

Squid Pax-6 and eye development

(Pax-6/olfactory organ/evolution mollusc/crystallin)

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ABSTRACT Pax-6 in vertebrates and its homolog *eyeless* in *Drosophila* are known to be essential for eye development. Here we investigate the role of Pax-6 in eye development in another major systematic group, molluscs. We demonstrate that alternatively spliced RNAs derived from a single Pax-6 gene in the squid (*Loligo opalescens*) are expressed in the embryonic eye, olfactory organ, brain, and arms. Despite significant sequence differences between squid Pax-6 and *Drosophila eyeless* in the region outside the paired- and homeodomains, squid Pax-6 is able to induce the formation of ectopic eyes in *Drosophila*. Our results support the idea that Pax-6 related genes are necessary for eye and olfactory system formation throughout the animal kingdom.

Eyes of very diverse type and structure ranging from simple light-sensitive receptors to complex image-forming eyes can be found in the animal kingdom (1–3). Most of the major animal groups comprise species with a simple eye spot. A more elaborate optical system can be found in only six of the animal phyla; these, however, contribute about 96% of the known species (2). Different explanations for the diversity of eyes have been proposed. The morphological differences of the various eyes have been considered as evidence that they did not share a common ancestor and thus are polyphyletic in origin. Indeed, it has been estimated that photoreceptors may have evolved independently 40–60 times (1). An alternative view suggesting a common evolutionary origin of the various eye types has also been proposed (4). Recent data based on the demonstration that the paired domain/homeodomain transcription factor, Pax-6/*eyeless*, has a critical role in eye development in vertebrates (5–10) and *Drosophila* (11) support the idea of a monophyletic origin of the eyes.

Heterozygous mutations in Pax-6 of vertebrates are associated with a variety of eye diseases, including aniridia in human and *Small eye (Sey)* in rodents (5, 6, 8). In homozygotes, Pax-6 mutations are lethal due to a complete absence of eyes and nose and severe defects in brain formation (see ref. 12 for a review). The curtailing of normal *eyeless* expression in the eye primordia of *Drosophila* leads to a reduction or complete absence of the compound eyes (11, 13). Targeted expression of *Drosophila eyeless* or mouse Pax-6 in various imaginal disc primordia of *Drosophila* results in supernumerary eyes (14). On the basis of these results it was proposed that *eyeless/Pax-6* is the master control gene for eye morphogenesis (11, 14, 15). Taken together these data suggest that two types of image-forming eyes, complex eyes of vertebrates and compound eyes of arthropods, share—at least partially—developmental pathways. Furthermore, this suggests that the last common ancestor

of these organisms at the protostome–deuterostome divergence possessed eyes in which a Pax-6 gene was already active (16).

Molluscs represent a third phylum in which highly complex eyes are present. Cephalopod molluscs (squid, octopus, cuttlefish) possess a well developed nervous system and are highly intelligent (17). The complex eyes of cephalopod molluscs and vertebrates have been considered a classical example of convergent evolution (18). The eyes in these two systematic groups are remarkably similar in general appearance and organization but they are formed by different mechanisms during development and differ in many details.

A possible strategy used to evolve complex image-forming eyes from the primitive eyes present in the last common ancestor is the use of similar developmental mechanisms with the same or closely related transcription factors. If this assumption is correct, one would expect that in cephalopod molluscs a Pax-6 homolog is involved in visual system development as it is in *Drosophila* and vertebrates. In this report, we present evidence corroborating this prediction by way of the structural and functional characterization of a Pax-6 homolog of the squid, *Loligo opalescens*.

MATERIALS AND METHODS

Squid Collection. Squids (*L. opalescens*) were collected at the Hopkins Marine Station, Stanford University (Pacific Grove, CA). For *in situ* hybridization, embryos were fixed overnight at 4°C in 3.7% formaldehyde, 0.1 M Mops (pH 7.2), 2 mM EGTA, and 1 mM MgSO₄, rinsed once in 0.9% NaCl, transferred to 90% methanol, and stored at –20°C before hybridization. Embryos used for RNA isolation were frozen at –70°C.

RNA Isolation and RNA Blot Analysis. Total RNA from indicated tissues was isolated by the acidic guanidinium thiocyanate-phenol-chloroform extraction method (RNazol B; Tel-Test, Friendswood, TX). Poly(A)⁺ RNA was purified from total RNA by using the Dynabeads mRNA purification kit (Dyna, Oslo). For Northern blot experiments, about 2 μg of poly(A)⁺ RNA from the indicated tissues were separated by agarose gel electrophoresis, transferred to Duralon UV membrane (Stratagene), and hybridized with ³²P-labeled PCR fragment (positions 70–361) in QuickHyb as recommended by the manufacturer (Stratagene). Squid β-actin PCR probe was used as a control.

Cloning and Characterization of the Squid Pax-6 cDNA. An initial Pax-6 PCR fragment was obtained with primers 5'-CC GCTCGAGGGITG(T/C)GTITC(G/A/T/C)AA-3' (5' oligonucleotide corresponding to the conserved GCVSK sequence in the paired domain, positions 210–223 in Fig. 1) and 5'-GTATCTAGAGTTC(A/C/G/T)CG(A/G/T)AT(T/C)TC-

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Abbreviation: RACE, rapid amplification of cDNA ends.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. U59830).

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CCA-3' (3' oligonucleotide corresponding to the WEIRD sequence in the paired domain, positions 357–371 in Fig. 1) using genomic DNA of the squid *Loligo vulgaris* as a template. The 5' and 3' primers had *Xho*I and *Xba*I restriction sites, respectively, which are underlined. The PCR was conducted in two steps: 5 cycles at low stringency with ramping (1 min at 94°C, in 1 min to 37°C, 1 min at 37°C, in 2.5 min to 72°C, 1 min at 72°C, and in 1 min to 94°C) and then 30 cycles without ramping (30 sec at 94°C, 30 sec at 50°C, 30 sec at 72°C) with final extension at 72°C for 2 min. A 162-bp fragment of the *Pax-6* gene, identified by low stringency Southern hybridization using a mouse *Pax-6* cDNA as a probe, was isolated, cloned in Bluescript KS+, and sequenced. This genomic fragment was used to screen a squid *Ommastrephes sloani pacificus* genomic library (5×10^5 plaques) made in λ EMBL3 (19). Four independent plaques were isolated. A 5-kb *Eco*RI restriction fragment hybridizing with a *Pax-6*-specific probe was identified, cloned, and sequenced. It contained an exon sequence (347 bp) encoding part of the paired domain (see Fig. 1) and its flanking intron sequences. The full-length *Pax-6* coding sequence was obtained by PCR using primers 5'-AAGATTCTCGGACGGTACTATGA-3' (5' primer, positions 222–244 in Fig. 1) and 5'-CCATTT(C/T)GCTC(G/T)TC(G/T)GTT(A/T)GA(A/G)AACCA-3' (3' primer, positions 873–899 in Fig. 1) and then 5'- and 3'-rapid amplification of cDNA ends (RACE) (BRL/GIBCO kits) using squid *L. opalescens* embryonic cDNA as a template. The cDNA for RACE reactions was synthesized using poly(A)⁺ RNA isolated from squid embryos as a template. cDNA2 was obtained in 5' RACE reaction using primers 5'-CTCATAGTACCGTC-CGAGAATCTT-3' (positions 222–245) and 5'-GAGTC-GAGTCTGGAAGGGGA-3' (positions 116–135).

In Situ Hybridization. Whole mount *in situ* hybridization was conducted as described (20). Digoxigenin labeling of the probes and detection were conducted using the Genius RNA labeling and detection kit (Boehringer Mannheim). Specimens were photographed on Kodak Ektachrome 160 film with a Nikon SMZ-U microscope. Whole mount hybridized embryos were embedded in JB-4 glycol methacrylate resin and sectioned with a glass knife. Sections were photographed on Kodak Ektachrome film with a Zeiss Axiophot microscope.

Ectopic Expression of Squid *Pax-6* cDNA in *Drosophila*. The full-length squid *Pax-6* cDNA1 (positions 2–1510 in Fig. 1) was first cloned as a *Not*I fragment in the Bluescript KS+ plasmid and then recloned in the GAL-UAS plasmid, pUAST (21). Orientation of the insert was confirmed by restriction mapping, and flies were transformed as described (14). Ectopic induction of squid *Pax-6* was accomplished by crossing the UAS-*Pax-6* transformants to the MS1096, dpp^{blinker}, and E132 GAL4 expressing lines, and subsequent analysis of the ectopic eyes was performed as described (14).

RESULTS

Isolation and Characterization of Squid *Pax-6* cDNAs. A squid *Pax-6* cDNA was cloned using a PCR approach (see *Materials and Methods*, Fig. 1). There is a single *Pax-6* gene in the squid genome as judged by Southern blot hybridization with several probes corresponding to different parts of the squid *Pax-6* cDNA (data not shown). By 5'-RACE reactions, we identified two variants of the squid *Pax-6* cDNA differing by their 5' exon(s), as was previously observed for the quail *Pax-6* and *Drosophila eyeless* cDNAs (11, 22). The squid cDNA1 has an ORF encoding 460 amino acids and contains a potential initiator methionine codon 11 codons upstream of the encoded paired domain. The deduced protein has a molecular mass of 50,568 Da. cDNA2 does not encode methionine residues within a stretch specifying 44 amino acid residues upstream of the paired domain, which also lacks methionine. Although we do not know at present whether there is an initiator methionine codon further upstream in

cDNA2, it could be that cDNA2 encodes a variant Pax-6 protein containing a homeodomain but not a paired domain as was observed previously for quail and *Caenorhabditis elegans* Pax-6 (22–25).

Comparison of squid Pax-6 with that from other species (refs. 7, 11, 22, and 24–27; see Fig. 1B) confirmed the high degree of conservation of this protein. The highest overall deduced amino acid sequence identities between Pax-6 of the squid and that of other species is 78% with a nemertine, 67% with vertebrates, and 63% with a sea urchin. In the region of the paired domain, squid Pax-6 shows 91–95% identity with its vertebrate, *Drosophila*, nemertine, and sea urchin homologs, and 78% identity with *C. elegans* Pax-6-related proteins; the homeodomain of squid Pax-6 shows 90–98% identities with Pax-6 from these different species. Sequences C⁻ terminal of the homeodomain are also conserved but to a lesser extent with amino acid sequence identities of 74%, 47% and 38% between Pax-6 of squid and nemertine, human, and sea urchin, respectively. This region has been called the PST domain and shown to possess a potent transactivator function (26, 28, 29). The squid Pax-6 linker region between the paired and homeodomain shows 51%, 42%, and 36% identity with nemertine, sea urchin, and human sequences, respectively. Regions upstream of the paired domain show no similarities at all.

At present the positions of only two introns have been identified in the squid *Pax-6* gene; one is located in codon 1 and the other between codons 116 and 117 within the region encoding the paired domain (Fig. 1). They coincide exactly with the positions of introns in the *Pax-6* gene of vertebrates, nemertine, *Drosophila*, and *C. elegans* (7, 11, 22, 24, 25, 27). The second intron found in the paired box of vertebrates, which is used for alternative splicing of an additional small exon (28), is missing in the squid as in all other invertebrate species analyzed so far. In *Drosophila* and *C. elegans* additional splice sites have been found in the 3' part of the paired domain that are absent in squid and nemertine.

Squid *Pax-6* Transcripts. To estimate the size of the squid *Pax-6* mRNA and investigate its expression profile, we conducted Northern blot hybridization tests using available squid adult tissues and embryos (Fig. 2). No hybridization signal was observed in the RNAs from adult tissues. However, a low level of expression in adult optic lobe and cornea was demonstrated by PCR with *Pax-6*-specific primers (data not shown). In contrast to the results obtained with adult tissues, four bands of hybridization indicating *Pax-6* RNAs with a length of \approx 5.6, 8, 11, and 12 kb were detected in the squid embryonic material. Because there appears to be only one *Pax-6* gene in the squid, these data suggest that the squid *Pax-6* primary transcript is alternatively spliced. The longest available cDNAs (1980 and 2000 nt) are much shorter than the smallest mRNA, suggesting that significant parts of 5'- and/or 3'-untranslated regions are missing from our clones. The squid *Pax-6* mRNAs are longer than their 2.7–3 kb homologs in vertebrates (7). The sea urchin *Pax-6* mRNA is at least 5 kb long judging from the cDNA sequencing data (26).

Developmental Expression Pattern of Squid *Pax-6*. To investigate *Pax-6* expression at early stages of squid development and obtain more detailed information on the localization of *Pax-6* mRNA in the embryos, we conducted whole mount *in situ* hybridization experiments. We could not detect expression of *Pax-6* at Arnold stage 15 (Naef stage VI) and earlier (not shown) (17, 30, 31). The first traces of *Pax-6* expression were detected at Arnold stages 16–17 (Naef stages VII–VIII) in the region of the rudimentary eye primordia (Fig. 3A). At these early developmental stages the squid embryos consist of three components: two layers of cells (ectoderm and mesendoderm) comprising the actual embryo, an inner syncytial epithelium (transitory formation, referred to as “yolk syncytium”), and a central mass of yolk (32). The retina anlage becomes evident at Arnold stage 17 (Naef stages VII–VIII) but

A

cDNA2
 CTAATGATACAGTGACAAAT
 N S T V T N

ACGACATTTTAACTCTAGCAGTGGGGCAAGTCCCAACGAAACAGTCTCCGACTGCATGGAATGGTCCACAAATCCGGTGATAGCGGAGGAACTCCAAGGGAGAAACCTACAGGGCAT
 T T F L N S S S G A S P N E Q S P T A W K W S T N P V I A E E T P R E K P T G H

cDNA1
 TTAAGCACTTGGAAACAGCACGAAGAAGTCCCTATGTCCTCGGTAGAGAGTGACCAGCATCCCAATGGGCATAGCGGTGTAATACAGTGGGAGGTGATTTCGTTAACGGACGTCCTCC
 M S C V E S D Q H P N G H S G V N Q L G G V F V N G R P L 120

TTCCAGACTCGACTCGTCAAAGAATAGTGGAGTTGGCACACAGTGGGGCTCGACCTCGGCATCTCTCGTATACTCCAAGTCTCCAACGGTCTCGTTAGCAAGATTCTCGGACGGTACT
 P D S T R Q R I V E L A H S G A R P C D I S R I L Q V S N G C V S K I L G R Y Y 240

ATGAGACGGGCTCCATAAGACCGCGCGATCGGGGCGAGTAAAGCAAGGTTGCCACCCAGAAAGTGGTACAGAAAATAGCCCAATCAAGAGGGAGTCCCTCGATATTTCGCATGGG
 E T G S I R P R A I G G S K P R V A T P E E V V Q K I A Q F K R E C P S I F A W E 360

AAATAGGGATCGATTGCTCAGAAAGGATTGGCACTCAGGATAATATACCAAGTGTCTCTATAACAGGGTCTCGTAATCTTCAGGCGAAATCAGAAAGTCTTCGGTCAGG
 I R D R L L S E G V C T Q D N I P S V S S I N R V L R N L A G E N Q K V L G R Y G 480

GGACTCAATGTATGATAAAGTGGTCTCTGACAGCCAGCATGGCCAGCCCAATCCCTGGTACGCACCTAATCCCTCCATGGCCGGAGTACGAGGCTCCCTCAAGTTATACTCAAC
 T T M Y D K L G L L N G Q A W P R P N P W Y A P N A S M A G L S A P S S Y T Q P 600

CAACGCACCTCCACCGTTCGCTGGGAAAAAGAGATGGAGAACATGAGTACCAATTCGGGACACGGTTCAGGGGATGGGAGCCGAATGGTGAAGTACGAGTACGACCAATTCGATCAGGT
 N A P P T V A G K K E M E N M S T N S D N G Q G D G S Q N G E T D E Q M R I R L 720

TGAAAAGGAATTCGCAACGGAATCGTACGTCACTCAGCAGCAAAATGAAGCTTTGGAAAAAGAAATTTGAACGTACCCATTCAGAGCTTTTTCGACGTGAGAGGTTAGCTCAGA
 K R K L Q R N R A I G G S K P R V A T P E E V V Q K I A Q F K R E C P S I F A W E 840

AAATAGATTTGCCGAAAGCTCGTATACAGGTGGTCTCGAATCGAAGGGCAAGTTCGGGACACGGTTCAGGGGATGGGAGCCGAATGGTGAAGTACGAGTACGACCAATTCGATCAGGT
 I D L P E A R I Q V W F S N R R A K W R R E E K L R N Q R R E A A N G S T R L P 960

CAATTAACAGCAGTTTCCACATTCATGTAACCTACCATCAATCAACCTCGGCATCAATGGCCGAATTCCTACAGGACCTTAAAGGCTGGATGTCAAGCGGTTTAGGCTCGATG
 I N S S F P H S M Y P T I N Q P L A S M A E S Y R D L K G W D V A S G L G S M P 1080

CCACCGTCCAAACTACTCCTTATCAATAACATCACTGGAACCCCGCTGCTTACAGTCCAAATGCTCTCATACTCGTGTATGTTACGATTATGTGTCAGGAACAGCAAGAGCT
 T V P N Y S L S N N I T G N P A C L Q S N A S S Y S C M L P D Y V S G T A R S Y 1200

ACGACCCCTTATCCCTCAGTAACTATTTCGATCGTCTGGCAATCCCGCGGCTGGTACGATCCCATGACACCCACAGTACCAACGGAGCTTCCACAGGTCTCATCTCACCCGGGG
 P L S L S N Y S R S C N P A A G A G M Q S H M T P H S T G N G A S T G L I S P G V 1320

TATCGTTCCTATTACAGTACCCGCGGAGGCCCTCAGGATATGAGCACTCACCATATGAGTTCGATGGCTTCACAATATTGGCCACGCCTTCACTGACAGTGGCAGAGCTCAGCGAAA
 S V P I Q V P G G G P Q D M S T H H M S S M A S Q Y W P R L Q 1440

TGGAGACGGAACGCTATTACTAATTAACCAAACTTTAATGTGATAATGTTGAGATTAATGACGCGAAGTGAAGTACTCACCATTTTTTCCCTCTTTTATTGAAGAGTTCAAAAACAAA
 CAACAACAACACCGATATTAGCTACCAATATTTCTTAATGGAGGAGAGATTACAAGAGACTTTGAAAAAAAAGGAATGAAAAAGAAAAACAAAAAACAACAAAACAAAC 1560

AATCGAATTTAATGATTTTGTGATGTAAGAAAAATTAATCATCATCATATTATTATTATTTTTTGTGTGAGTGTATGCTTGTATACAGACATTTATGACTAGTGGTGGCGGT
 GATGTTGTTGAAGGAAGGAACAAGGAGGAGAAAAGCGGTGAGCGAAGAAGGCCCTTTTTTTTTTTTTTGTAGCCAAAAATAAATGAAAAAAGATCACGAAGAGTCTTAATAT 1680

TCGTGATGAGGTTTTCATTGTTTACTCGAATTATGTCGGAAGAAAGAATAAACGAG 1800

1920

B

	PAIRED DOMAIN	%id
Loligo	GHSVNLQGGVFNVRPLDSTRQIVELAHSGARPCDISRILQVSNQCVSKILGRYYETGSIRP	100
Homo	S K	96
Drosophila	S G K	91
Lineus	S T	93
Paracentrotus	S	90
Caenorhabditis	T A K D K C L C S T	78
Loligo	RAIGGSKPRVATPEVVQKIAQFKRECPISFAWEIRDRLSEGVCTQDNIPVSSVINRVLNLA	
Homo	S S Y S	
Drosophila	A S Y Q M N	AQ
Lineus	G H Y D A N	S
Paracentrotus	H TR HY A K I N E	S
Caenorhabditis	SD E EDY DQ K ADNI NNET	AK
Loligo	LQRNRTSFTAQIEALEKEFERHYDPVFAERERLAQKIDLPEARIQVWFSNRRKWRREE	100
Homo	QE A	95
Drosophila	ND DS G C	90
Lineus	N	98
Paracentrotus	Q E	96
Caenorhabditis	QV S Q	93
Loligo	KLRNQRREAN-----GSTRIPINSSFFH---SNVFTINQFLASH----AESYRDLKGV	100
Homo	Q S -----TPSHI S ST--- V Q P T TTPVSSFTSG MLGRDST	47
Lineus	D -----GS I N--- S H I T ---G T *	74
Paracentrotus	QQ EVGGVHTQS SH LS GYSSNNV V Q P S A-----TMVPR	38
Loligo	DVASGLSMPTEVPNYSLSNITGNPAQLSNASSYSCHLPDVSQTARSYDPLSLSNYSRS	
Homo	ALHTYSAL PM SFMA LPMQ P-UP QD TSP VNG TYPFHQMT	
Paracentrotus	TAPDSYSAI PM SF MASG--P IQMQRDTR TR HQPH YDSL ST-PPNHTM	
Loligo	SCNPAAGQSHMTPHSTINGASTGLISPGVSVIQQVGGGQDMSTHHMSMASQYWPRLQ	
Homo	MNSQPMG-----TSGTT V SE D-----M	
Paracentrotus	HGH PABRGMHQ S MNSACNV V SS A THPS ----- I	

C-TERMINAL DOMAIN

		%id
Loligo	KLRNQRREAN-----GSTRIPINSSFFH---SNVFTINQFLASH----AESYRDLKGV	100
Homo	Q S -----TPSHI S ST--- V Q P T TTPVSSFTSG MLGRDST	47
Lineus	D -----GS I N--- S H I T ---G T *	74
Paracentrotus	QQ EVGGVHTQS SH LS GYSSNNV V Q P S A-----TMVPR	38

FIG. 1. (A) Nucleotide and deduced amino acid sequences of the squid *L. opalescens* embryonic *Pax-6* cDNA. The paired domain and homeodomain are boxed. Two arrows indicate the positions of two known introns in the *L. opalescens Pax-6* gene. (B) Comparison of the amino acid sequences between paired-, homeo- and C-terminal domains of vertebrate and invertebrate species. The squid sequence is shown in full; for other sequences only differing amino acids are shown. A - indicates gaps that were introduced to maximize similarities in the C-terminal domain; an * marks the end of the known nemertine sequence. The percent identities are shown for the squid sequence.

it is still not enclosed by the ectodermal fold. At Arnold stages 19–20 (Naef stages VIII–IX), when the optic vesicle is formed by internalization of the eye placode, *Pax-6* expression was increased and clearly seen in the regions of the developing eyes, optic lobe, arms, and mantle (Fig. 3B). The expression of *Pax-6* in the arms and mantle of the squid embryo indicates that this transcription factor has a role in the development of these tissues, an observation that deserves further study. Differentiation of the lens primordia and formation of the iris

fold begin after Arnold stages 19–20. At Arnold stages 26–28 (Naef stages XIV–XVIII), *Pax-6* expression was detected predominantly in the eyes, chemosensory olfactory organ (33–35), arms, and brain (Fig. 3 C and G). In the olfactory organ, arms, and suckers, *Pax-6* was expressed mainly in the outer layers (Fig. 3D) that are rich in nerve endings, while in the brain expression was detected mainly in the cerebral ganglion (Fig. 3 E and F). In the eye, *Pax-6* expression was detected at the surface of the eye, the developing iris and the

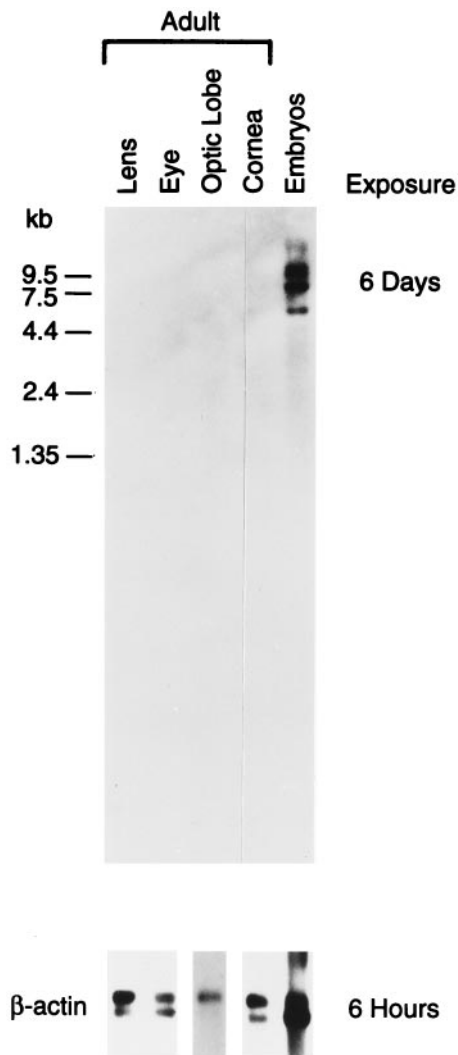


FIG. 2. Northern blot analysis of the squid *Pax-6* gene in different adult tissues and embryos.

anterior part of the anterior lens segment (Fig. 3 *G* and *H*). We could not detect any expression in the inner lentigenic cells that give rise to the lens posterior segment or in the retina. *Pax-6* mRNA is also present in the fold of tissue posterior to the eye (see bracket, Fig. 3*H*) which will move forward in development to surround the eyeball and form the cornea (17). *Pax-6* mRNA staining is not yet seen on the developing corneal fold above the eye in this section. To eliminate the possibility of poor probe penetration we removed the eye, cut it open and hybridized it as whole mount. The pattern of hybridization was indistinguishable from that obtained with intact embryos (data not shown). Whole mount hybridization using two mixed antisense probes from S-crystallins (*Lops12* and *Lops7*; refs. 36 and 37) that belong to the major squid lens and cornea-specific proteins and are synthesized in the lentigenic cells of the embryos (38), showed as expected expression in the inner and outer lentigenic cells (Fig. 3 *I* and *J*).

Ectopic Expression of Squid *Pax-6* in *Drosophila*. Functional studies of *Pax-6* in squid are complicated due to the absence of mutants, transgenic methods, and appropriate cell lines. Thus, as a first approach for investigating the functional role of squid *Pax-6*, we decided to test whether it can induce ectopic eyes in *Drosophila*. We used the GAL4 system (21) to target squid *Pax-6* cDNA1 expression to various imaginal discs. The results demonstrated (Fig. 4) that squid *Pax-6* is able to induce ectopic *Drosophila* eyes on wings, antennae, and legs, as was previously

demonstrated for *Drosophila eyeless* and mouse *Pax-6* (14). All *Drosophila* eye-specific structures including cornea, pigment cells, cone cells and photoreceptors with rhabdomeres were formed in the ectopic eyes induced by squid *Pax-6* cDNA.

DISCUSSION

We have isolated cDNAs corresponding to the squid *Pax-6* gene. Squid *Pax-6* shows the highest overall amino acid identity (78%) with *Pax-6* from nemertines, consistent with nemertines being coelomate animals and molluscs and nemertines having a close evolutionary relationship (27, 39). Despite the fact that only the paired- and homeodomain regions of *Pax-6/eyeless* are well conserved between squid and *Drosophila*, squid *Pax-6* can induce the formation of ectopic eyes in *Drosophila* as was previously demonstrated for *Drosophila eyeless* and mouse *Pax-6* (14). It has been shown for vertebrate *Pax-6* that the C-terminal PST domain is necessary for function of the protein (28, 29). Although squid *Pax-6* and *Drosophila eyeless* C-terminal domains show appreciable differences in sequence and have different lengths (168 and 368 amino acids, respectively), both are proline-, serine- and threonine-rich (33% and 37%, respectively). It seems reasonable to propose that structural features such as secondary or tertiary folding, rather than direct similarity in amino acid sequences, are responsible for the common functional properties of squid *Pax-6* and *Drosophila eyeless*, as was proposed for the C-terminal domains of vertebrate *Pax-3* and *Drosophila* Gooseberry and Paired proteins (40).

The expression of squid *Pax-6* in the developing brain, olfactory organ, and eyes shows parallels and differences with the expression pattern of *Pax-6* in vertebrates, *Drosophila*, and nemertines. Despite remarkable similarity in general appearance of the eye in vertebrates and cephalopods, they are formed differently in the course of development. In the squid, the eye develops from a thickened ectodermal monolayer, which forms a multilayered oval mitotic placode on the dorsal surface of the head lobe. The eye vesicle develops by the internalization of this placode (17). The developing eye vesicle of squids is sealed off by the three layers of the primary eye fold: outer and inner ectodermal layers and a layer of mesoderm that separates the ectodermal layers. The outer ectodermal layer will produce the iris and outer lens segment, while the inner ectodermal layer will give rise to the inner lens segment. The lens is formed by projection of lentigenic processes and consists of two parts separated by a septum (41). The cornea has a very different ectodermal origin from the rest of the eye in the squid and is formed as a new skin fold from the edge of the forward growing arms (42). In vertebrates, the optic vesicle appears as an evagination of the diencephalon and the lens develops from the overlying ectoderm (see refs. 43 and 44 for reviews). During eye development in vertebrates, *Pax-6* expression occurs in the optic vesicle, the overlying surface ectoderm, and, at later stages of development, in the developing retina, iris/ciliary body, lens, and cornea (7, 9, 45). In *Drosophila*, *eyeless* is expressed in the eye portion of the eye-antennal imaginal disc, with higher expression being observed in the undifferentiated cells anterior to the morphogenetic furrow than behind the furrow where cells start to differentiate (11). Thus, both in vertebrates and *Drosophila* *Pax-6/eyeless* is involved in the formation of a morphogenetic field that will give rise to the eye. Explanation and transplantation experiments with squid eye rudiments from Arnold stages 15–17 (Naef stages VI–VIII) demonstrated that they possess the ability for autonomous eye differentiation (46), and our data are consistent with the idea that *Pax-6* is necessary for establishing the “eye field,” and that eye determination in squid is primarily a process intrinsic to the differentiation capacities of the blastoderm (46).

Our data also show that there are some notable differences between the expression pattern of *Pax-6* in the developing eyes of squid and vertebrates, and that the expression pattern of

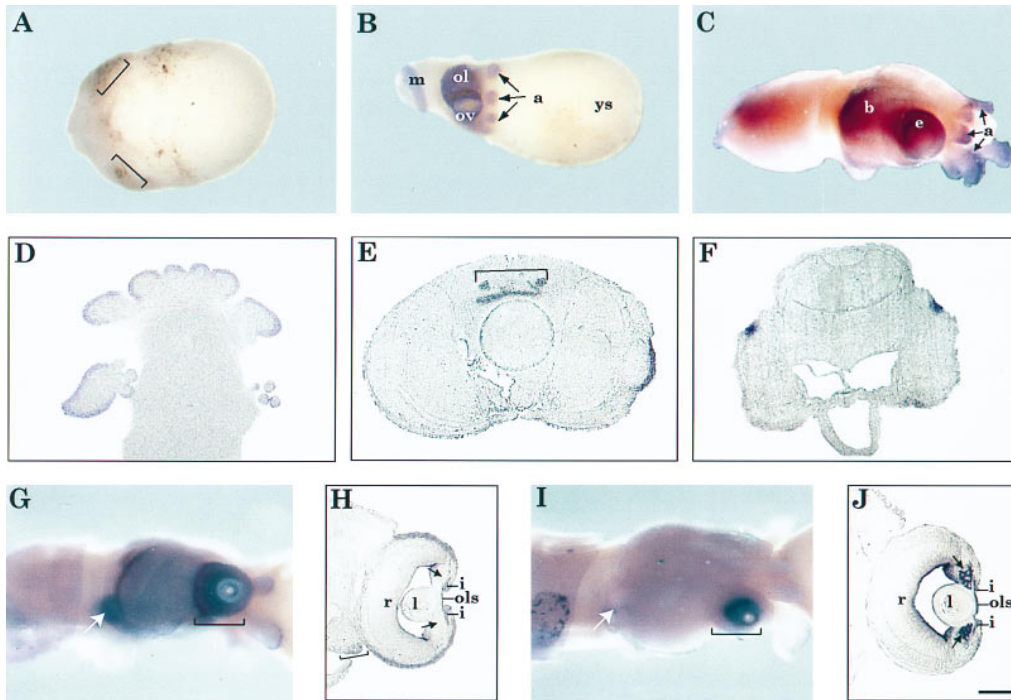


FIG. 3. Expression of *Pax-6* during *L. opalescens* embryonic development. (A–C, G, and J) Whole mount *in situ* hybridization of squid embryos using *Pax-6* (A–C and G) and S-crystallin (I) antisense riboprobes. Sense probes were used as controls (data not shown). (D–F, H, and J) Frontal, 10- μ m plastic sections of embryos hybridized as whole mount with *Pax-6* (D–F and H) and S-crystallin (J) antisense probes (20). (A) Stage 17 embryo. Brackets indicate the eye primordia where first traces of *Pax-6* expression are detected. (B) Stage 20 embryo. Arrows point to the arms. (C) Stage 27 embryo. The yolk sac was removed. Arrows point to the arms and suckers. (D) Section through the arms and suckers. (E) Section through the brain adjacent to the posterior edge of the eyes. Bracket indicates the cerebral ganglion. (F) Section through the posterior end of the brain. (G) Stage 27 embryo. Bracket indicates eye and arrow indicates olfactory organ, where expression of *Pax-6* is detected. (H) Section through the eye of an embryo as in G. Brackets indicate areas from which the cornea will develop as a fold from the edge of forward growing arms and arrows point to lentigenic cells which do not express *Pax-6*. (I) Stage 27 embryo, hybridized with S-crystallin probe. Bracket indicates eye where expression of S-crystallin is detected. Note that there is no expression in the olfactory organ (arrow). (J) Section through the eye of an embryo as in I. Brackets and arrows are as in H. Label at the inner edge of the eye chamber is background staining also observed with the sense probe. (Bar in J = 300 μ m in A and B, 150 μ m in C, 100 μ m in D–F, 200 μ m in G and I, and 50 μ m in H and J.) Abbreviations: a, arms; b, brain; e, eye; ep, eye primordia; i, iris; l, lens; m, mantle; ol, optic lobe; ols, outer lens segment; ov, optic vesicle; r, retina; ys, yolk sac.

squid *Pax-6* in the developing eye is more similar to that of *eyeless* in *Drosophila* than of *Pax-6* in vertebrates. *eyeless* is actively expressed in undifferentiated precursor cells of the eye imaginal disks in *Drosophila* and its expression decreases significantly in differentiating cells posterior to the morphogenetic furrow (11). In vertebrates, *Pax-6* is expressed in

presumptive neural retina and in amacrine and ganglion cells at later stages of retina development (7, 9, 47). In the squid, expression of *Pax-6* was not detected in differentiating retina cells (Fig. 3H). Moreover, in vertebrates *Pax-6* is expressed in the developing lens (7, 9, 45) and has been implicated in transcription of many crystallin genes (see ref. 48 for a review).

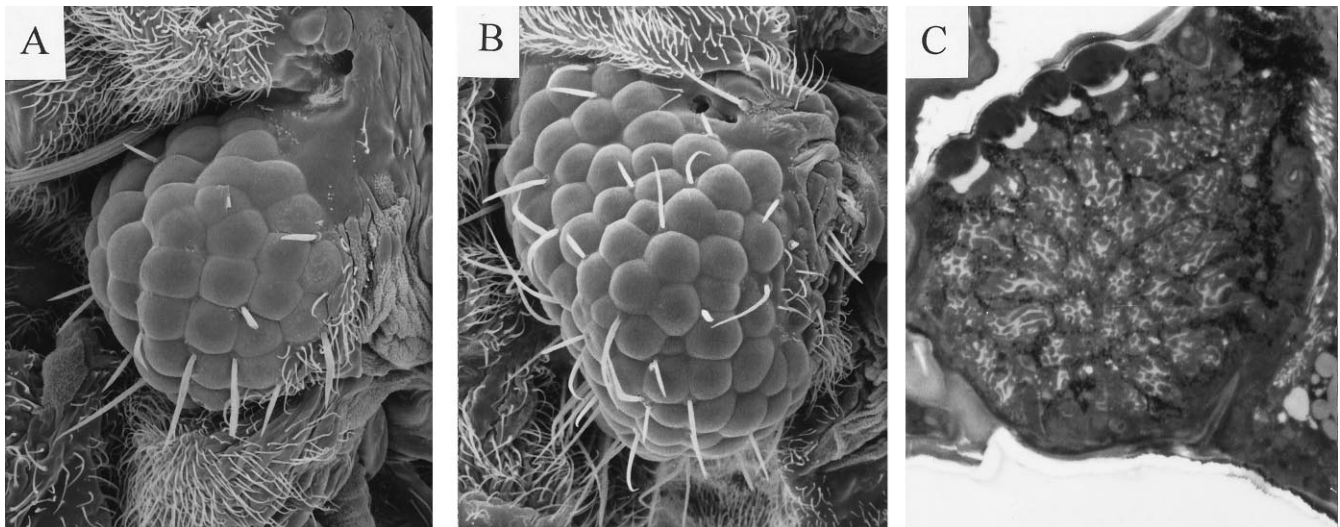


FIG. 4. The ectopic expression of *L. opalescens Pax-6* cDNA1 by means of the GAL4 system (21) induces the formation of ectopic eye in *Drosophila*. (A and B) Scanning electron micrograph of an ectopic eye induced on the wing by misexpression of *L. opalescens Pax-6* cDNA1 (A) and *Drosophila eyeless* (B). (C) Section through a *L. opalescens Pax-6*-induced ectopic eye stained with Azur II and methylene blue.

However, we did not find squid *Pax-6* mRNA in the inner lentigenic cells, a major site of S-crystallin synthesis (38, 49). Nonetheless, *Pax-6* may still be involved in the regulation of S-crystallin synthesis in the developing anterior segment of the squid lens, where expression of both *Pax-6* and two S-crystallin genes (*Lops7* and *-12*) were observed. We have noted potential *Pax-6* binding sites in the promoters of several squid S-crystallin genes (data not shown). Thus, a possible role of *Pax-6* in crystallin gene regulation in the squid remains to be established. Together, our results suggest that squid *Pax-6* is not as critical for later stages of retina and lens development as it is in vertebrates.

The expression of *Pax-6* in the developing vertebrate nose and olfactory bulbs (7), nemertine cerebral (chemosensory) organ (27), and squid olfactory organ suggests that also these structures may have a common ancestral origin. It is noteworthy that development of the olfactory and visual systems has a number of common features. In vertebrates, both the visual and olfactory systems are ectodermally derived and depend upon determinative interactions with particular regions of the brain (43). Of special interest here is that expression of *Pax-6* is critical for the development of both sensory systems (9). In *Drosophila*, the olfactory and visual organs derive from the same eye-antennal imaginal disc; moreover, both systems express a number of identical genes during development (for example, *irregular chiasm C-roughest* for cell death, *lozenge* for pattern formation, *retinal degeneration B* for maintenance and physiology, *norpA* for phototransduction and odorant reception; see ref. 50 for a review). Further experiments comparing the roles of *Pax-6* in the developing olfactory and visual systems seem warranted.

It is not clear at present whether squid *Pax-6* initiates the development of ectopic eyes in *Drosophila* directly or indirectly by activating *eyeless*. Moreover, it is not known how many common genes acting downstream of *Pax-6* are involved in the cascade leading to eye development in different systematic groups. At least two other candidate genes have been identified. One is *eyes absent* which is necessary for *Drosophila* eye development (51); two homologous genes for *eyes absent* are expressed in vertebrate lens and retina (52), and one homolog was recently identified in the squid (S.I.T., unpublished data). The homeobox gene, *sine oculis*, is also essential for eye development in *Drosophila* (53) and acts after *eyes absent* (54). The *sine oculis* homolog, *Six-3*, is expressed in the vertebrate eye (55). *sine oculis* homologs have not been identified yet in the squid.

Our data support the idea that morphologically distinct eyes of different species have arisen through elaboration of a common conserved *Pax-6*-dependent mechanism (11, 14, 15) that is operative at early stages of eye development and that the anatomical differences among eyes arose later in evolution. Consequently, we believe that eyes in cephalopods and vertebrates have a common evolutionary origin and are products of parallel rather than convergent evolution (56).

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