PAX-6 IN DEVELOPMENT AND EVOLUTION

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ABSTRACT
Pax-6 is a member of the Pax gene class and encodes a protein containing a paired domain and a homeodomain. The molecular characterization of Pax-6 genes from species of different animal phyla and the analysis of Pax-6 function in the developing eyes and central nervous system of vertebrates, Drosophila melanogaster, and Caenorhabditis elegans suggest that Pax-6 homologues share conserved functions. In this review, we present recent data on the structural and functional characterization of Pax-6 homologues from species of different animal phyla. We discuss the implications of these findings for our understanding of the development and evolution of eyes and nervous systems.

INTRODUCTION
The molecular and genetic analyses of the development of vertebrates, Drosophila melanogaster, Caenorhabditis elegans, and other organisms have yielded a wealth of information about the underlying mechanisms of development. The theme emerging from these studies is that conserved regulatory proteins are involved in the development of animals of strikingly different architecture and embryogenesis. The Hox genes, which are a subset of the family of homeobox-containing genes, are dramatic examples of functional conservation (Gehring et al 1994). Hox genes are common to most or all animals, are organized in clusters, and define positional information along the anteroposterior axis. In addition to the Hox genes, many other homeobox genes appear to have homologous counterparts in diverse species and may also have conserved functions (Manak & Scott 1994). The molecular characterization of Pax-6 genes in vertebrates, D. melanogaster, and C. elegans and the analysis of Pax-6 function in...
developing eyes and central nervous systems suggest that Pax-6 homologues share conserved functions (Halder et al 1995b). Recent data on the structural and functional characterizations of Pax-6 homologues from species of different animal phyla suggest that a Pax-6–dependent mechanism may be a common theme underlying the development and evolution of eyes and nervous systems in the animal kingdom.

THE PAX GENE FAMILY

The study of the genetic basis and molecular mechanisms underlying vertebrate and invertebrate development has resulted in the identification of different families of transcriptional regulators, one of which is the Pax gene family (reviewed in Noll 1993). The characteristic feature of this family is the presence of the so-called paired box. This 384-base pair (bp) DNA sequence encodes a 128–amino acid–long DNA-binding domain, the paired domain, which was originally identified in the D. melanogaster segmentation genes paired, gooseberry, and gooseberry neuro (Bopp et al 1986, Frigerio et al 1986). In addition to the identification of a paired box in two other D. melanogaster genes, Pox meso and Pox neuro (Bopp et al 1989), the paired box was detected in other organisms as diverse as mouse, sea urchin, nematodes, and humans (Burri et al 1989, Dressler et al 1988). The paired box (Pax) genes are grouped into six different classes depending on (a) whether or not they encode, besides the obligatory paired domain, a complete or a partial homeodomain, a conserved octapeptide, or class-specific amino acids, and (b) whether or not the genes share a similar intron-exon structure (Walther et al 1991). An overview of the different classes with their features and constituent members is given in Figure 1.

In D. melanogaster, mutations in the Pax genes paired, gooseberry, gooseberry-neuro, Pox meso, Pox neuro, and eyeless lead to developmental defects in segmentation, neurogenesis, and eye development (Noll 1993, Quiring et al 1994).

Several mutations affecting the Pax genes in the mouse have been described (reviewed in Tremblay & Gruss 1994). Mutations in Pax-1 interfere with normal development of the axial skeleton and result in the undulated phenotype. Pax-3 mutants, called splotch, lead to neural crest and neural tube defects. The eye, nose, and nervous system of Small eye mutants are severely affected by mutations in Pax-6.

Mutations in Pax genes are associated with three congenital disorders in humans: mutations in PAX-2 with autosomal dominant renal anomalies, vesicoureteral reflux, and optic nerve colobomas (Sanyanusin et al 1995); mutations in PAX-3 with Waardenburg’s syndrome type 1 and Klein-Waardenburg syndrome (Baldwin et al 1992, Hoth et al 1993, Morell et al 1992, Tassabehji...
**Figure 1** Schematic representation of the six classes of Pax genes and classification of the known mouse and *D. melanogaster* Pax genes. The gene products contain the obligatory paired domain, and in addition some classes contain the octapeptide and/or a complete or partial homeodomain. (*) *prd* lacks the octapeptide but is nevertheless placed in class II based on the similarities in the DNA-binding domains. *prd*: paired, *gsb*: gooseberry, *gsbn*: gooseberry neuro, *ey*: eyeless, *toy*: twin of eyeless.


A description of the developmental and functional analysis of Pax genes in mouse, humans, other vertebrates, and *D. melanogaster* can be found in the following review articles and in references therein: Noll (1993), Strachan & Read (1994), Stuart et al (1994), and Tremblay & Gruss (1994).

**PAX-6: A HIGHLY CONSERVED TRANSCRIPTION FACTOR**

**Primary Sequences**

*Pax-6* genes have been isolated from species of many different animal phyla; the first to be isolated were from humans, mouse, and zebrafish. The human gene was localized with long-range physical mapping (Davis et al, 1989, Gessler et al, 1989, Lyons et al, 1992, Ton et al, 1991) and subsequently isolated with positional cloning, while the mouse (Walther and Gruss, 1991) and zebrafish genes (Krauss et al, 1991b, Püschel et al, 1992) were isolated by using cross-hybridization to other, previously characterized Pax genes. Other vertebrate *Pax-6* genes have been isolated from quail (Martin et al, 1992), chicken (Goulding et al

The first invertebrate Pax-6 gene to be cloned was from *D. melanogaster* and corresponded to the *eyeless* locus (Quiring et al 1994). Recently, a second Pax-6 gene from *D. melanogaster*, designated *twin of eyeless* (*toy*), was isolated (T Czerny, G Halder, P Callaerts, WJ Gehring & M Busslinger, unpublished data). The identification of *D. melanogaster eyeless* stimulated our group to search for homologous genes in other species of different animal phyla. A PCR-based approach has led to the isolation of Pax-6 homologues from the flatworm *Dugesia tigrina* (M Munoz, P Callaerts, WJ Gehring & E Salo, unpublished data), the ascidian *Phallusia mammillata*, (S Glardon, P Baumgartner, P Callaerts, G Halder & WJ Gehring, unpublished data), the ribbon worm *Lineus sanguineus* (Loosli et al 1996), and the squid *Loligo opalescens* (Tomarev, P Callaerts, L Kos, R Zinovieva, G Halder, WJ Gehring & J Piatigorsky, unpublished data).

Recently, other groups have isolated Pax-6 homologues from the sea urchin *Paracentrotus lividus* (Czerny & Busslinger 1995) and from the nematode *Caenorhabditis elegans* (Chisholm & Horvitz 1995, Zhang & Emmons 1995).

All Pax-6 genes isolated so far encode proteins containing an N-terminally located 128–amino acid (aa)–long paired domain, a linker region of variable length, a 60-aa-long paired-type homeodomain, and a C-terminal proline-serine-threonine (PST)–rich region. The conserved octapeptide that is present in several Pax proteins (Figure 1) is not found in any Pax-6 protein. Mouse and human Pax-6 genes, which we use as a reference for sequence comparisons, encode identical proteins of 422 aa. In these proteins, the N-terminally located paired domain is preceded by only a short stretch of amino acids. Mouse and human Pax-6 proteins have a 78-aa linker separating the paired and homeodomain and a 153-aa C terminus. The length of the other vertebrate Pax-6 proteins varies between 416 aa (quail) and 437 aa (zebrafish). Compared with that of mouse, the overall sequence identity of Pax-6 of other species ranges from 97% (zebrafish) over 99% (quail) to 100% (rat, chicken). The invertebrate Pax-6 proteins are generally longer than their vertebrate counterparts, with the smallest protein being 441 aa (sea urchin) and the longest 836 aa (*D. melanogaster eyeless*). The invertebrate Pax-6 proteins have linkers and C termini that vary considerably in length. The linker region of *eyeless*, for example, is three times as long as the vertebrate linkers.

The paired domains of all Pax-6 proteins display a high degree of sequence conservation. A compilation of Pax-6 paired domain sequences is shown in Figure 2A. The vertebrate Pax-6 proteins share an identical paired domain, with the exception of zebrafish Pax-6, in which a single amino acid is changed. Comparison of the invertebrate Pax-6 proteins with mouse Pax-6 shows that the paired domain sequence identities are mostly over 90% (Figure 2A). This
is true for the *D. melanogaster*, *L. sanguineus*, *P. lividus*, and *L. opalescens* genes. A comparison of the paired domain of these Pax-6 proteins with the paired domain of the other Pax proteins has led to the proposal that the Pax-6 paired domain contains eight amino acids that are Pax-6 specific: serine 21, alanine 34, isoleucine 42, glutamine 44, arginine 66, cysteine 91, isoleucine 114, and alanine 128 (Loosli 1995) (shaded in Figure 2A). Asparagine 47 is an important determinant for DNA-binding specificity (Czerny & Busslinger 1995) (see below) and is shared with Pax-4 only. These amino acids are conserved in all Pax-6 paired domains except for *P. mammillata*, which has a 1-aa substitution, and *C. elegans* and *D. tigrina*, which have 4-aa substitutions in the paired domain. The Pax-6 paired domain of *P. mammillata* is 87% identical, that of *C. elegans* 80%, and that of *D. tigrina* 77% (Figure 2A). The sequence identities of these paired domains are significantly lower when compared with other Pax genes (e.g. *D. tigrina* Pax-6 paired domain is 69% identical with the paired domain of mouse Pax-2 and 56% identical with that of *D. melanogaster* paired), which identifies them as true Pax-6 homologues, although they lack some Pax-6–specific amino acids.

The homeodomain of the Pax-6 genes is also highly conserved (Figure 2B). The vertebrate Pax-6 homeodomains are identical, while their invertebrate counterparts have more than 90% sequence identity to the mouse sequence. The only exception is the homeodomain of *D. tigrina* Pax-6, which is only 73% identical but still significantly more similar than other paired-type homeodomains, identifying it unambiguously as a Pax-6 homeodomain. Most variations in Pax-6 homeodomains are seen at positions 10, 11, and 36 of the homeodomains (Figure 2B). These amino acids are located in the first two helices of the homeodomain and were previously identified as highly variable in a compilation of over 300 homeodomains (Gehring et al 1994). The amino acids of the recognition helix are identical in all Pax-6 homologues, with the exception of alanine 43, which is substituted by a threonine in *D. tigrina* Pax-6. All homeodomains found in Pax genes have a serine at position 50 (position 9 in the recognition helix). The amino acid at this position is a major determinant for the in vitro and in vivo DNA-binding specificity of the homeodomain (Capovilla et al 1994; Hanes & Brent 1989; Percival-Smith et al 1990, 1992; Schier & Gehring 1992; Treisman et al 1989). Sequence comparison of Pax-6 homeodomains and other Pax homeodomains has resulted in the proposal of ten Pax-6–specific amino acids: leucine 1, glutamine 2, asparagine 4, serine 7, arginine 33, lysine 37, proline 41, arginine 58, and glutamic acids 59 and 60 (Loosli 1995) (shaded in Figure 2B). These amino acids are conserved in nearly all Pax-6 homeodomains, with the exception of *L. opalescens* and *D. tigrina* Pax-6, in which only nine out of ten and five out of ten amino acids are conserved, respectively.
Figure 2  Sequence comparison of (A) the paired domains and (B) the homeodomains of Pax-6 genes of different species. Only Homo and Mus are shown for the vertebrate sequences. The other vertebrate sequences are very similar or identical. The secondary structure of the domains are shown at the top of the figure. The filled arrowheads behind the species’ name and in the sequences indicate the Pax-6 genes for which intron-exon boundaries have been determined and where they are located. The open arrowhead in the vertebrate sequence (Homo, Mus) shows the location of the splice site where via alternative splicing a 42-bp fragment is inserted into the paired box. The shaded bars indicate the Pax-6 specific amino acids (Loosli 1995). Percentages of sequence identity were determined by comparison with mouse Pax-6 and are indicated behind the sequences.

Additional sequence similarities are found in N- and C-terminal flanking sequences of the Pax-6 homeodomain (Figure 2B). On average, eight amino acids are conserved in a stretch of twelve amino acids immediately preceeding the homeodomain. The notable exception is D. tigrina Pax-6, in which only two of these amino acids are conserved. C-terminal of the homeodomain, six out of seven amino acids are conserved. Again, the exception is D. tigrina Pax-6, with only one amino acid conserved.

There is an additional region of sequence conservation in the linker of most Pax-6 proteins, namely an undecapeptide with consensus sequence MYDKL-RMLNGQ (Figure 2A). In most cases this motif is found just a few amino acids C-terminal of the paired domain. This sequence is not present in the P. mammillata and D. tigrina Pax-6 linkers and is poorly conserved in C. elegans (4 aa out of 11 identical). A functional significance for this peptide is unknown. However, in a functional assay in D. melanogaster, P. mammillata Pax-6 was able to induce ectopic eyes, thereby demonstrating that the undecapeptide is unnecessary for Pax-6 function in this assay (S Glardon, P Baumgartner, P Callaerts, G Halder, WJ Gehring, unpublished data). The PST-rich C termini of the Pax-6 proteins are highly variable both in length and in amino acid sequence. Nevertheless, sequence conservation is found in vertebrate, sea urchin, and squid Pax-6 genes.

**Alternative Splice Forms**

Alternatively spliced Pax-6 transcripts have been identified in mouse, humans, zebrafish, and chicken. These transcripts have an additional exon of 42 bp that gives rise to a 14-aa insertion between glutamine 44 and valine 45 of the paired domain (Glaser et al 1992, Goulding et al 1993, Püschel et al 1992, Walther & Gruss 1991) (see Figure 2A). A similar exon has not been found in any invertebrate Pax-6 gene, including the urochordate P. mammillata Pax-6; thus, this 14-aa insertion seems to be vertebrate specific. This insertion results in proteins with different binding specificities (see below). A different function for the two proteins is suggested by the different expression levels of the short and extended forms of Pax-6 in the eye and brain of mouse and by the occurrence of a human ocular phenotype caused by aberrant, increased expression of the extended form (Epstein et al 1994b, Richardson et al 1995).

A protein encoded by the Pax-6 gene that contains the homeodomain as the DNA-binding domain and lacks the paired domain has been found in the quail neuroretina (Carrière et al 1993). Zhang & Emmons (1995) recently demonstrated that the Pax-6 gene of C. elegans encodes a second transcript (in addition to the transcript containing the paired domain and homeodomain) that is initiated at a second, internal promoter and that lacks the paired box. In D. melanogaster and L. opalescens, two transcripts have been detected in which different 5′ exons
are used, thereby giving rise to proteins with different sequences N-terminal
to the paired domain (Quiring et al 1994; S Tomarev, P Callaerts, L Kos, R

Genomic Organization
In addition to the high sequence conservation, the significantly similar ge-
nomic structure of the different Pax-6 genes (see Figure 2) provides further
evidence that the genes are orthologous. A splice site in the first codon of the
paired domain is conserved between the Pax-6 genes of humans, mouse, quail,
D. melanogaster, P. mammillata, L. opalescens, and probably L. sanguineus
but is absent in C. elegans (Dozier et al 1993; other references are listed in
Figure 2). A splice site between the codons of serine 116 and valine 117 of
the paired domain is present in the Pax-6 genes of humans, mouse, quail,
D. melanogaster, P. mammillata, L. sanguineus, and C. elegans. The Pax-6 genes
analyzed so far have one additional nonconserved splice site in the paired box
(two in the case of C. elegans). The vertebrate-specific 42-bp exon is inserted
in a splice site between the codons of glutamine 44 and valine 45 by alternative
splicing. This splice site is missing in invertebrates. D. melanogaster and P.
mammillata Pax-6 share another splice site between the codons of arginine 56
and tyrosine 57. This splice site is conserved in Pax-2, -5, and -8 (Walther et al
1991; S Glardon & WJ Gehring, unpublished observation). The C. elegans Pax-
6 gene has splice sites between the codons of glycine 48 and cysteine 49 and
of arginine 74 and valine 75 of the paired domain. The linker region of Pax-6
contains one splice site in D. melanogaster, humans, quail, P. mammillata, and
L. sanguineus and two in C. elegans.

A highly conserved splice site has been found in codon 19 (glutamic acid)
of the homebox of Pax-6 in all species analyzed. A second splice site be-
tween codons 46 and 47 of the homebox (in the recognition helix) is con-
served in quail, humans, L. sanguineus, P. mammillata, and C. elegans Pax-6.
The region 3’ to the homebox contains two splice sites in D. melanogaster,
quail, and C. elegans Pax-6. P. mammillata Pax-6 has three splice sites in this
region.

Because acquisition and loss of introns are rare evolutionary events, the con-
servation of splice sites in both the paired domain and homeodomain strongly
supports the notion that the Pax-6 genes found in the different species are true
homologues.

DNA-Binding Specificity and Structure
Most DNA-binding analyses of proteins containing both a paired domain and
a homeodomain, for example D. melanogaster Paired and the various Pax-6
proteins, have been done with the separate DNA-binding domains. However,
Pax-6 binding to the mouse NCAM-L1 promoter occurred efficiently only when both domains were present in the protein, suggesting that the domains act synergistically to confer Pax-6 binding affinity for this sequence (Chalepakis et al 1994). Similarly, full in vivo activity of *D. melanogaster* Paired also depends on the presence of both DNA-binding domains (Bertuccioli et al 1996). Below, we describe DNA binding and structure of the paired domain and homeodomain.

**PAIRED DOMAIN DNA BINDING** DNA binding of paired domain–containing proteins was first demonstrated for the paired domain of the *D. melanogaster* Paired protein, which binds in vitro to the e3 site, found in the *even-skipped* promoter (Hoey & Levine 1988; Treisman et al 1989, 1991). Functional target sites have been identified for Pax-5, Pax-8, and Pax-6 (Adams et al 1992, Czerny et al 1993, Holst et al 1994, Singh & Birshtein 1993, Zannini et al 1992, Zwollo & Desiderio 1994) (see Figure 3 for Pax-6 references). Consensus binding sites for Paired, Pax-2, Pax-5, Pax-6, and Pax-8 have been derived by comparing natural binding sites (Cvekl et al 1994, 1995a,b; Czerny & Busslinger 1995; Czerny et al 1993) and by PCR-based binding site selection (Epstein et al 1994a, Sun & Desplan 1996) (see Figure 3A,B). Initial analysis of natural Pax-5 binding sites using full-length paired domains yielded only a weak consensus sequence. The subsequent use of a protein lacking the C terminus of the Pax-5 paired domain resulted in the identification of a strong consensus sequence for Pax-5 that consisted of two distinct nonpalindromic half sites. Other paired domain–containing proteins all recognize this Pax-5 consensus sequence, although with different affinities (Czerny et al 1993), suggesting that the different paired domains can bind to similar DNA sequences. Indeed, comparison of the consensus sequences and optimized binding sites shows that the different binding sites are almost identical and share a conserved core motif (see Figure 3A). The Pax-6 binding site defined by PCR selection and the Pax-5 recognition sequence differ essentially in one nucleotide only (see Figure 3A). Pax-6 binds its recognition sequence with high affinity ($K_d = 2.5 \times 10^{-9}$ M) (Epstein et al 1994a). The paired domain binds as a monomer to its nonpalindromic recognition sequence from one side of the DNA helix and thereby interacts with two successive major grooves; the N-terminal subdomain recognizes the more extensive 5′ consensus half-site motif, whereas the C-terminal part of the paired domain interacts with the 3′ consensus motif. The two interactions with these half sites together determine the overall affinity of a given binding site (Czerny et al 1993).

**PAIRED DOMAIN STRUCTURE** Czerny et al’s (1993) model, in which the bipartite paired domain binds to two half sites in adjacent major grooves on the
DNA, has recently been confirmed by X-ray crystallography (Xu et al. 1995) of a complex containing the paired domain from the D. melanogaster Paired protein and a 15-bp DNA duplex. This optimized Paired binding site was isolated by in vitro selection and amplification and is very similar to the optimized sites defined for the other paired domains (see Figure 3A). Modeling of the Pax-6 paired domain with the Swiss model program (Peitsch et al. 1996) has shown that the predicted structure of the Pax-6 paired domain is nearly identical (G Halder, P Callaerts, G Capitani, J Groppe & WJ Gehring, unpublished observations) to the structure of the Paired paired domain (Xu et al. 1995). The description of the Paired paired domain structure below is thus a likely description for the Pax-6 paired domain as well.

The paired domain consists of globular N- and C-terminal domains called PAI and RED, respectively (Jun & Desplan 1996). These domains are separated by a linker, and each domain contains a helical region similar to the homeodomain (see Figures 2A and 4A). The N-terminal PAI domain contains a short run of an antiparallel β-sheet followed by a type II β-turn, three helices with a fold resembling a homeodomain, and a C-terminal tail. The β-sheet (amino acids 4–6 and 10–12) interacts with the sugar phosphate backbone of the DNA. The β-turn (residues 13–16) fits directly into the minor groove of the DNA and makes critical base contacts (Figure 4). This interaction is stabilized by protein-protein and protein-DNA contacts of flanking regions. The N-terminal PAI domain contains three α-helices (residues 20–32, 37–43, and 47–60), with helix 2 and 3 forming a helix-turn-helix (HTH) unit. Helix 2 makes extensive phosphate contacts, and helix 3, the recognition helix, fits directly into the major groove. Side chains from this helix contact base pairs 4–8 of the binding site. The C-terminal tail (residues 65–72) of the N-terminal domain makes minor groove contacts. Conserved residues at the end of helix 3 help fix the position of the extended polypeptide chain. Residues 65–67 run parallel to and make contacts with one strand of the backbone. Residues 68–72, which are invariant in all paired domains, fit directly into the minor groove, where they contact a region adjacent to the one contacted by the β-turn.

DNA-binding specificity of the paired domains of Pax-5 and Pax-6 is determined by amino acids 42, 44, and 47 of the paired domain (Czerny & Busslinger 1995). Isoleucine 42 and glutamine 44 are Pax-6 specific, while asparagine 47 is shared with Pax-4. All other Pax proteins, including Paired, have a glutamine 42, arginine 44, and histidine 47 (see Figure 2A). Crystal structure analysis of the Paired paired domain has shown that residue 42 is near the C-terminal end of helix 2 and that residue 44 is in the HTH motif and interacts with the loop between the two strands of β-sheet. Residue 47 is the first residue of helix 3 and forms a hydrogen bond with the base at position 4 of the binding site (see Figure 3). Swapping experiments have shown that these three residues are
**Figure 4** Line drawings of Paired paired domain–DNA complex and Paired homeodomain dimer–DNA complex. Cylinders indicate α-helices, and arrows indicate β-sheets. (A) Paired paired domain–DNA complex showing the N- and C-terminal globular domains. Helices 1 and 2 in the N-terminal PAI domain are packed in an antiparallel arrangement. Helices 2 and 3 form a helix-turn-helix unit. A similar arrangement is seen in the C-terminal RED domain. Helix 3 (the recognition helix) makes contact in the major groove of the DNA. (Reproduced with permission of Cell Press.) (B) Paired homeodomain dimer–DNA complex. The two homeodomains bind in a head-to-head arrangement on the palindromic DNA site. Each homeodomain consists of three helices that are preceded by the N-terminal arm. The N-terminal arm of one homeodomain interacts with the beginning of helix 2 of the other homeodomain, and the N-termini of the recognition helices (helix 3) approach each other. (Redrawn after a figure kindly provided by C. Desplan.)
crucial for the distinction by Pax-6 and Pax-5 of a guanine versus a thymidine at position 4 of the respective binding sites.

A short linker region (residues 73–78) connects the PAI and RED domains. The C-terminal RED domain of the paired domain contains three helices (residues 79–88, 96–106, and 117–124), which in the case of the Paired paired domain, do not make any DNA contacts. This observation corroborates previous studies indicating that the N-terminal PAI domain provides the important DNA contacts and is sufficient for DNA binding (Cai et al 1994, Chalepakis et al 1991, Czerny et al 1993, Treisman et al 1991). However, the C-terminal RED domain does play a role in DNA recognition by other paired domains. The specificity of BSAP (Pax-5) is determined by both the N- and C-terminal domains; the N-terminal PAI domain makes more extensive DNA contacts (Czerny et al 1993). A Pax-6 splicing variant (Pax-6 5a) contains a 14-aa insertion in the PAI domain, which disrupts its DNA binding and results in the RED domain binding to a binding site other than the Pax-6 PAI consensus (Epstein et al 1994b) (see Figure 3C).

Xu et al (1995) proposed a model for binding of Pax-5 and Pax-6 proteins based on the HTH structure of the RED domain. According to this model, the linker between the N- and C-terminal domains makes contact in the minor groove and the recognition helix of the RED domain (helix 6) binds in the adjacent major groove, where it interacts with base pairs 16–20 of the optimized binding sites for Pax-5 and Pax-6. This model for Pax-6 binding is based on the predicted HTH motif of the C-terminal RED domain, which is similar to the homeodomain and the Hin recombinase, and on the amino acid sequence similarities between this recombinase and those members of the Pax family that use the C-terminal domain in DNA recognition. Modeling constraints imposed by the length of the linker and by the position of the additional base pairs recognized by Pax-6 also support this model.

THE HOMEODOMAIN The crystal structure of the Paired homeodomain has been deciphered (Wilson et al 1995) and is very similar to the structures of other homeodomains, which were determined by solution NMR (Otting et al 1990, Qian et al 1989) and X-ray crystallography (Hirsch & Aggarwal 1995, Kissinger et al 1990, Klemm et al 1994, Wolberger et al 1991). In summary, the homeodomains consist of three α-helical regions folded into a tight globular domain. Helix 1 is preceded by a flexible N-terminal arm and separated by a loop from helix 2, which forms a HTH motif with helix 3 (helix 3/4 in Antennapedia). Helix 3 (the recognition helix) and the N-terminal arm make base-specific contacts in the major groove and in the minor groove, respectively. The core binding motif contacted by the N-terminal arm and helix 3 consists of the TAAT sequence. The amino acid at position 9 of helix 3 (position 50 in the homeodomain) interacts with the 2 bp immediately 3’ to this core sequence.
Researchers have shown that different amino acids at position 9 can confer different DNA-binding specificities (Hanes & Brent 1989, Percival-Smith et al 1990, Schier & Gehring 1992, Treisman et al 1989).

All the previous structure determinations were done for homeodomains that bind to DNA as monomers. However, the paired class homeodomains preferentially bind cooperatively as dimers to palindromic sequences composed of two TAAT half sites (Wilson et al 1993). The spacing between the half sites, the magnitude of the cooperative interaction, and the identity of the base pairs at the center of the palindromic sites are determined by residue 9 of helix 3. The presence of a serine at position 9 in helix 3 results in a more compact structure of the cooperative dimer of DNA and in preferential binding to a P$_2$ site (2 bp spacing) (Figure 3D). A less compact cooperative dimer that preferentially binds to a P$_3$ site (3 bp spacing) (Figure 3D) is observed when a glutamine or a lysine is present at position 9. Nevertheless, homeodomains with a serine at position 9 also bind to a P$_3$ site but with an affinity of about one half that of P$_2$ (Wilson et al 1993). D. melanogaster Paired, which has a serine at position 9, preferentially dimerizes on P$_2$ sites. In contrast, the Pax-6 homeodomain, which also has a serine at position 9, preferentially dimerizes on P$_3$ sites (Czerny & Busslinger 1995). Furthermore, the importance of the central base pairs in the palindromic binding site was emphasized by the fact that replacement of a CG dinucleotide (in case of a P$_2$ site) by GC almost completely abolished cooperative binding of the Pax-6 homeodomain (Czerny & Busslinger 1995).

The crystal structure of the Paired homeodomain dimer reveals that each homeodomain consists of a flexibly disordered N-terminal arm (residues 1–9), helix 1 (residues 10–22), a connecting loop of residues (23–27), helix 2 (residues 28–37), a turn of three residues (38–41), and helix 3 (the recognition helix extending from residue 42 to the C terminus of the homeodomain) (Figures 2B and 4B). Helices 1 and 2 are arranged antiparallel relative to each other, and helix 3 is positioned perpendicular to the first two helices. Helices 2 and 3 form a HTH motif (Figure 4B). The recognition helix is inserted into the major groove of the DNA, with two residues (valine 47 and asparagine 51) making direct contacts with two bases. Position 50 of the homeodomain, which is critical for conferring different DNA binding specificities to homeodomains, interacts with specific base pairs via water as was first demonstrated for the Antennapedia homeodomain by NMR spectroscopy (Billeter et al 1993). The N-terminal arm of the Paired homeodomain makes extensive DNA backbone contacts and may contribute to the specific recognition of the TAAT core motif. The cooperative homeodomain dimer binds to the P$_3$ site in a head-to-head arrangement; the N-terminal arm of one homeodomain interacts with the beginning of helix 2 of the other, and the N termini of the recognition helices approach each other (Figure 4B). The DNA is bent, primarily at the center, by about 20° (Wilson et al 1995).
Thus, paired class homeodomains have achieved cooperativity in DNA binding without the assistance of other DNA-binding domains, thereby enabling the recognition of target sequences that are long enough to ensure specificity. The ability to dimerize cooperatively on the P3 sequence is specific for the paired class of homeodomains. Most of the interactions between the two homeodomains are made by main chain atoms or by the Cβ atoms of side chains. The confinement of cooperativity to the paired class of homeodomains can be explained by the lack of side chains that would prevent intimate association of the homeodomains. Nearly all nonpaired class homeodomains have arginine residues at position 28, 43, or both that abolish cooperative binding of the homeodomain (Wilson et al 1995). The cooperativity is facilitated by conformational changes in both DNA and protein (reciprocal induced fit), which are induced by the binding of a single homeodomain to one half site.

Transactivation
Plaza et al (1993) showed that quail Pax-6 can bind to and transactivate its own promoter in a cell culture assay. Pax-6 of mouse, chicken, and guinea pig binds to and transactivates the promoters of mouse αA crystallin, chicken αA and δ1 crystallin, and guinea pig ζ crystallin in transient transfection assays (Cvekl et al 1994, 1995a,b; Richardson et al 1995). Sea urchin and mouse Pax-6 are able to stimulate Pax-6–responsive reporter constructs in transient transfection assays (Czerny & Busslinger 1995) in which transactivation is critically dependent on Pax-6 concentration with low concentrations resulting in activation and high concentrations leading to repression. This effect could reflect self-squelching of the Pax-6 transactivation domain. Fusion of the proline-, serine-, and threonine-rich C-terminal domain of sea urchin and mouse Pax-6 to the DNA-binding domain of the yeast GAL4 transcription factor and subsequent activation of GAL4-responsive genes allowed the direct demonstration of a potent transactivating function associated with the C-terminal region of Pax-6 (Czerny & Busslinger 1995). Similar results were obtained with GAL4-human Pax-6 fusion proteins (Glaser et al 1994). Furthermore, the transactivating properties of Pax-6 are similar regardless of whether Pax-6 binding sites are located in the promoter or in an enhancer 2 kb away (Czerny & Busslinger 1995). These results indicate that Pax-6 acts as an enhancer-binding transcriptional activator.

PAX-6 IN VERTEBRATE DEVELOPMENT

Expression Patterns
The expression patterns in the vertebrates analyzed to date are very similar. Expression patterns were analyzed by Northern blotting and by in situ
hybridization for mouse (Grindley et al. 1995, Schubert et al. 1995, Stoykova & Gruss 1994, Walther & Gruss 1991), zebrafish (Krauss et al. 1991a,b, Püschel et al. 1992), humans (Gérard et al. 1995, Ton et al. 1991), chicken (Li et al. 1994), quail (Martin et al. 1992, Plaza et al. 1995b, Turque et al. 1994), and rat (Matsuo et al. 1993). Detailed antibody studies, which are required for high-resolution analysis of Pax-6–expressing cells have only been performed in goldfish (Hitchcock et al. 1996). Notwithstanding the similarities in vertebrate central nervous system development, differences in organizational complexity (e.g., cortex formation in the cerebral hemispheres in the higher vertebrates) have to be kept in mind.

The expression patterns of Pax-6 described in this section are based on the detailed analyses of expression in mouse from Walther & Gruss (1991), Püschel et al. (1992), Stoykova & Gruss (1994), and Grindley et al. (1995). These references are not mentioned further in the following description.

The first expression of Pax-6 in embryonic development is observed at the stage when the first somites are formed and the neural folds begin to close in the cervical region, i.e., as early as embryonic day 8 (E8.0) in mouse (Grindley et al. 1995, Walther & Gruss 1991), stage 10 (day 22–23) in humans (Gérard et al. 1995), 10 h at 28.5°C (100% epiboly) in zebrafish (Krauss et al. 1991b, Püschel et al. 1992), and stage 7+ (two-somite stage) in chicken (Li et al. 1994).

Expression is first seen in the presumptive prosencephalon (forebrain) and rhombencephalon (hindbrain), in the developing spinal cord, and in a broad region of head surface ectoderm covering the prosencephalon. Later in development, Pax-6 transcripts are present in the telencephalon and diencephalon (both derived from forebrain) and in the myelencephalon (part of the hindbrain). Expression in the mesencephalon (midbrain) is observed from day E11.5 onward in the tegmentum. The roof of the mesencephalon is devoid of Pax-6 transcripts. Expression in the other part of the hindbrain, the metencephalon, is first observed at day E15.5. In the neural tube, Pax-6 expression extends along the entire anteroposterior axis up to the rhombencephalic isthmus, which delineates the midbrain-hindbrain boundary, and is mainly restricted to mitotically active cells in the ventral ventricular zone. The head surface ectoderm will give rise to the nasal placodes, which will develop the olfactory epithelium, and to the eye placodes, which develop into the lens and cornea. Pax-6 expression in the head surface ectoderm becomes restricted to the placodes as they form and continues to be expressed in the developing lens, cornea, and olfactory epithelium. Pax-6 is also expressed in the neural parts of eye and nose, namely the optic vesicle and the olfactory bulb. Further details of these expression patterns are described in the sections on eye and nose development. The expression patterns in the different compartments of the central nervous system and their further refinement during development are summarized below (see also Figure 5A).
Figure 5  Pax-6 expression in mouse. (A) Schematic representation of the expression of Pax-6 in mouse embryonic brain at day E13 p.c. [Redrawn after Stoykova & Gruss (1994).] cb, cerebellum; et, epithalamus; fc, frontal cortex; fi, fovea isthmi; lv, lateral ventricle; ob, olfactory bulb; oe, olfactory epithelium; sc, spinal cord; vt, ventral thalamus.  (B) At day E10.5 of embryonic development, Pax-6 expression is observed in the lens pit (lp) and in the developing optic stalk (os).  (C) Transverse section showing Pax-6 expression at day E13.5 of embryonic development in lens (le), neuroretina (nr), optic stalk (os), and future cornea (arrowhead).  (Figures B and C were kindly provided by L St-Onge and P Gruss.)
FOREBRAIN  Pax-6 is first expressed at day E8.0 in a broad domain in the neuroepithelium of the prosencephalon, which includes the prospective optic vesicle, the telencephalon, and the diencephalon. Expression in the telencephalon (E10.5 to E18.5) is restricted to the ventricular zone of the lateral and dorsal neural epithelium. The basal telencephalon is devoid of Pax-6 transcripts. In the developing diencephalon, Pax-6 is strongly expressed in the mantle zone of the ventral thalamus and anterior hypothalamus, with a sharp border at the level of the zona limitans intrathalamica, which delineates the dorsal from the ventral thalamus. In the dorsal thalamus, the expression is mostly confined to the internal germinative layer and to a thin layer of cells above the zona limitans. Pax-6 transcripts are detected in the ventricular zone of the epithalamus and in the precommissural and commissural zones of the pretectum. The posterior commissure delineates the caudal limit of expression of Pax-6 in the diencephalon.

In the adult brain, Pax-6 is expressed in discrete areas of the forebrain: the olfactory bulb (see later), the lateral and medial septal nucleus, the horizontal and vertical limb of the diagonal band nucleus (Broca), the nuclei of the basolateral complex of the amygdala, some cells of the ventral pallidum, the entopeduncular nucleus, and the zona incerta and its extension into the thalamic reticular nucleus.

MIDBRAIN  At day E13, the expression of Pax-6 in the tegmentum of the mesencephalon extends laterally into the presumptive region of the differentiating substantia nigra. Strong expression is also observed in the area of the differentiating dorsal raphe. In the young adult brain (4 weeks), Pax-6 expression is observed in the dorsolateral part of the substantia nigra reticularis, in the rostral part of the midbrain central gray, in some scattered cells in the ventral tegmental area, and in the deep mesencephalic nucleus.

HINDBRAIN  The initial expression of Pax-6 in the hindbrain (rhombencephalon) at E8.0 resolves into expression in discrete areas of the metencephalon (pons and cerebellum) and myelencephalon (medulla). Pax-6 is expressed in the ventricular zone and the external germinative layer (EGL) of the developing cerebellum at E13.5. The EGL later produces the granular cell layer of the cerebellum, where Pax-6 is still expressed in the mature brain. Pax-6 is also expressed in pontine and inferior olivary nuclei. In the young adult brain, Pax-6 is expressed in different nuclei in the pons and medulla (pontine nuclei in the trapezoid body, reticulotegmental nucleus, posterior dorsal tegmental nucleus, ventral and dorsal cochlear nuclei, prepositus of the hypoglossal nucleus) and in both ventricular and external granular layers of the cerebellum. The expression in primary cell cultures from cerebellum is confined to granular neurons and glial astrocytes (Kioussi & Gruss 1994, Yamada et al 1994). The expression in the myelencephalon is similar to that observed in the spinal cord.
THE SPINAL CORD  The onset of Pax-6 expression in the spinal cord is coincident with neural tube closure. No signal is seen in the neural groove. At day E8.5, Pax-6 is expressed in a broad mid-lateral band of mitotically active cells in the neural tube. Pax-6 expression is absent from the dorsalmost and ventralmost cells and will extend along the entire anteroposterior axis. At day E9.0, this expression becomes confined to the ventral half of the neural tube (basal plate) but is excluded from the ventralmost floorplate and adjacent cells. On the dorsal side, the expression extends into the dorsally located alar plate, where no clear-cut expression border can be observed.

Differentiation in the spinal cord results in the formation of distinct layers. A single layer of pseudostratified mitotically active cells, the ventricular zone, is located near the neural tube lumen and is covered by the more lateral intermediate zone, which consists of postmitotic cells.

Pax-6 expression is mainly restricted to the ventricular zone. Its expression is excluded from the intermediate zone, with the exception of cells in the ventral intermediate zone, which form two columns on both sides of the floor plate.

Still later in development (E15.5), when virtually all neurons are formed and regression of the ventricular zone is complete, Pax-6 expression is restricted to a subset of ependymal cells around the wider part of the neural canal. It is also weakly expressed in a subset of postmitotic cells located ventrally.

REGULATION OF PAX-6 EXPRESSION IN THE CENTRAL NERVOUS SYSTEM  Pax-6 is expressed in both ventricular and external granular layers of the adult mouse cerebellum in granule neurons and astrocytes. The neurotrophins nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are known to affect cellular differentiation in the nervous system. The application of NGF and BDNF to cerebellar primary cultures resulted in a moderate increase of Pax-6 expression, suggesting that Pax gene expression is modulated by neurotrophins (Kioussi & Gruss 1994).

In the spinal cord, Pax-6 expression is confined to the ventricular zone of the mid-lateral region. This dorsoventrally restricted expression pattern develops after neural tube closure by progressive down-regulation in cells adjacent to the floor plate and roof plate. The neural plate rapidly develops a typical dorsoventral patterning in the absence of the notochord, suggesting that the ventral cell types are determined prior to notochord formation (Pituello et al 1995, Yamada et al 1993). Nevertheless, microsurgery experiments (notochord removal and implantation of a supernumerary notochord) performed in chicken embryos demonstrated that the correct Pax-6 expression pattern and the expression of the ventral traits in the spinal cord depend on the notochord (Goulding et al 1993). Recent evidence suggests that signaling molecules such as vhh-1 and activin A or other activin-like molecules play a role in this process (Pituello
et al 1995, Roelink et al 1994). Changes in Pax-6 expression concomittant with altered dorsoventralization of the neural tube were also observed in the mouse mutants Brachyury and open brain (Günther et al 1994, Rashbass et al 1994).

EYE The vertebrate eye develops as a two-component system, with the neural ectoderm on the one hand and the head surface ectoderm on the other. The neural ectoderm evaginates laterally at the base of the telencephalon and becomes the optic pit, which will form the optic vesicle. Invagination of the optic vesicle results in the bilayered optic cup. The inner layer of the optic cup forms the neuroretina, and the outer layer the pigmented retina. The head ectoderm forms the lens placode. The thickened epithelium of the placode invaginates to form the lens vesicle. The interaction between lens placode and optic vesicle is important for continued eye development. In the current model of lens induction different stages are distinguished in which (a) the head ectoderm acquires a lens-forming competence, (b) early lens-determining signals cause a lens-forming bias, (c) the optic vesicle provides the final signal for lens determination, and (d) lens differentiation starts (Grainger 1992).

Pax-6 expression in the vertebrate eye has been studied in mouse (Ton et al 1991, 1992; Walther & Gruss 1991), zebrafish (Krauss et al 1991b, Püschel et al 1992), goldfish (Hitchcock et al 1996), quail (Martin et al 1992), chicken (Li et al 1994), newt and axolotl (Del Rio-Tsonis et al 1995), and guinea pig (Richardson et al 1995). The expression is comparable in all species examined, and the following description is based on the expression in mouse.

Pax-6 expression is observed early in mouse development (E8.0) in a broad region of head surface ectoderm covering the prosencephalon and in an extensive part of head neural ectoderm, including the optic pit, which is the first morphologically detectable indication of the eye region. A day later, Pax-6 is expressed in the epithelial layer of the optic vesicle and in the developing optic stalk (Figures 5B,C). Later, the expression level is highest around the rim of the developing optic cup but is consistently weaker in the back of it and in the more proximal structures, such as the optic stalk. Whereas in the early optic cup both epithelial layers (presumptive neuroretina and pigmented retinal epithelium) express Pax-6 at equal levels, in later stages the neuroretina continues to express Pax-6 at high levels, but the pigmented retinal epithelium shows only low expression near the rim of the optic cup. The rim is the future pars indica retinae and pars ciliaris retinae, which constitute the neuroectodermal component of the presumptive iris/ciliary body and overlap the lens. Expression in the adult neuroretina is restricted to the ganglion cell layer, the bipolar nerve layer, and the amacrine neurons, as was documented for quail, zebrafish, and goldfish (Hitchcock et al 1996, Krauss et al 1991b, Martin et al 1992, Püschel et al 1992).
The expression in the surface ectoderm becomes progressively restricted to the developing lens placode, nasal placode (see below), and immediately adjacent tissues. In chicken, the expression in the head surface ectoderm precedes the expression in the neuroepithelium in contrast to the other vertebrates analyzed where the onset of expression coincides (Li et al 1994). The initial expression of Pax-6 in the lens placode becomes further restricted, which leads to a separated expression in the lens and nasal placodes. The expression of Pax-6 in the head surface ectoderm and subsequently in the lens placode is correlated with the lens-forming competence of these tissues (Grainger 1992, Grindley et al 1995). The lens forms as an invagination of the thickened surface ectoderm and eventually detaches from the surface. The lens continues to express Pax-6 as the lens fiber cells start to differentiate. Richardson et al (1995) found that in adult guinea pig lens, Pax-6 staining was highest in cell nuclei of the lens epithelium, reduced in the equatorial region where cells are differentiating into elongated lens cortical fibers, and weak or absent in older layers. The overlying surface ectoderm, which corresponds to the developing cornea, also continues to express Pax-6 until the last stage analyzed, E15.5.

Del Rio-Tsonis et al (1995) have found evidence that may link continued Pax-6 expression in the newt dorsal iris with the capacity of lens regeneration. Hitchcock et al (1996) have demonstrated Pax-6 expression in goldfish neuronal progenitors during both continued retinal growth and regeneration. During regeneration, Pax-6 is expressed in the mitotically active cells of the germinative zone of the retina and in the regeneration blastema but not in rod precursors. Pax-6 expression could also be a prerequisite for the ability of nonlens tissues to transdifferentiate into lens (Okada 1991). Indeed, the prospective pineal gland, embryonic iris, retina, and pigmented retinal epithelium at the anterior of the eye, which have the capacity to transdifferentiate, all express Pax-6 (Grindley et al 1995, Walter & Gruss 1991).

REGULATION OF Pax-6 EXPRESSION IN THE EYE   Pax-6 expression in the quail neuroretina is regulated via two promoters, P₀ and P₁, and a neuroretina-specific enhancer (Plaza et al 1993, 1995a,b). The activity of the enhancer is restricted to the P₀ promoter. The transcripts of the two promoters show a different temporal pattern: Whereas the transcripts of P₁ appear first and then slowly decrease, those of P₀ appear later, increase, remain constant until hatching, and decrease thereafter. Transcripts from both promoters are detected in all tissues in which Pax-6 is expressed, with the exception of the retinal pigmented epithelium and of the pancreas, in which only transcripts from P₁ are found. The identification of c-Myb binding sites in the P₀ promoter, the transactivation of this promoter by c-Myb, and the colocalization of both transcripts in the developing neuroretina strongly suggest that c-Myb regulates quail Pax-6 (Plaza et al 1995c).
NOSE  Extensive similarities in formation and \textit{Pax-6} expression exist between lens and nasal placodes. Like the lens, the nasal cavities form as a result of invagination of the head surface ectoderm. The ectodermal nasal placodes, which are situated anterior to the optic placodes, invaginate to form the nasal cavities and the olfactory epithelium. Early interactions in nose development have not been studied extensively, but the available data suggest that they have some mechanisms in common with lens induction (Grainger 1992, Jacobson 1966). The parallels in \textit{Pax-6} expression suggest that \textit{Pax-6} could be involved in one of these parallel mechanisms.

The expression of \textit{Pax-6} in the head surface ectoderm gradually becomes restricted to the forming placodes, so at the time of lens and nasal pit formation, the two expression domains are clearly separated. The expression continues in the placodal epithelium during the formation of nasal pits and can subsequently be detected in the developing olfactory epithelium. \textit{Pax-6} is also expressed in the developing olfactory bulb, which forms from a protrusion of the rostroventral telencephalon. In the adult mouse, the expression is confined to the internal granular and to the glomerular layer of the olfactory bulb. In zebrafish, expression is still observed in the anterior part of the olfactory bulb one week after hatching (Krauss et al 1991b).

PITUITARY  The pituitary consists of the adeno- and neurohypophysis, which have different embryological origins. The adenohypophysis (anterior pituitary) develops from Rathke’s pouch, an outpocketing of the roof ectoderm of the stomodeum in close association with the infundibulum. The latter is an evagination of the floor of the diencephalon, which will give rise to the neurohypophysis. \textit{Pax-6} is expressed in Rathke’s pouch from E11.5 and reaches peak levels at E12.5. Weak labeling is still observed at E15.5 (shortly before the different parts of the pituitary start differentiating) in the epithelium that lines the remaining lumen of Rathke’s pouch and in the anterior lobe. At the end of embryonic development, low-level expression can still be observed.

PANCREAS  \textit{Pax-6} expression has been observed in the pancreas of mouse, chicken, and quail, where it is restricted to the \(\alpha\)- and \(\beta\)-cells in the endocrine pancreas (Li et al 1994, Turque et al 1994, Walther and Gruss 1991). Weak expression was also observed in human pancreas by means of Northern blotting (Ton et al 1991).

\textit{Mutations}

MOUSE SMALL EYE  \textit{Small eye (Sey)} is a semidominant, homozygous lethal mutation of the mouse \textit{Pax-6} gene (Hogan et al 1986, 1988). \textit{Sey} mutant mice have been proposed as a model for human aniridia (see below) on the basis of mapping, mode of inheritance, and phenotype (Glaser et al 1990,
Hogan et al 1988). Several alleles have been isolated that all show comparable phenotypes, although the severity of the phenotypes can vary. Sey is a spontaneous mutation, SeyNeu was recovered in an ethylnitrosourea mutagenesis experiment, Harwell Sey (SeyH) is a radiation-induced homozygous prenatal lethal, and Dickie Sey (SeyDey) arose as a spontaneous mutation. The last two correspond to small deletions or rearrangements extending into nearby genes. Sey and SeyNeu are point mutations resulting in truncated Pax-6 proteins (see Table 1).

In heterozygous condition, the Sey and SeyNeu alleles result in a pronounced size reduction of the developing lens. Histological analysis of embryos shows the presence of many vacuolated, degenerating cells in the lens, a folding of the retinal epithelium around the anterior of the lens, and in some cases an incomplete separation of the lens from the overlying ectoderm. In addition to these phenotypes, a different eye phenotype reminiscent of human Peters’ anomaly has been seen in Sey heterozygous mice (Hanson et al 1994). These mice develop cataracts at variable times after birth. One quarter of Sey heterozygous offspring develop bilateral hydrocephaly of the lateral ventricles and dies at an age of eight weeks (Hogan et al 1988). A detailed analysis of developmental brain abnormalities in SeyNeu heterozygotes (Schmahl et al 1993) has revealed frequent aberrations in the germinative epithelia within telencephalon, diencephalon, and metencephalon. The alleles SeyDey and SeyH display additional defects. In SeyDey embryos, the developing optic cup is smaller, and overall eye anlagen development is retarded. Both alleles result in mice that have small eyes with coloboma, anterior eye chamber defects, and a reduced body size and viability.

All Sey alleles are homozygous lethal. Two of them (SeyDey, SeyH) have their lethal phase around blastocyst implantation, which probably results from the loss of other nearby loci important for early development. Sey and SeyNeu mice develop to term but die soon after birth, completely lacking eyes and nasal cavities and with severe brain defects. It has been suggested that the primary cause of neonatal death is the inability of the animals to breath while they are suckling due to the absence of a nose.

A detailed morphological analysis of homozygous embryos (Grindley et al 1995) has revealed that the lens placode is absent in 9.5-day-old embryos and that consequently no lens pit and lens vesicle are formed. A region of dense tissue is visible between the ectoderm and the optic vesicle, probably corresponding to a condensation of mesenchyme cells. The underlying optic vesicle is uniformly broad in contrast to the proximal constriction observed in wild-type embryos. In wild-type embryos, the optic vesicle interacts with the lens pit, thereby forming a bilayered optic cup that produces the neuroretina and the pigmented retina. In the mutant, the optic cup does display a bilayered—but
abnormal—neuroepithelial structure. The mutant optic stalk retains a lumen, unlike the normal optic stalk, that forms the optic nerve consisting of a dense bundle of axons. In a study of chimeric mouse embryos containing wild-type and Sey mutant cells, Quinn et al (1996) have shown that Pax-6 has a direct, cell-autonomous effect in the developing optic cup, with an early role in both layers of the optic cup at a time prior to the appearance of differentiating retinal cells. Quinn et al (1996) suggested that Pax-6 expression defines early cellular properties, such as cell surface characteristics of the optic vesicle.

Early in development (E10.5), no nasal pits can be distinguished (Hogan et al 1986). Subsequently, these animals develop only two small frontonasal protuberances, which fuse with the maxillary processes. The developmental defects observed in the developing optic cup, lens, and nasal placodes result in the absence of eyes and nasal structures in newborn animals. Quinn et al (1996) have shown that the defects observed in the lens and the nasal epithelium are, as is the case for the optic cup, the result of cell-autonomous requirement of Pax-6 in these tissues.

In addition to the lack of the nasal placodes, the olfactory bulbs are missing. The absence of the olfactory bulbs is due to a lack of germinal epithelium differentiation in the forebrain. This lack of germinative epithelium differentiation results in extension of the germinative epithelium and concomitant defects in telencephalic stratification. An abnormal organization is visible in the ventricular zone, the subventricular zone, the intermediary zone, the cortical plate, and the marginal zone (Schmah et al 1993). Developmental defects are also seen in the diencephalon and metencephalon. The mesencephalon is intact in SeyNeu homozygotes (Glaser et al 1994). The germinal epithelium of the diencephalon is enlarged. In the metencephalon, the external granular layer is enlarged, and the germinal epithelium is highly packed and separated from the underlying parts of the cerebellum by a band of highly vacuolated fibers. The malformations observed in the brain are consistent with the expression of Pax-6 in the germinative epithelia of the lateral and dorsal telencephalic wall and the diencephalic wall, in the olfactory bulb, and in the external granular layer of the cerebellum.

The glandular tissue of the adenohypophysis and the intermediate pituitary appear normal notwithstanding the fact that Pax-6 is expressed there. Possible explanations for this discrepancy are that Pax-6 does not have a function in these structures, that it is partially redundant with another factor expressed in the developing adenohypophysis, or that the mutant analyzed is not a complete loss of function.

In summary, the developmental defects observed in Sey mice correspond well with the expression patterns of Pax-6 in the eye, nose, and brain—with the exception of the mesencephalon, the adenohypophysis, the intermediate pituitary, and the pancreas in which no phenotype is observed.
## Table 1  Pax-6 mutations

<table>
<thead>
<tr>
<th>Allele/patient code</th>
<th>Defect in</th>
<th>Molecular description</th>
<th>References</th>
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<tbody>
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<tr>
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<tr>
<td>Human Peters' 5 anomaly</td>
<td>PP</td>
<td>Whole gene</td>
<td>Deletion of 11 p13</td>
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<tr>
<td>Human eye anterior chamber defects</td>
<td>Bh</td>
<td>Paired domain</td>
<td>1-bp Substitution in exon 5a SA site; increase in PAX-6 with extended PD</td>
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<tr>
<td>Family 3</td>
<td>Paired domain</td>
<td>1-bp Substitution in exon; R26 → G26; structural disruption</td>
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<tr>
<td>Mouse</td>
<td>S&lt;sub&gt;e&lt;/sub&gt;y</td>
<td>Linker</td>
<td>1-bp Substitution; G194 → stop; truncation of protein before HD</td>
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<td>PST-rich</td>
<td>Mutation in SD site; truncation in PST-region</td>
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<td>S&lt;sub&gt;e&lt;/sub&gt;y&lt;sub&gt;H&lt;/sub&gt;</td>
<td>Whole gene</td>
<td>Deletion of P&lt;sub&gt;ax&lt;/sub&gt;-6 and adjacent genes</td>
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<td>S&lt;sub&gt;e&lt;/sub&gt;y&lt;sub&gt;W&lt;/sub&gt;</td>
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<td>PST-rich</td>
<td>Mutation in SD site; internal deletion</td>
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<td>Regulatory</td>
<td>Insertion of doc transposon in eye-specific enhancer</td>
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<td>Regulatory</td>
<td>Insertion of blastopia transposon in eye-specific enhancer</td>
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<td>vab-3 (e648)</td>
<td>Paired domain</td>
<td>W101 → stop; truncation in PD</td>
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<td>Paired domain</td>
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<td>Missense mutation G19 → R19</td>
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<td>vab-3 (e41)</td>
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<td>Mutation in SD site; truncation in PD</td>
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<td>mab-18 (e1976)</td>
<td>Homeodomain</td>
<td>Two missense mutations: I129 → T129; L232 → H232</td>
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<td>mab-18 (bx23)</td>
<td>Linker</td>
<td>556-bp deletion of exon 8 and flanking sequences</td>
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- familial aniridia.
- sporadic aniridia.

Abbreviations: (WAGR) Wilm's tumor, aniridia, genitourinary abnormalities, mental retardation syndrome; SA site: splice acceptor site; SD site: splice donor site.

UCHIDA RAT (rSey)  

rSey (Uchida rat) is inherited as an autosomal dominant mutation (Fujiwara et al. 1994) (see Table 1). The phenotype is very similar to the mouse Sey phenotype. The heterozygotes have small eyes, while the homozygotes lack eyes and nose and die perinatally. Initial development appears normal in heterozygotes, but later a smaller lens and an abnormal retina and iris are observed. Microscopic observation disclosed partial retinal dysplasia and vacuolar degeneration of the iris stroma. In homozygous rSey embryos, the optic vesicle is formed but with an abnormal structure. Lens and nasal placodes do not develop. The neuroepithelium of the forebrain and optic vesicle degenerates. These abnormalities result in a complete lack of eyes and nasal cavity. The absence of lens differentiation in the homozygotes is due to a lack of Pax-6 function in the head ectoderm. In addition, the lateral nasal prominence is missing. Matsuo et al. (1993) proposed that this craniofacial defect is due to an impaired migration of anterior midbrain neural crest cells that normally migrate to the frontonasal area. Since Pax-6 is not expressed in neural crest derivatives, this effect on migration is indirect.

ANIRIDIA  

Aniridia is a congenital, bilateral panocular disorder with a reported incidence between 1:64,000 and 1:96,000 (Nelson et al. 1984). Two thirds of aniridia cases are familial, with autosomal dominant inheritance. The inheritance pattern in the remaining third of cases is sporadic, and most of these are new point mutations. A small proportion (<5%) (V van Heyningen, personal communication) is associated with deletions of chromosome 11 p13, which often encompass the Wilms’ tumor predisposition gene 700 kb upstream and centromeric of PAX-6. Children with such deletions are susceptible to developing malignant childhood kidney tumor. This contiguous gene syndrome (WAGR, Wilms tumor, aniridia, genitourinary abnormalities and mental retardation) is often associated with variable developmental delay and other abnormalities, depending on the extent of the deletion.

The characteristic clinical manifestations of heterozygous aniridia are a complete or partial absence of the iris and iris hypoplasia. The ophthalmic complications associated with aniridia include poor vision, iris coloboma, glaucoma, cataracts, ectopia lentis, corneal opacification, optic nerve hypoplasia, and nystagmus (MacDonald & Sasi 1994, Nelson et al. 1984). Just as is the case for Sey mice, which show a similar range of eye defects, there is considerable variation in the aniridia phenotype. The molecularly identified aniridia mutations range from deletions of PAX-6 to point mutations that are spread throughout the gene (see Table 1). All except one of the point mutations result in truncated proteins, which abolish gene function. The fact that mutants affect various parts of the protein and that the phenotypes of point mutations and deletions are indistinguishable suggests that haploinsufficiency through loss of one copy of
PAX-6 rather than the accumulation of dominant or dominant negative forms of the PAX-6 protein is the molecular basis of aniridia (Strachan & Read 1994). The phenotype of heterozygous aniridia and its variability suggest that the eye in particular is sensitive to PAX-6 dosage. The possible PAX-6 autoregulatory loop acting in the developing eye (Grindley et al 1995, Plaza et al 1993) may explain why the eye is so sensitive to Pax-6 dosage (Glaser et al 1994).

Hodgson & Saunders (1980) described a case of presumed homozygous aniridia in humans for a nonconsanguineous union between two people with congenital aniridia. At 37 weeks of gestation, a stillborn infant was delivered with a small brain and a complete absence of eyes, nose, and adrenal glands. Another case was a transheterozygous infant MH with identified PAX-6 mutations that was born by caesarean section at 43 weeks (Glaser et al 1994). The ocular phenotypes of the parents (JH and WH) are discussed in the next section. The child had microcephaly, bilateral anophthalmia with fused eyelids, a small malformed nose, and other craniofacial malformations and died on the eighth day of life. A detailed examination after death confirmed the absence of the eyes, periorcular tissues, optic nerves, and chiasma and also revealed a small brain with multiple abnormalities. The corpus callosum was absent, the cerebral hemispheres were greatly reduced in size, the olfactory bulbs were absent, the hypothalamus was truncated abnormally with an expanded infundibulum and pituitary stalk, the cerebral aqueduct was malformed, and the pons and cerebellum were hypoplastic. The adrenal glands developed normally. Histological analysis of the brain revealed microscopic abnormalities throughout the brain: polymicrogyria in the cerebral cortex, ectopic foci of glial cells and choroid plexus in the leptomeninges, replacement of cortical neurons by glial cells in frontal and temporal lobes, heterotopic islands of germinal or ependymal cells extending outward from the lateral ventricles into cortical white matter, abnormal cortical stratification, dysplasia of cerebellar elements, and a reduction in the substance of the pyramidal tracts. This range of defects is comparable to the defects observed in homozygous SeyNeu mice. In general, there is a good correspondence between the pattern of CNS defects and the expression pattern of PAX-6. Areas with high PAX-6 expression are absent or greatly reduced. Exceptions are the midbrain, myelencephalon, and ventral thalamus, which show high expression but are hardly affected in mutants. This disparity may reflect partial redundancy with other genes. The one exception is the striatum, which does not express high levels of PAX-6 but is nevertheless, severely dysplastic. This phenomenon could be due to a dependence of striatal development on axonal connections from the cortex and the thalamus.

PETERS’ ANOMALY AND OTHER HUMAN OCULAR PHENOTYPES PAX-6 is also involved in other clinical syndromes of the eye. The milder anterior eye chamber
defects in these syndromes probably reflect the underlying molecular mechanisms of PAX-6 function, especially the importance of PAX-6 gene dosage. The different tissues of the developing eye in which PAX-6 is expressed may require different concentrations, thereby accounting for the variability in phenotypes observed in aniridia, Peters' anomaly, and the other syndromes described below.

Peters' anomaly is a congenital defect of the anterior chamber of the eye. The most frequent clinical manifestation consists of central corneal opacity (leukoma) overlying a defect in the posterior (inner) layers of the cornea. Adhesions are often seen between the anterior (outer) surface of the lens and the posterior corneal defect or the iris. In some pedigrees, Peters' anomaly was shown to segregate in an autosomal dominant fashion. Most cases, however, are sporadic. Hanson et al (1994) described a child with sporadic Peters' anomaly in which one copy of PAX-6 is deleted (see Table 1). They also described affected members of a family with dominantly inherited anterior segment malformations, including Peters' anomaly, that are heterozygous for a missense (arginine to glycine) mutation in the paired box. The deletion suggests that aniridia and Peters' anomaly are different phenotypic manifestations due to loss of function of one copy of PAX-6. The similar phenotypes for the deletion and the missense mutation also suggest that the missense mutation leads to loss of function.

During the study of patients with aniridia and related ocular disorders, Epstein et al (1994b) observed a family (Bh) in which the typical manifestations of aniridia were absent in mother and daughter (Bh mutation) (see Table 1). Abnormalities were observed in the anterior aspect of the iris derived from neural crest, including small radial defects, decreased vasculature and crypt density, and thinning of the iris stroma. The two patients are also affected with bilateral juvenile cataracts, peripheral corneal opacification, glaucoma, and pendular nystagmus. The mutation affects the splice acceptor site of the small, alternatively spliced 42-bp exon 5a, which upon insertion, results in an extended paired domain. In vitro experiments suggest that this mutation increases splicing efficiency significantly and thereby changes the ratio of the alternatively spliced forms (with and without extension) and causes the described phenotype. Richardson et al (1995) obtained additional evidence for the importance of a correct ratio between the two splice forms and showed that in adult mouse brain both forms are similarly abundant, whereas in the adult lens the shorter form predominates.

The parents of the child MH described in the previous section had distinct ocular phenotypes. The mother (JH) had the typical characteristics of aniridia, while the father (WH) had a milder phenotype consisting of perinatal cataracts, decreased visual acuity, a fine nystagmus, moderate unilateral exotropia, and a late-onset corneal pannus (Glaser et al 1994). The irises and foveas of the father appeared normal. The WH mutation affects the PST-rich region of PAX-6, resulting in a premature termination of the protein. The JH mutation results in
a protein truncated in the paired domain (see Table 1). The child (MH) inherited both mutations but retained only the residual activity of the WH mutation, which resulted in the severe phenotype described above.

**PAX-6 IN INVERTEBRATE DEVELOPMENT**

*Phallusia mammillata*

The ascidian *P. mammillata* is a urochordate with a life cycle containing both a larval and an adult phase (Satoh 1994). The ascidian tadpole larva is regarded as a prototype of the ancestral chordate. The larva has a primitive notochord, a brain and a dorsal nerve cord, a single statocyte (a gravity sense organ), and a single ocellus (primitive eye), which is composed of a small number of pigment cells, sensory cells, and lens cells. At the end of the short larval stage, the larvae attach and undergo metamorphosis, during which the larval structures degenerate. The adults are sessile organisms with a barrel-shaped body and a nervous system containing a single cerebral ganglion from which several nerves elongate to various body parts (Satoh 1994).

The ascidian *Pax-6* gene is expressed from the neurula stage onward. Initial expression is found in a limited number of cells that give rise to the brain and the dorsal nerve cord. In the late tailbud stage, transcripts are observed in the brain and the nerve cord. The gene is expressed in the cells immediately adjacent to the pigment cell of the ocellus and the statocyte. These *Pax-6*-expressing cells presumably give rise to sensory neurons. This expression pattern suggests that besides a function in the central nervous system, *Pax-6* could also play a role in the development of the neural component of the ocellus and the statocyte (S Glardon, P Baumgartner, P Callaerts, G Halder & WJ Gehring, unpublished data).

*Paracentrotus lividus*

Sea urchins (e.g. *Paracentrotus lividus*) are echinoderms with a typical pentamerous radial symmetry. Their nervous system consists of a nerve ring and radial nerves. Numerous sensory cells are present throughout the epidermis but are particularly prevalent on the suckers of the tube feet. Photoreception has been associated with certain nerves (Barnes 1982). Little is known about *Pax-6* expression during sea urchin development. The gene is first expressed at gastrulation stage. In the adult sea urchin, *Pax-6* is expressed in the mesodermal layer and at the tip of the tube feet (Czerny & Busslinger 1995).

*Loligo opalescens*

The squid *L. opalescens* is a representative of the class of cephalopods. The cephalopod nervous system has the highest degree of complexity among invertebrates. Their brain, central nervous system, and sense organs, particularly
the eye (which has a structure strikingly similar to that of vertebrates), are well
developed. The arms, and especially the sucker epithelium, are covered with
tactile and chemosensory cells (Barnes 1982).

The onset of \textit{Pax-6} expression in the squid is early in development in the
region of the rudimentary eye primordia. At a later stage, the optic vesicle
forms as the result of internalization of the eye placode and \textit{Pax-6} expression
increases and is seen in the regions of the developing eyes. At the same stage,
expression is also seen in the developing arms and mantle (see Figure 6A) while
later additional expression is observed in the olfactory organ and the brain. The
expression in the olfactory organ, arms and suckers is predominantly in the outer
cell layers which are rich in nerve endings, while the expression in the brain is
located in the medial region and in a bilateral group of cells at the dorsal edge of
the optic lobe. In the eye, expression is detected mainly in the outer layers which
give rise to the lens, iris and cornea (see Figure 6B) (S Tomarev, P Callaerts,
L Kos, R Zinovieva, G Halder, WJ Gehring & J Piatigorsky, unpublished
data).

\textit{Lineus sanguineus}

The nemertines, or ribbonworms, are mostly unsegmented, bilaterally symmet-
rical, coelomate, wormlike marine animals. They typically have an eversible
proboscis, a closed circulatory system, and a gut with separate mouth and anus.
Their sense organs include eyes of the primitive pigment cup type, cephalic
grooves and paired cerebral organs with possible chemosensory function, sen-
sory epithelial cells, and a frontal organ consisting of a group of ciliated cells lo-
cated at the anterior end. Besides sexual reproduction, which is observed in most
nemertines, some nemertines, including \textit{L. sanguineus}, can reproduce asexu-
ally by fragmentation and subsequent regeneration of the missing structures.
All nemertine species have the capacity to regenerate body parts (Gibson 1972).
The expression of \textit{Lineus Pax-6} was analyzed in regenerating \textit{L. sanguineus} and
in metamorphosing larvae of a closely related species, \textit{Lineus viridis} (Loosli
1995, Loosli et al 1996). During regeneration, \textit{Pax-6} expression is observed in
the regenerating cerebral organ and brain and in dorsolateral spots that correlate
well with the location of regenerating eyes. Expression in the closely related
\textit{L. viridis} was observed in the entire brain and in the cerebral organ.

\textit{Drosophila melanogaster}

The \textit{D. melanogaster} Pax-6 homologue \textit{eyeless} is expressed throughou-
development of the central nervous system and in the developing eye (Quiring
et al 1994; P Callaerts, G Halder & WJ Gehring, unpublished data). Shortly
after gastrulation, at germband extension stage, neuroblasts in the head, which
will give rise to parts of the brain, and neuroblasts in the ventral nerve cord
Figure 6  Pax-6 expression in invertebrates.  (A) Pax-6 in embryo of the squid *L. opalescens*. Whole mount in situ hybridization shows expression in the eye and optic stalk (e), arms (a), and region of the future mantle (left). (B) Section through a squid eye showing expression in the future iris and cornea (arrowhead). l = lens. (C) Dorsal view of a whole mount in situ hybridization of a stage 15 embryo. Expression of *D. melanogaster* eyeless is observed in the optic anlagen (o, arrows) and in parts of the brain (rest of staining). Anterior is to the left. (D) Expression of *D. melanogaster* eyeless in a third larval stage eye-antennal imaginal disc. Strong expression is seen in the eye part of the disc (left). The antennal part shows no expression. (E) Pax-6 (*vab-3*) expression in early embryonic development of *C. elegans*. The transcripts are confined to presumptive hypodermal cells and neuroblasts in the anterior of the embryo (left). (F) Expression of a *vab-3*-GFP reporter construct in hypodermal and neuronal cells in the head of an L1 stage larva. hyp 5, hypodermal syncytium 5; HO, lateral hypodermal cell HO; H1, p, posterior progeny of lateral hypodermal cell H1; BAG, BAG neurons. [Figures A and B provided courtesy of S Tomarev, L Kos & J Piatigorsky. Figures E and F provided courtesy of A Chisholm & R Horvitz and reproduced with permission of Nature, MacMillan Ltd.]
express *eyeless*. The expression in the ventral nerve cord is restricted to three neuroblasts per hemisegment. The cellular progeny (ganglion mother cells and neurons) of these neuroblasts continue to express *eyeless* throughout the larval stages and in the adult. The expression in the embryonic brain is restricted to distinct parts, including the optic lobes.

*Eyeless* is continuously expressed in the eye primordia until the onset of differentiation (Quiring et al 1994) (Figure 6C,D). *D. melanogaster* eye development starts in the embryo when the dorsally located optic anlagen are formed (Wolff & Ready 1993). These will develop into eye imaginal discs (as part of the eye-antennal disc) that through metamorphosis will give rise to the adult eyes. Each eye consists of around 750 hexagonal ommatidia or facets, with each facet containing 8 photoreceptor cells, 1 pseudocone, and 11 accessory cells comprising 4 cone cells and 7 pigment cells (Wolff & Ready 1993). The imaginal discs grow during the three larval stages during which *eyeless* is continuously expressed in the eye imaginal discs. Beginning early in the third larval stage, cells at the posterior end of the disc begin to assemble into ommatidial precursors. This transition from a proliferative, unpatterned epithelium to differentiating ommatidial clusters is marked by the morphogenetic furrow, which is an indentation in the eye disc that will move from posterior to anterior, thereby gradually patterning the whole eye imaginal disc (Wolff & Ready 1993). In the eye disc of the third larval stage, *eyeless* expression is confined to the unpatterned part of the epithelium anterior to the morphogenetic furrow, and its expression level drops after the furrow (Quiring et al 1994) (Figure 6D). A similar pattern is observed with antibody studies (G Halder, P Callaerts & WJ Gehring, unpublished data). Whether *eyeless* is expressed again at a later stage of eye development is unknown.

Several mutant alleles of *eyeless* (*ey*) have been studied (Lindsley & Zimm 1992), of which only a couple are still available at present. Two alleles, *ey* and *eyR*, have been characterized molecularly (Quiring et al 1994) (see Table 1). Both mutations are caused by the insertion of a transposable element in an intron containing an eye-specific cis-regulatory element; this insertion curtails normal expression of the gene in the eye primordia. These mutations behave as strong hypomorphs in the eye but show no apparent defects in the parts of the central nervous system where *eyeless* is expressed. A complete understanding of the gene’s function will require the analysis of the developmental defects caused by a null mutation.

The mutant defects observed in the adult are a partial to complete loss of the compound eye and of sensory bristles surrounding the eye. Some flies show antennal duplications (Lindsley & Zimm 1992). Extra maxillary structures are also observed. An additional external defect is the tetragonal instead of hexagonal organization of the ommatidia, which is caused by a failure of the
The eye part of the eye-antennal imaginal disc in the third larval instar is reduced in size, and massive apoptotic cell death is observed, which is instrumental in producing the ey phenotype (Fristrom 1969, Ransom 1979; G Halder, P Callaerts & WJ Gehring, unpublished data).

The second Pax-6 gene of D. melanogaster, twin of eyeless (toy) is expressed earlier than eyeless (T Czerny, G Halder, P Callaerts, WJ Gehring & M Busslinger, unpublished data). At the cellular blastoderm stage, toy is expressed in a broad dorsolateral region at the anterior of the embryo. Later, its expression in the central nervous system partially overlaps that of eyeless. toy expression in the embryonic optic anlagen and in the eye imaginal disc is very similar if not identical to ey. No mutant in toy has been identified.

**Caenorhabditis elegans**

*C. elegans* is a small, free-living soil nematode. The body of the hermaphrodite *C. elegans* contains a fixed number of somatic cells, whose entire lineage has been mapped. The nematodes’ nervous system consists of a brain comprising a nerve ring with attached ganglia; anteriorly extending ring nerves; and dorsal, lateral, and ventral nerves extending posteriorly (Barnes 1982, Gilbert 1994). Pax-6 has two separable genetic functions (Chisholm & Horvitz 1995, Zhang & Emmons 1995). The genetic locus vab-3 encodes a Pax-6 protein containing both the paired domain and the homeodomain, whereas the locus mab-18 encodes Pax-6 proteins containing only the homeodomain portion. The mab-18 transcript is expressed from an internal promoter. The vab-3 transcript is widely expressed in the precursors of the head hypodermis cells and neurons and subsequently in the progeny of these cells (Chisholm & Horvitz 1995) (Figure 6F). When these cells begin to differentiate, vab-3 is expressed in a broad domain across the developing head (Figure 6E). The mab-18 transcript is expressed in the structural cells and neurons of a peripheral posterior sense organ called ray, in neurons in the preanal ganglion, in the head hypodermis, and in a pair of bilaterally symmetrical neurons in the head anterior of the nerve ring (Zhang & Emmons 1995).

vab-3 mutations (see Table 1) result in variable deformities of the larval and adult head, in head neuron transformations, and in additional defects in gonadogenesis and male tail development. The head deformities result from defects in the embryonic migration or determination of head hypodermal cells. The head and neural phenotypes are consistent with the expression pattern of vab-3 in those structures (Chisholm & Horvitz 1995). The genetic locus mab-18 comprises two mutations that affect one pair of a set of nine pairs of male-specific genital sensillae known as rays. The mutations (see Table 1)
result in the anterior transformation of the entire ray 6 sublineage to a type 4 sublineage, in which the specificity of the cell associations of the structural cell and neurons has changed. In addition one of the alleles has a gonad-cell-migration defect. The ray phenotype and the other phenotypes are consistent with the continued expression of *mab-18* in the ray sublineage and in the other cell types (Zhang & Emmons 1995).

*Dugesia tigrina*

The free-living freshwater planarian *D. tigrina* is an acoelomate, bilaterally symmetrical animal. Like many other flatworms, it has considerable regenerative capacities. During regeneration, a blastema of undifferentiated cells forms, out of which the tissues regenerate. The net-like nervous system and the sensory organs comprise a brain, chemosensory structures, and eyes. The flatworm *D. tigrina* has a primitive pigment cup eye consisting of a small number of pigment cells and photoreceptors. *Pax-6* transcripts are detected in the flatworm in the regenerating and fully grown eye. Some evidence has been obtained for low-level expression elsewhere in the body (M Munoz, P Callaerts, WJ Gehring & E Salo, unpublished data).

**PAX-6 IN REGULATORY HIERARCHIES**

**Vertebrates**

PAX-6 In their analysis of the quail *Pax-6* gene (*Pax-QNR*), Plaza et al (1993) have shown that the gene product can bind to multiple binding sites in its own promoter and subsequently transactivate it in quail neuroretinal and embryonic cells. *Pax-6* function is also required for the maintenance of its own transcription in the surface ectoderm (Grindley et al 1995). Thus, autoregulation of *Pax-6* plays an important role in the developing eye.

**Lens crystallins** The spatial and temporal expression patterns of *Pax-6* and the mutant phenotypes suggest that *Pax-6* participates in later stages of lens development and might be required for growth, differentiation, and maintenance of the lens and cornea. Crystallins are the major soluble proteins that occur in the lens and are required for its proper optical properties (Wistow & Piatigorsky 1988). In a set of transient transfection and protein-DNA binding experiments, different crystallin genes were shown to be targets of *Pax-6*. The first crystallin gene to be identified as a *Pax-6* target was the chicken *αA*-crystallin (Cvekl et al 1994). The promoter of this gene is a complex array of positive and negative *cis*-acting elements—including the transcription factors USF, CREB and/or CREM, AP-1 factors, and *Pax-6*—involved in the control of the high expression of *αA*-crystallin in the lens and repression in non-lens tissues. *Pax-6*
PAX-6 contributes to the lens-specific expression and acts in concert with USF and CREB and/or CREM to activate the chicken αA-crystallin gene.

Pax-6 acts synergistically with CREB-like proteins to drive high expression of mouse αA-crystallin in the lens (Cvekl et al 1995a) and to bind to and activate the lens-specific enhancer of the chicken δ1-crystallin gene (Cvekl et al 1995b). Similarly, Pax-6 also transactivates the lens-specific regulatory sequence of the guinea pig ζ-crystallin gene (Richardson et al 1995). Richardson et al (1995) also showed that Pax-6 is continuously expressed in the lens. Its expression is intense in nuclei of lens epithelial cells, moderate in the equatorial region (lens cells differentiate into elongated lens cortical fibers), and weak or absent in fiber cells in older layers. Pax-6 reaches maximal expression prior to the maximum levels of ζ-crystallin in the lens, which is in agreement with a regulatory interaction.

Another gene for which evidence is available that it could be a direct target for Pax-6 is neural cell adhesion molecule (N-CAM) L1. Chalepakis et al (1994) have identified three in vitro binding sites for Pax-6 in the promoter region of L1, but the biological significance of this finding has not been tested yet. The presence of binding sites and the partial overlap in expression in the developing neuroretina, the olfactory system, the developing neocortex, and other parts of the brain suggest that Pax-6 could play a direct role in regulating L1 expression.

Drosophila melanogaster  
ECTOPIC EXPRESSION The expression pattern of eyeless in the eye primordia in D. melanogaster and the mutant phenotype with a partial or complete loss of the compound eyes were suggestive for a possible early and determinative function in eye development. In order to test whether eyeless acts high up in the eye developmental pathway, Halder et al (1995a) ectopically expressed the eyeless gene by means of the GAL4-system (Brand & Perrimon 1993). The outcome of this experiment was the formation of supernumerary compound eyes in which the gene was ectopically expressed, namely on legs, wings, and antennae. The essentially normal compound eyes are also light-sensitive, and the photoreceptors respond to illumination with depolarization. These data demonstrate that eyeless functions as a master regulatory gene in the context of the developing imaginal discs and that a single gene can activate the eye developmental pathway in which a few thousand genes act. The induction of ectopic compound eyes by overexpressing mouse and squid Pax-6 (Halder et al 1995a; S Tomarev, P Callaerts, L Kos, R Zinovieva, G Halder, WJ Gehring & J Piatigorsky, unpublished data) shows that not only the sequences of Pax-6 are conserved but also their biochemical activity and presumably the sequence
of the DNA-binding sites. An important question is what the target genes of *eyeless* are. Several genes acting early in *D. melanogaster* eye development, such as *sine oculis* (Cheyette et al 1994, Serikaku & O’Tousa 1994) and *eyes absent* (Bonini et al 1993), are candidate target genes. However, no evidence is available for a direct regulatory interaction of *eyeless* with any of these.

**EYE-SPECIFIC GENES AND PROMOTERS** Czerny & Busslinger (1995) have identified the P3 site (see above) as the optimal binding site for cooperative binding and transactivation by the Pax-6 homeodomain. Wilson et al (1995) noted that the P3 sequence is conserved in the eye-specific promoters of *D. melanogaster* and *Drosophila virilis* rhodopsins 1–4, *Calliphora vicina* rhodopsin 1, bovine and human rhodopsin, and *D. melanogaster ninaC, trp* and arrestin. The P3 sites in the *Drosophila* sp. rhodopsin promoters are required for proper rhodopsin expression (Fortini & Rubin 1990, Mismer & Rubin 1989). These data suggest that the expression of rhodopsins and other eye-specific genes could be directly regulated via Pax-6 binding to the P3 sites.

**PAX-6 IN THE ANIMAL KINGDOM: A COMPARISON**

*Pax-6* is expressed in the developing nervous systems and, when they have them, in the developing eyes (regardless of type) of all animals studied. *Pax-6* expression is observed in the complex image-forming eyes of *D. melanogaster*, squid, and mammals, and also in the more primitive pigment cup eyes of *L. sanguineus* and *D. tigrina*. In all cases, *Pax-6* is first expressed in a broad region in the head and then becomes subsequently confined to the area of the future eyes. The expression of *Pax-6* generally precedes the differentiation of the eye, suggesting that it plays a role in forming the eye morphogenetic field, in proliferation of the eye primordia, and in initiating the diverse eye developmental pathways.

The expression in the brain and in the rest of the central nervous system (e.g. mouse spinal cord and *D. melanogaster* ventral nerve cord) is restricted to subdomains that, with the exception of the mouse, have not been mapped in detail. Nevertheless, one general observation is that in the brain the expression domains are large early in development and become restricted to subsets of cells in the adult. Hence, *Pax-6* could be involved in the specification of spatial domains in the embryonic brain of vertebrates (Puelles & Rubenstein 1993) and of the other species. Additional parallels between vertebrate and *D. melanogaster* brain regionalization (Hirth et al 1995) suggest that similar mechanisms are involved in brain development of both. The expression of *Pax-6* in subsets of cells in the adult brain suggests that *Pax-6* may have a role in their differentiation, maintenance, and functional assembly.
An intriguing observation is the expression of Pax-6 in structures such as the vertebrate nose, the chemosensory cerebral organs of L. sanguineus, and the squid chemosensory organ. Whether some of the structures expressing Pax-6 in the other species, such as the sea urchin tube feet (which are rich in sensory cells), also contain chemosensory structures remains to be seen.

Despite the above-mentioned similarities in expression patterns, there are also differences. The most conspicuous example in this respect is C. elegans, in which Pax-6 is expressed in structures such as the male tail and the ray sensory organ besides its expression in the nervous system. In vertebrates, expression is also detected in the endocrine cells of the pancreas and in the adenohypophysis. This suggests that besides a possible functional conservation in eye, brain, and chemosensory organ development, Pax-6 has been recruited for additional functions in different species.

**PAX-6 IN EVOLUTION**

*Molecular Conservation and Phylogeny*

Pax-6 genes have been identified in species as diverse as flatworms and humans. The striking molecular feature of these genes is the unusually high degree of amino acid sequence conservation in the DNA binding domains of the encoded proteins and the conservation of several intron-exon boundaries identifying them as true orthologs. A compilation of the amino acid changes in the DNA-binding domains shows that most of the variability is situated in regions that are functionally less important for DNA binding (see Figure 2).

The regions of the proteins outside of the DNA-binding domains show a much smaller degree of sequence conservation, e.g. when Pax-6 from flatworms, flies, and humans are compared. An evolutionary tree based on Pax-6 sequences supports the most recent molecular phylogenies derived by ribosomal RNA sequence comparison (Conway Morris 1993). The most interesting finding is that the new position of nemerteans, placed close to molluscs instead of flatworms, is corroborated by the analysis of Pax-6 sequences.

The C-terminal PST-rich domains of the vertebrate, echinoderm, and mollusc Pax-6 genes contain a region of significant sequence similarity. This conservation suggests that the last common ancestor of vertebrates, echinoderms, and molluscs had a Pax-6 ancestral gene containing this sequence in the C-terminal domain. In this respect, it will be interesting to determine the Pax-6 C-terminal sequence of the nemertean L. sanguineus which was proposed to be a slightly diverged descendant of the last common ancestor of invertebrates and vertebrates (Loosli 1995).

A small exon that can lead to an extended paired domain upon insertion in the paired box has been found only in humans, mouse, and zebrafish. Therefore,
this variation could be an innovation restricted to the vertebrate evolutionary line.

The Pax-6 gene of the urochordate *P. mammillata* has diverged significantly from those of the vertebrates. A similar strong divergence has also been observed for ascidian *Hox* genes (Ruddle et al. 1994). The fact that the evolutionary clock seems to run faster for *P. mammillata* Pax-6 could thus imply that the functional requirements for Pax-6 are reduced. This in turn could reflect a reduced function of the ocellus which in some ascidians is missing completely. Similarly, the absence of eyes in *C. elegans* may explain the diverged sequence in the paired domain. It will be interesting to see whether the Pax-6 sequences of nematodes which have eyes have diverged to a lesser extent than those of *C. elegans*.

Pax-6–specific amino acids in the paired domain are largely conserved between species, which could indicate that all triploblastic lineages possess or once possessed a clear-cut Pax-6 gene. A coelenterate Pax gene has been identified that lacks the Pax-6–specific amino acids in the paired domain but in which the homeodomain is clearly Pax-6-like (H Groeger, P Callaerts, WJ Gehring & V Schmid, unpublished data). This observation could imply that the separation of the Pax 4/6 line from the Pax 2/5/8 line (Noll 1993) is coincident with the divergence of diploblastic and triploblastic lineages.

**Pax-6 and the Evolution of the Eye**

The expression of Pax-6 in the developing eyes of vertebrates, flies, squid, ribbonworms, and flatworms is striking, because their eyes differ profoundly in structure and development (see Figure 7). Simple visual structures such as eye spots were estimated to have evolved between 40 and 65 times independently (Salvini-Plawen & Mayr 1977). The three largest animal phyla—molluscs, arthropods and vertebrates—have evolved distinct solutions (for which no evolutionary relationship is evident) to the problem of obtaining images. The compound eye of arthropods is so different in structure and development from other species that no structural homology to single-lens eyes can be claimed. The single-lens eyes of vertebrates and molluscs are similar in structure, but the differences in embryonic development argue against structural homology. Therefore, the supposed independent evolution of single-lens eyes has become the textbook example of convergence—natural selection leads to a similar result, single-lens eyes, from different starting points.

Eakin (1979) proposed that at the level of the photoreceptors, a strong dichotomy exists between the protostome and the deuterostome lineage, with the microvilli-type photoreceptor associated with the former and the ciliated-type photoreceptor associated with the latter. The description of numerous exceptions to this rule, however, suggests a monophyletic rather than a polyphyletic origin.
Figure 7  Schematic representation of eyes of different animal phyla. Light grey shading marks photoreceptive cells, while dark grey shading indicates pigment cells or pigment layer. (A) Pigment cup eye of the flatworm *Planaria gonocephala*. ep, epidermis; pc, pigment cells; ph, photoreceptors. (B) Pigment cup eye of the nemertean *Drepanophorus spectabilis*. ec, eye cup; Fp, fibrous photoreceptors; np, nodular photoreceptors; on, optic nerve; pc, pigment cells. (C) Single-lens eye of mammals. ch, choriodea; co, cornea; ir, iris; le, lens; re, retina; sc, sclera. (D) Single-lens eye of squid. co, cornea; ir, iris; le, lens; rep, retina with intermingled pigment cells. (E) Compound eye of flies. cl, corneal lens; pc, pigment cells; ph, photoreceptors; ps, pseudocone. [Figure was redrawn after Plate (1924), Gilbert (1994), Remane et al (1985), Wolken (1975).]
of photoreceptors. Many authors now suppose that various photoreceptor cell types derived from a single ciliary precursor (reviewed in Willmer 1994).

Furthermore, on the molecular level, the visual pigments responsible for catching photons are very similar. The visual pigments consist of a vitamin A–related chromophore covalently attached to the protein component opsin. Opsins are seven-transmembrane-domain proteins common to all metazoans. A rhodopsin has also been identified in the protist *Chlamydomonas reinhardtii* that is clearly distinct from bacterial rhodopsins and shows several motifs of animal opsins (Deininger et al 1995). The similarity of the proteins suggests that they represent a single protein family and share a common ancestry. This led Goldsmith (1990) to write: “The fact that all photoreceptor cells use homologous proteins as their photoreceptor pigments, however, raises the question of which other genes may have homologous representatives in structurally diverse photoreceptor organs.”

The structural and functional characterizations of *Pax-6* genes in animals of different phyla identify a gene with homologous representatives. The genes encode a conserved transcription factor acting as a key regulator in the eye developmental pathway of mammals, flies, and possibly other species. Several models can be proposed to explain this association of *Pax-6* with the developing eyes. In one, the ancestral *Pax-6* gene may have played a role in patterning anterior neural regions (see also Chisholm & Horvitz 1995, Hanson & van Heyningen 1995, Nilsson 1996). In already separated lineages, the gene would then gradually change its role to include new target genes that participate in the development of the eye. In this way, selection may have resulted in the independent recruitment of *Pax-6* into independently evolving eye developmental pathways after the separation of phylogenetic lineages. This model of evolution would have to explain why *Pax-6* was always specifically recruited into the eye developmental pathways and not any other anteriorly expressed regulatory gene.

In a second model, the *Pax-6* ancestral gene may have become associated with a specialized target gene involved in, for example, photodetection. Such an association has been termed a seminal regulatory interaction (SRI) (Scott 1994). In time, other useful genes could become targets of *Pax-6*. Multiple target genes of *Pax-6* involved in the development of the visual system would then lock in the structure of *Pax-6*, which could not change anymore without disrupting the entire developmental pathway. However, the structures of the visual system could be modified by the recruitment of additional genes into the eye developmental pathway. The identification of the transcription factor *sine oculis* in *D. melanogaster* (Cheyette et al 1994, Serikaku & O’Tousa 1994) and its mouse homologue *Six3* (Oliver et al 1995), which both act during early eye development, suggests that part of the mechanism for eye development was
in place before the phylogenetic lineages separated. According to this model, the morphologically diverse eyes would have a common evolutionary origin (Halder et al 1995b), wherein a common ancestor Pax-6 was already involved in the development of a primitive visual system. The identification of additional conserved transcription factors has led to the proposal that unexpected parallels could also exist between lens-forming cells in D. melanogaster and vertebrates and between interneurons connected to photoreceptor cells in both vertebrates and nematodes (Halder et al 1995b).

**Pax-6 and the Evolution of the Nervous System**

Homologous membrane proteins, such as ion channels and membrane receptors, and neurotransmitters, such as acetylcholine, are used in the nervous systems of vertebrates and invertebrates. This wide phylogenetic distribution implies that part of the molecular diversity found in the various nervous systems pre-dates the divergence of the major phyla (Arbas et al 1991). Parallels also exist for transcription factors involved in nervous system development such as D. melanogaster achaete-scute complex (AS-C) genes and vertebrate Mash1, which are expressed in dividing neuronal precursors (Campos-Ortega & Jan 1991, Lo et al 1991). The finding that the D. melanogaster genes orthoden-ticle (otd) and empty spiracles (ems) are important for the regionalization of the brain and the observation that the vertebrate counterparts Otx 1/2 and Emx 1/2 show similar regionalized expression patterns suggest that not only the sequence but also the function of these transcription factors in brain development and regionalization could be conserved (Hirth et al 1995, Puelles & Rubenstein 1993).

Early in development, Pax-6 is expressed in a broad domain of the nervous system of vertebrates, flies, squids, and other species. This suggests that, as in the examples mentioned above, Pax-6 functions in the developing nervous systems may be conserved and that Pax-6 may participate in regionalization of the brain in various animals.

**CONCLUSIONS AND PERSPECTIVES**

The characterization of the mouse and human Pax-6 genes and the subsequent cloning of the D. melanogaster Pax-6 homologue eyeless formed the starting point for the identification of Pax-6 genes from species of diverse animal phyla. Pax-6 genes have now been found in all triploblastic animals studied. Future research will reveal whether diploblastic animals have a Pax-6 homologue and may shed light on the divergence of the different Pax-gene classes.

The expression patterns of Pax-6 display several intriguing parallels, although the animals analyzed differ significantly in body plan. The emerging theme is
that Pax-6 expression is associated with developing eyes and brains. Pax-6
expression is also observed in the developing chemosensory structures of ver-
tebrates, ribbonworms, and squid. However, Pax-6 expression in some species
is also observed in structures other than those summarized above, which could
reflect newly acquired functions of Pax-6. Important questions for the future
are how similar the expression patterns in the brain really are and whether the
similarities in expression patterns also reflect similar functions. Interestingly,
a comparable example of conservation of regulatory function is found in the
heart. Insect and mammalian hearts have very different morphologies, but in
the development of both hearts, at least two common regulatory proteins are
used, namely Csx and MEF2, and the fly homologues tinman and D-mef2 (see
references in Scott 1994).

In the case of D. melanogaster, the Pax-6 homologue eyeless acts as a master
regulator for eye development in the context of the imaginal discs. This and
the early expression of eyeless and the other Pax-6 genes in the developing eyes
suggests that Pax-6 genes act very high up in the developmental hierarchies. An
important issue for the future is the identification of downstream and interacting
genes. This will allow us to understand not only what the cellular context and
the requirements are for Pax-6 in order to direct eye development but also
how Pax-6 actually regulates eye development. The discovery of conserved
genes playing a role in the development of diverse eyes has led us (Halder et al
1995b) to propose that the different eyes may have a monophyletic origin. At
the same time, however, these new data raise the issue of how the action of
homologous regulators is interpreted differently to build the distinct eyes or,
in other words, how subsequent evolution has modified the ancestral visual
structure in the numerous ways leading to the different eye types observed
today. It will be informative to compare the regulatory cascade required to
form a D. melanogaster compound eye with that of a mouse eye, to determine
how much is conserved and how many new genes have been recruited into these
developmental pathways, obviously leading to the formation of different types
of eyes.

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