early neurula (Fig. 4C-F), transcripts appear in the ectoderm cells at the anterior end of the embryo and the expression in the anterior neural plate becomes localized in two bilateral spots. At mid neurula (Fig. 4G-K), the lateral edges of the neural plate are rolling up dorsally to form the hollow neural tube; near the anterior end of the neural tube, the two spots of AmphiPax-6 expression are merging medially. In the mid-neurula, expression continues in the anterior ectoderm and transcripts are also now present in endoderm cells located dorsolaterally on either side of the foregut where the left and right Hatschek’s diverticula are just beginning to evaginate (diagrammed in Fig. 5A).

By the late neurula stage, the neural canal is somewhat dilated at the anterior end of the neural tube and forms the cerebral vesicle (region between the arrowheads in Fig. 5H). In the late neurula (Fig. 4L-P), expression continues in the anterior neural tube, where transcripts are conspicuous in several anteroventral cells of the frontal eye, probably in the type 1 and/or type 2 receptor cells of Lacalli (1996), and in numerous cells constituting the posterior half of the cerebral vesicle, including the lamellar organ, a putative homolog of the vertebrate pineal eye (epiphysis). In the posterior half of the cerebral vesicle, most of the neural cells express AmphiPax-6, which continue to be expressed throughout gastrulation. The expression domain also extends past the brain to the pharynx and gut by the posterior neurula stage. By mid neurula, the expression of AmphiPax-6 is restricted to the neural tube, including the olfactory organ and the cerebral vesicle, and in the otic vesicles. At very late neurula, the expression is restricted to the otic vesicle and the cerebral vesicle, which is maintained throughout the rest of development. At early larval stage, the expression is restricted to the fore gut and hind gut. By mid larval stage, the expression is restricted to the posterior part of the hind gut, which is a feature common to all Pax-6 homologs.
irrespective of their dorsoventral position. No expression is detectable in any neural tube cells posterior to the cerebral vesicle. At the late neurula stage, AmphiPax-6 expression continues in the anterior ectoderm and is detectable in the posterior cells lining Hatcheck’s left diverticulum, whereas the expression in the right diverticulum has disappeared.

In the 2-gill-slit larva (Fig. 4Q,R), the expression pattern is similar to that already described for the late neurula. Neural expression is detected in some cells of the frontal eye and in the posterior half of the cerebral vesicle. Transcripts are also conspicuous in the anterior ectoderm and in the posterior cells of Hatschek’s left diverticulum, which now opens to the exterior as the preoral ciliated pit (diagrammed in Fig. 5C). No transcripts are detectable in the two photoreceptor cells of Hesse differentiating in association with the primary pigment spot. By the 3-gill-slit stage (5 days old), none of the larval tissues express AmphiPax-6 at levels detectable by whole-mount in situ hybridization.

DISCUSSION

Sequence conservation of AmphiPax-6

The amphioxus Pax-6 gene AmphiPax-6 codes for a protein containing a paired domain and a homeodomain showing high sequence identities in the range of 80 to 100% with the
respective domains of vertebrate and invertebrate Pax-6 genes as well as considerable sequence identity outside of these conserved DNA-binding motifs, especially at the C terminus (Fig. 2A-C). Moreover, AmphiPax-6 is clearly distinct from proteins encoded by the evolutionarily closely related Pax genes of the 2/5/8 subclass (Noll, 1993) (Fig. 2A). Therefore, AmphiPax-6 is orthologous to the other invertebrate and vertebrate Pax-6 genes.

Within the paired domain, one of the AmphiPax-6 clones (J2) lacks the amino acids CVSKILGR (positions 49-56) corresponding to nearly the complete recognition α-helix 3 of the N-terminal DNA-binding motif (Figs 1, 2A) (Czerny et al., 1993; Xu et al., 1995). The omission would be expected to alter the DNA-binding specificity of this Pax-6 form, possibly shifting the binding activity toward the C-terminal DNA-binding motif of the paired domain or toward the homeodomain. A comparable restriction in DNA sequence specificity is also known within the paired domain of some other chordate Pax genes: alternative splicing in the paired domain of Pax-8 results in an extra serine residue between R56 and Y57 and in the paired domain of Pax-6 in a 14-amino acid insertion between Q44 and V45 (Fig. 2A) (Epstein et al., 1994; Kozmik et al., 1997). An isoform containing a 14-amino acid insertion in the paired domain, within the vertebrates already present in fish (Püschel et al., 1992), seems to be absent in amphioxus, as we were unsuccessful in obtaining it, either by PCR (data not shown) or by library screening. Hence, this isoform appears to be vertebrate-specific, having evidently arisen in the vertebrate evolutionary line after the separation of the cephalochordate and vertebrate lineages. Three of the five AmphiPax-6 clones (1, 4, 2 and J2) have a 17-base insertion that results in a protein lacking the conserved C-terminal region (Figs 1, 2C). The isoform with the altered C terminus could well have different transactivation properties. Isoforms with different C termini and presumably altered functions have previously been described for human Pax-8 (Kozmik et al., 1993) and human Pax-2 (Ward et al., 1994).

For the phylum Chordata, sequence data for Pax-6 proteins are now available for all three subphyla: namely, Urochordata (tunicates), Cephalochordata (amphioxus) and Vertebrata. A comparison of amino acid sequences between vertebrate Pax-6 and AmphiPax-6 proteins reveals that the paired domain is 92% identical, the homeodomain is 100% identical and the conserved motif near the C terminus is present (Fig. 2). When tunicate and vertebrate Pax-6 proteins are similarly compared, amino acid identities in the paired and homeodomains are lower (87% and 95%, respectively), and no conserved C-terminal domain was detected (Fig. 2) (Glardon et al., 1997). This comparison adds to a growing body of genetic evidence that amphioxus and the vertebrates are sister groups (Garcia-Fernandez and Holland, 1994; Gee, 1996; Wada and Satoh, 1994).

**Ectodermal AmphiPax-6 expression**

During amphioxus development, conspicuous transcripts of AmphiPax-6 are detected in the anterior ectoderm from the early neurula stage (Fig. 4C-E) until the early larval stages (Fig. 4Q.R). The head surface ectoderm of amphioxus includes an especially high concentration of primary sensory neurons (i.e. cells sending an axon directly toward the central nervous system) (Baartrup, 1981; Fritzsch, 1996). The sensory modalities of these neurons are not known, but it has been suggested that they might be chemoreceptors (Stokes and Holland, 1995). This AmphiPax-6 expression pattern is comparable to that of vertebrates, where the Pax-6-expressing ectoderm on the head surface gives rise to nasal placodes (future olfactory epithelium) and eye placodes (future lens and cornea) (Grindley et al., 1995; Walther and Gruss, 1991). Since amphioxus has no lens or cornea, AmphiPax-6 expression in the head surface ectoderm may be involved in the development of an olfactory epithelium. This is in agreement with the developmental expression of Pax-6 in nemerteans.

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**Fig. 5.** (A-G) Anterior ends of developing and postlarval amphioxus showing development of Hatschek’s left diverticulum into Hatschek’s pit (probable homolog of vertebrate adenohypophysis). Mid neurula (A), late neurula (B) and early larva (C) in dorsal view; early larva (D), larva beginning metamorphosis (E), larva in late metamorphosis (F) and postmetamorphic animal (G) in side view. Circular arrow in E indicates anterior edge of mouth rotating into plane of page, and arrows in F indicate the rotation of the epidermal region anterior to the mouth, thus moving the preoral ciliated pit from left side to ventral side, where it becomes the prebuccal cavity. Preoral ciliated pit in D-F is stippled, and triangle indicates small region destined to become Hatschek’s pit in postmetamorphic animals. G includes notochord and nerve cord with black dots indicating anterior pigment spot and organs of Hesse. (H) Diagrammatic side view of nerve cord of postlarval amphioxus; cerebral vesicle delimited by the two arrowheads; the four cell groups proposed as photoreceptors are the frontal eye (fe), lamellar organ (lo), Joseph cells (jc), and organs of Hesse (oh). Abbreviations (in alphabetical order) are: aps, anterior pigment spot; fe, frontal eye; hld, Hatschek’s left diverticulum; hrd, Hatschek’s right diverticulum; hp, Hatschek’s pit; ic, infundibular cells; jc, Joseph cells; lo, lamellar organ; mo, mouth; no, notochord; nc, nerve cord; oh, organs of Hesse; ph, Pharynx; bpc, prebuccal cavity; pocp, preoral ciliated pit; re & n, receptor cells and neurons; rn, rostral nerve; vmo, velar mouth.
chemoreceptor cells and in the squid olfactory organs (Loosli et al., 1996; Tomarev et al., 1997).

**Pax-6 and peptide endocrine cell differentiation**

In vertebrates, Pax-6 expression is necessary for the normal development of pancreatic islet cells (St-Onge et al., 1997), and Pax-6 contributes to glucagon and insulin transcription in vivo (Sander et al., 1997). In amphioxus, some cells lining the midgut contain endocrine peptides (including glucagon and insulin) and were proposed to represent homologs of pancreatic islet cells of vertebrates (Reinecke, 1981). However, AmphiPax-6 expression is not detectable in these endocrine peptide cells in the amphioxus midgut. Therefore, the Pax-6-dependent control of pancreatic cell differentiation seems to be a vertebrate innovation.

**AmphiPax-6 expression in a possible homolog of the vertebrate anterior hypophysis**

During the neurula stage of amphioxus, two evaginations (Hatschek’s right and left diverticula) form at the anterior end of the pharynx and pinch off (Fig. 5A,B). There is general agreement that the right diverticulum becomes a minor coelomic space, but the nature of the left diverticulum is much more controversial. To facilitate exposition, we will accept the majority view (Goodrich, 1917) that the left diverticulum represents a premandibular coelom and is thus mesodermal. The developmental fate of Hatschek’s left diverticulum is peculiar and complicated. In the beginning it fuses with the epidermis to form a concavity (the preoral ciliated pit) on the left side of the head (Fig. 5C,D). Then, during metamorphosis, the concavity enlarges and rotates from the left side to the ventral side of the body (Fig. 5E-G). Most of the concavity becomes the prebuccal cavity of the postlarval amphioxus, but a small region becomes Hatschek’s pit (Fig. 5G), which is probably homologous to the vertebrate anterior hypophysis according to cytological and endocrinological data (Nozaki and Gorbman, 1992; Welsch and Welsch, 1978; Zhang et al., 1982).

During vertebrate development, the anterior hypophysis originates, at least in large part, from Rathke’s pouch, which is typically an invagination of epidermal cells. Moreover, in some lower vertebrates, Rathke’s pouch establishes a temporary connection with the premandibular coelom (Holmes and Ball, 1974), although it is not known whether this temporary union adds a component of mesodermal cells to the invaginating ectoderm. If so, Rathke’s pouch of some vertebrates would resemble the preoral ciliated pit of amphioxus in being a composite of a surface invagination and elements of a premandibular coelom.

At first glance, the expression of Pax-6 homologs in amphioxus and vertebrate rudiments of the anterior hypophysis appears to fit well with the homology between these structures. However, when looked at in detail, the data are more equivocal, because AmphiPax-6 expression appears to be limited to mesoderm cells of the preoral ciliated pit, while vertebrate Pax-6 expression in Rathke’s pouch seems to be expressed in epidermal cells (Walther and Gruss, 1991). The meaning of this discrepancy might be clarified by additional study of more specific markers of the anterior hypophysis (e.g. Pit-1) during amphioxus development.

**AmphiPax-6 expression in the central nervous system and photoreceptors**

The amphioxus central nervous system comprises a dorsal, hollow neural tube with a small cerebral vesicle (region between arrowheads in Fig. 5H) at its anterior end. It is likely that the cerebral vesicle is homologous to the vertebrate diencephalic forebrain (N.D. Holland et al., 1996; Lacalli et al., 1994), and the remaining part of the neural tube is homologous to the vertebrate hindbrain and spinal cord (Holland et al., 1992, 1994), although it remains possible that the small region just posterior to the cerebral vesicle is homologous to the vertebrate midbrain (Lacalli, 1996).

In amphioxus, expression of AmphiPax-6 in the central nervous system is detectable by in situ hybridization in the cerebral vesicle, but not in more posterior parts of the central nervous system. In the cerebral vesicle, the expression in the anterior half is limited to a few cells of the developing frontal eye, but expression in the posterior half is ubiquitous (considerably more extensive than the rudiment of the lamellar organ, the proposed homolog of the vertebrate epiphysis (pineal)). It is likely that AmphiPax-6, like mouse Pax-6 (Grindley et al., 1997), is involved in patterning the diencephalon. The absence of detectable AmphiPax-6 expression in more posterior regions of the amphioxus central nervous system contrasts with the more widespread neural expression in Drosophila (Quiring et al., 1994), tunicates (Gardon et al., 1997) and vertebrates (Osumi et al., 1997; Pituello, 1997) in which Pax-6 transcripts are detectable in specific regions throughout the central nervous system posterior to the brain. Since Pax-6 is expressed in the spinal cord of tunicates, which are the most basal group within the phylum chordata, the absence of AmphiPax-6 expression in the amphioxus hindbrain and spinal cord may represent the loss of a primitive chordate feature.

On the basis of fine structure and limited behavioral studies, four different cell groups in the amphioxus neural tube (Fig. 5H) are currently believed to be photoreceptors (Lacalli, 1996; Lacalli et al., 1994; Ruiz and Anadon, 1991). First is the frontal eye, comprising the anterior pigment spot and associated receptor cells and neurons located anteroventrally in the cerebral vesicle. Second is the lamellar organ, which is located dorsally in the posterior half of the cerebral vesicle. Third are the Joseph cells, which are located dorsally and extend from the level of the lamellar organ to a point several hundred micrometers posteriorly. And fourth are the bicellular organs of Hesse, which are located ventrally and extend from the level of the most posterior Joseph cells almost to the caudal extremity of the nerve cord.

In the embryos and early larvae of amphioxus, AmphiPax-6 is expressed in receptor cells and/or neurons of the frontal eye and in a more posterior region of the cerebral vesicle where the lamellar organ is developing. These expression data help support (1) the homology between the amphioxus lamellar organ and the vertebrate epiphysis (pineal), and (2) the homology between the amphioxus frontal eye and vertebrate lateral eyes, all expressing Pax-6 (Callaerts et al., 1997; Walther and Gruss, 1991). Interestingly, a single morphogenetic field gives rise to the bilateral symmetric eye anlagen of vertebrates (Adelmann, 1929a,b; Li et al., 1997) and Sonic hedgehog (Shh) has been proposed as the prechordal
plate signal for resolving the retina field, as lack of Shh gene function leads to cyclopia (Belloni et al., 1996; Chiang et al., 1996; Roessler et al., 1996). Nevertheless, the present result of AmphiPax-6 expression does not help resolve the outstanding question of whether one eye, as the frontal eye of amphioxus, or two eyes is the more primitive condition in the evolutionary line leading to vertebrates (Lacalli et al., 1994).

The failure to detect AmphiPax-6 expression in the other proposed photoreceptor of amphioxus evidently does not result from poor penetration of reagents during whole-mount in situ hybridization, since the earliest photoreceptor cells of the organs of Hesse are present, but contain no detectable transcripts of AmphiPax-6 at larval stages (e.g. Fig. 4Q) in which other cells express the gene strongly. The Joseph cells do not appear until a later larval stage (Lacalli, personal communication), but cytologically they are virtually the same cell type as the photoreceptor cells of the organs of Hesse (Joseph, 1904; Ruiz and Anadon, 1991).

During development, these cells evidently originate from already differentiated neurons that are re-programmed to become ‘ganglionic’ photoreceptors (Salvini-Plawen and Mayr, 1977), a rare type of photoreceptors that has not yet been studied at the molecular genetic level. The exceptional nature of the Joseph cells and organs of Hesse was first noticed by Joseph (1904), who proposed that they differ from the other amphioxus photoreceptors in being recent (cenogenetic) adaptations. If these photoreceptors are indeed relatively recent adaptations, it becomes less surprising that their development should be directed by a gene cascade not triggered by Pax-6.

Possible implications for photoreceptor evolution in metazoans

20 years ago, Eakin (1979) concluded that ciliary photoreceptors arose once in animal evolution and in some lineages gave rise to rhabdomeric photoreceptors. Conversely, Ghiselin (1988) suggested that the first photoreceptors to appear were rhabdomeric. In either scenario, photoreceptors would have a single origin in animal evolution. In contrast, Salvini-Plawen and Mayr (1977) postulated that rhabdomeric and ciliary photoreceptors arose independently from undifferentiated epithelial cells in dozens of separate phyletic lines. In addition, they also recognized a relatively rare class of ganglionic photoreceptors (see above). Recently, the extreme polyphyly claimed by Salvini-Plawen and Mayr (1977) have been challenged by the discovery that PAX-6 triggers development of both ciliary and rhabdomeric photoreceptors in a variety of animal phyla (Callaerts et al., 1997). This commonality of Pax-6 as a master gene for eye development has been taken as evidence of a single phylogenetic origin for animal photoreceptors (Gehring, 1996a,b).

In amphioxus, the cephalic photoreceptors (frontal eye and lamellar organ) obey this general rule, but the more posterior photoreceptors (Joseph cells and organs of Hesse) apparently develop from a genetic program that is not triggered by Pax-6. It will be interesting to see whether other ganglionic photoreceptors (e.g. in rotifers, gastrotrichs and a few nematodes) also lack Pax-6 expression and lie outside of the main monophyletic line of animal photoreceptors.

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