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Evolution

Isolation of a *Pax-6* homolog from the ribbonworm *Lineus sanguineus*

(paired box/homeobox/eye morphogenesis/eye evolution/cerebral organ)

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ABSTRACT **The *Pax-6* genes of vertebrates and *Drosophila* encode transcription factors with highly conserved paired- and homeodomains. They are expressed in the nervous system and the developing eyes. Loss-of-function mutations in mammals and flies lead to a reduction or absence of the eyes. By ectopic expression of *Pax-6* in *Drosophila* ectopic eyes can be induced, indicating a determinative role in eye morphogenesis. We have isolated a *Pax-6* homolog of the ribbonworm *Lineus sanguineus*. This gene shares extensive sequence identity and several conserved splice sites with the mammalian and *Drosophila* genes. During head regeneration the *L. sanguineus* *Pax-6* homolog is expressed in the central nervous system, in the cerebral organ, and in the eye region. These findings support the hypothesis that *Pax-6* was present in primitive metazoa before the evolutionary separation of vertebrates and arthropods and suggest a fundamental role in eye and central nervous system development.**

The members of the *Pax* gene family of transcription factors are characterized by the 130-amino acid-long, paired domain involved in DNA binding (1–4). A second DNA-binding domain, a paired-type homeodomain, is present in some *Pax* genes. The sequence, gene structure, and function are highly conserved in the *Pax-6* homologs of vertebrates and invertebrates. The murine and human Pax-6 proteins are identical over the entire length of 422 amino acids (5, 6). The zebrafish *Pax-6* gene is 97% identical at the amino acid level to the mammalian *Pax-6* homologs (7, 8). The *Drosophila melanogaster Pax-6* homolog, which is encoded by the eyeless (*ey*) gene, shares 94% sequence identity in the paired domain and 90% in the homeodomain with the mammalian *Pax-6* genes (9). The *Caenorhabditis elegans vab-3* gene encodes the most highly diverged *Pax-6* homolog isolated so far (10). The sequence similarity of the *vab-3* paired domain to that of the vertebrate and *Drosophila Pax-6* genes is in the range of 80%, whereas the *vab-3* homeodomain shares over 90% identity with that of the other known *Pax-6* genes. The presence of conserved splice sites in the paired box and in the homeobox of the human (11), quail (12), *Drosophila* (9), and *C. elegans* (10, 13) *Pax-6* genes suggests that the vertebrate, *Drosophila*, and *C. elegans Pax-6* genes are orthologous. In humans (6), mouse (5), quail (14), chicken (15), zebrafish (7, 8), and *Drosophila* (9) *Pax-6* is expressed in the developing central nervous system and eye anlagen. Loss-of-function mutations in humans, mouse, rat, and *Drosophila* reveal an essential role for *Pax-6* in eye development of these species. In heterozygous individuals the human Aniridia syndrome is characterized by complete or partial absence of the iris and malformation of the lens, cornea, retina and optic nerve (6). The eyes are reduced in mice and rats heterozygous for the Small eye (*Sey*) mutation (16, 17), whereas homozygous mutant embryos lack the eyes and nose completely. Flies homozygous for the *ey* mutation show a reduction or complete absence of the compound eyes (9, 18, 19).

Ectopic expression of the *Drosophila* as well as the mouse *Pax-6* gene in various imaginal discs during *Drosophila* development results in the formation of supernumerary eyes in the fly, indicating that *Pax-6* is a master control gene of eye development shared between insects and mammals (20). Furthermore, these results support the orthology of the vertebrate and insect *Pax-6* genes. The finding of this highly conserved transcription factor as a key regulator of eye morphogenesis in vertebrates and flies indicates a common evolutionary origin, suggesting that an ancestral *Pax-6* gene was involved in eye morphogenesis and probably central nervous system development of the last common ancestor of the vertebrates and invertebrates (9).

We pursued this hypothesis by examining a lower metazoan with simple eyes, the ribbonworm (nemertine) *Lineus sanguineus*. The nemertine phylum consists of ≈900 species of mostly marine worms (21). The nemertines share the spiral cleavage of the zygote with several invertebrate phyla—namely, with platyhelminthes, annelids, and molluscs. However, sequence comparison of several homeobox genes isolated from *L. sanguineus* revealed a high sequence similarity to vertebrate homeoboxes, suggesting an unexpectedly close relationship of *L. sanguineus* to vertebrates (M.K.-C., F.L., J. Bièrne, and W.J.G., unpublished work). It is tempting to speculate that this indicates a close relationship of nemertines to both the vertebrates and invertebrates, suggesting that nemertines could represent moderately diverged descendants of the common ancestors of vertebrates and invertebrates.

Here we describe the isolation of the *L. sanguineus Pax-6* (*LsPax-6*) gene. The conservation of the amino acid sequence both in the paired and the homeodomain, and the presence of three conserved splice sites, suggest that *LsPax-6* is orthologous to the *Drosophila, C. elegans*, and vertebrate *Pax-6* genes. The expression pattern in regenerating heads of *L. sanguineus* and in developing worms of the closely related *Lineus viridis* suggests a role in the central nervous system, the cerebral (chemosensory) organ, and probably in eye development, similar to that in insects and vertebrates.

MATERIALS AND METHODS

General Methods. The screening of the genomic library and Southern blot analysis were done as described in Sambrook *et al.* (22). For the establishment of genomic libraries, DNA was

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Abbreviations: RT-PCR, reverse transcription PCR; RACE-PCR, rapid amplification of cDNA ends by PCR; *Ls Pax-6, Lineus sanguineus Pax-6* gene.

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isolated from *L. sanguineus* and *L. viridis* as described by Hempstead *et al.* (23), partially digested with *MBo* I (22) and cloned into the λFIX II vector (Stratagene). Total RNA was isolated from regeneration blastemas after 5, 7, and 17 days of regeneration according to Chomczynski and Sacchi (24).

Amplification of Paired Box Fragments from Genomic DNA. Degenerate primers specific for the paired box were designed by P. Callaerts and W.J.G. The sense primers corresponding to amino acids GCVSK were CCG CTC GAG GGI TGY GTI TCN AA and CCG CTC GAG GGI TGY GTI AGY AA containing an *Xho* I site; the antisense primer corresponding to amino acids WEIRD was GTA TCT AGA GTC NCG DAT YTC CCA containing an *Xba* I site where D is A, G, or T; N is A, G, C, or T; Y is C or T. Conditions for the PCR were as follows: 4 μg of genomic DNA, 0.5 μM (each) primer, 0.5 mM (each) dNTP, 0.25 unit of *Taq* polymerase and 5 μl of 10× PCR buffer in 50 μl; 5 cycles of 1 min at 94°C, 1 min at 37°C, and 3 min at 72°C and then 30 cycles of 1 min at 94°C, 1 min at 50°C, and 3 min at 72°C. Aliquots of the PCR reactions were analyzed by Southern blot hybridization at low stringency (25) with a mouse *Pax-6* cDNA probe. Hybridizing bands were cloned into Bluescript.

Reverse Transcription-PCR (RT-PCR). One microgram of total RNA isolated from regeneration blastemas was reverse-transcribed (26). To RT-PCR amplify fragment RT1 (Fig. 1*B*) the following primers were designed: a gene-specific sense primer corresponding to amino acids SKPRV of the paired domain CCG CTC GAG CAG CAA GCC CAG AGT GG containing an *Xho* I site and degenerate antisense primers corresponding to amino acids WFSNR of the homeodomain: GGC TCT AGA GCK RTT NGA RAA CCA and GGC TCT AGA GCK RTT RCT RAA CCA containing an *Xba* I site where K is G or T; R is A or G; N is A, T, G, or C. The cycling conditions were as follows: 40 cycles for 1 min at 94°C, 1 min at 50°C, 3 min at 72°C. Positive PCR products were identified by Southern blot analysis at high stringency using a 33-11 probe (Fig. 1*B*) and cloned into Bluescript. RT-PCR amplification of fragment RT2 (Fig. 1*B*) was done with two gene specific primers: the sense primer was CAC AGT GGC GTC AAC CAA CTC G, the antisense primer was TAT GTT TCT CCC ATT GTT GCT ATG G. The cycling conditions were as follows: 30 cycles for 1 min at 94°C, 1 min at 65°C, 3 min at 72°C.

Whole-Mount *In Situ* Hybridization. Worms were anesthetized in 8% magnesium chloride and photographed, rinsed in phosphate-buffered saline (PBS) and treated with 0.1 M cysteine chloride for 15 min to remove the mucus on their surface. Whole-mount *in situ* hybridization was done as described (27) with the following modification: proteinase K treatment was extended to 15 min. The worms were then washed four times for 30 min in 150 mM NaCl/1% Nonidet P-40/0.5% sodium deoxycholate/0.3% SDS/1 mM EDTA/50 mM Tris·HCl, pH 8. Nonspecific adsorption was prevented by treatment with 1% blocking agent (Boehringer Mannheim) in PBS/0.1% Tween 20 for 2 hr. Clone RT1 was used as a template for the synthesis of digoxigenin-labeled sense and antisense RNA probes.

RESULTS

Isolation of Two Paired Box Fragments by PCR. To isolate a *Pax-6* homolog of *L. sanguineus*, paired box fragments were PCR-amplified from genomic DNA using degenerate PCR primers. To ensure that also a diverged *Pax-6* paired box could be PCR amplified, degenerate PCR primers were designed which are directed against two regions that are highly conserved in all known paired domains. Therefore, these primers are not *Pax-6* specific. PCR amplification resulted in the isolation of two different paired box fragments from the genome of *L. sanguineus* (clones 33-11 and 33-13). Both fragments are 174 bp long and contain an uninterrupted open reading frame, which in the case of clone 33-11 shows 93% sequence identity at the amino acid level to the paired domain of the human and mouse *Pax-6* genes. Clone 33-13 shows 82% sequence identity to the paired domain of the human and mouse *Pax-1* genes, suggesting that the corresponding gene represents a *L. sanguineus Pax-1* homolog.

Isolation of *LsPax-6*. Using clone 33-11 five genomic phages were isolated by screening a genomic library at high stringency. These phages contain the paired box but no homeobox sequences. To clone the homeobox sequences that are separated from the paired box by a large intron, RNA isolated from

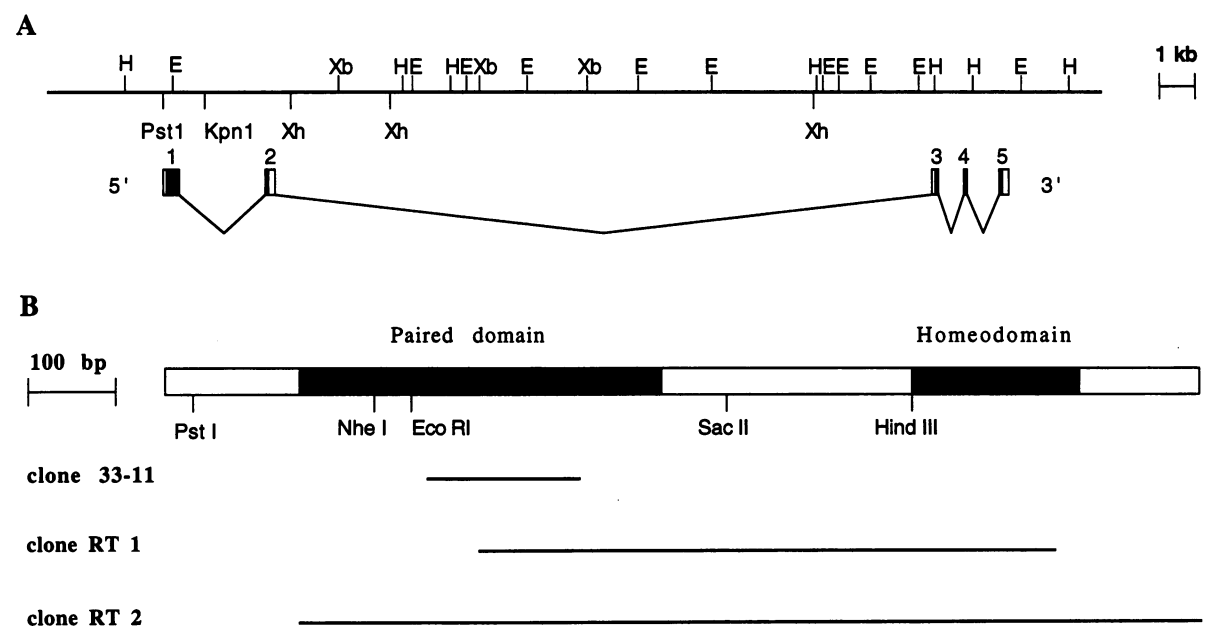

FIG. 1. Structural organization of the *LsPax-6* locus. (*A*) Genomic organization and restriction map of the *LsPax-6* locus. The paired box is indicated in black; the homeobox is hatched. E, *Eco*RI; H, *Hin*dIII, Xb, *Xba* I; Xh, *Xho* I. (*B*) Schematic structure of the open reading frame. The extent of the genomic PCR clone (33-11) and the RT PCR clones RT1 and RT2 is indicated. The open reading frame may not be full length because the 5′ and 3′ termini are deduced from genomic sequences only.

regeneration blastemas was amplified by RT-PCR using a gene-specific sense primer and degenerate antisense primers specific for the paired-type homeodomain. A single PCR product of 600 bp (clone RT1, Fig. 1*B*) was amplified, spanning the paired and the homeobox. Subsequently, RT1 was used to isolate genomic phages containing the *LsPax-6* homeobox. By sequencing the relevant parts of these genomic phages the open reading frame could be extended in both directions (Fig. 1*B*), which allowed us to design gene-specific primers for amplifying the respective sequences of the *LsPax-6* transcript by RT-PCR (clone RT2, Fig. 1*B*). The sense primer corresponds to amino acids 2 to 8 of the paired domain, the antisense primer corresponds to the amino acids PIAT-MGETY at the 3′ end of the identified open reading frame. PCR amplification resulted in a single PCR product with the expected length of 970 bp (clone RT2). Sequence analysis of both ends and restriction analysis showed that clone RT2 corresponds to the respective genomic sequences. The isolation of clone RT2 by RT-PCR shows that there exists an *LsPax-6* transcript which harbors both the complete paired- and homeobox. Attempts to isolate the 5′ and 3′ end of the *LsPax-6* transcript by RACE-PCR were not successful. Therefore, the putative 5′ and 3′ termini are deduced from genomic sequences only, and the open reading frame may not be full length.

Conservation of the Sequence and Gene Structure of *Ls-Pax-6*. Both the paired domain and the homeodomain of *LsPax-6* (Fig. 2) are most similar to the respective domains of the known vertebrate and invertebrate *Pax-6* genes (Fig. 3 *A* and *B*). The sequence identity to all other known *Pax* genes harboring a paired- and a homeodomain is considerably lower in both domains (in the range of 70% and less).

The paired domain of the *LsPax-6* differs in four positions (positions 25, 51, 106, and 107; Fig. 3*A*) from all other known *Pax-6* genes. At three of these positions (positions 25, 51, and 106) the substitutions in *LsPax-6* represent conservative exchanges.

The paired domain is separated from the paired-type homeodomain by a 92-amino acid linker region. This region is shorter in the sea urchin (72 amino acids) (28) and the vertebrate (76 amino acids) *Pax-6* genes, whereas in the *Drosophila Pax-6* gene it is considerably longer (244 amino acids). The linker regions of the known *Pax-6* genes share only little sequence homology, with the exception of the MYD-KLGLLNGQ motif that is highly conserved in the vertebrate, *Drosophila*, sea urchin, and *Lineus Pax-6* linker regions and absent in all other known *Pax* genes (Fig. 3*C*), suggesting that this motif is *Pax-6* specific.

The homeodomain of *LsPax-6* and the *Pax-6* genes from other species differ at positions in the first and second α-helix and in the turn of the helix–turn–helix motif, whereas the recognition helix is identical (Fig. 3*B*). The four amino acids preceding the homeodomain and the seven amino acids following the homeodomain are identical in all known *Pax-6* genes (Fig. 2).

The vertebrate, *Drosophila*, and sea urchin *Pax-6* genes harbor a carboxyl-terminal region that is considerably longer than the identified 42-amino acid carboxyl-terminal to the

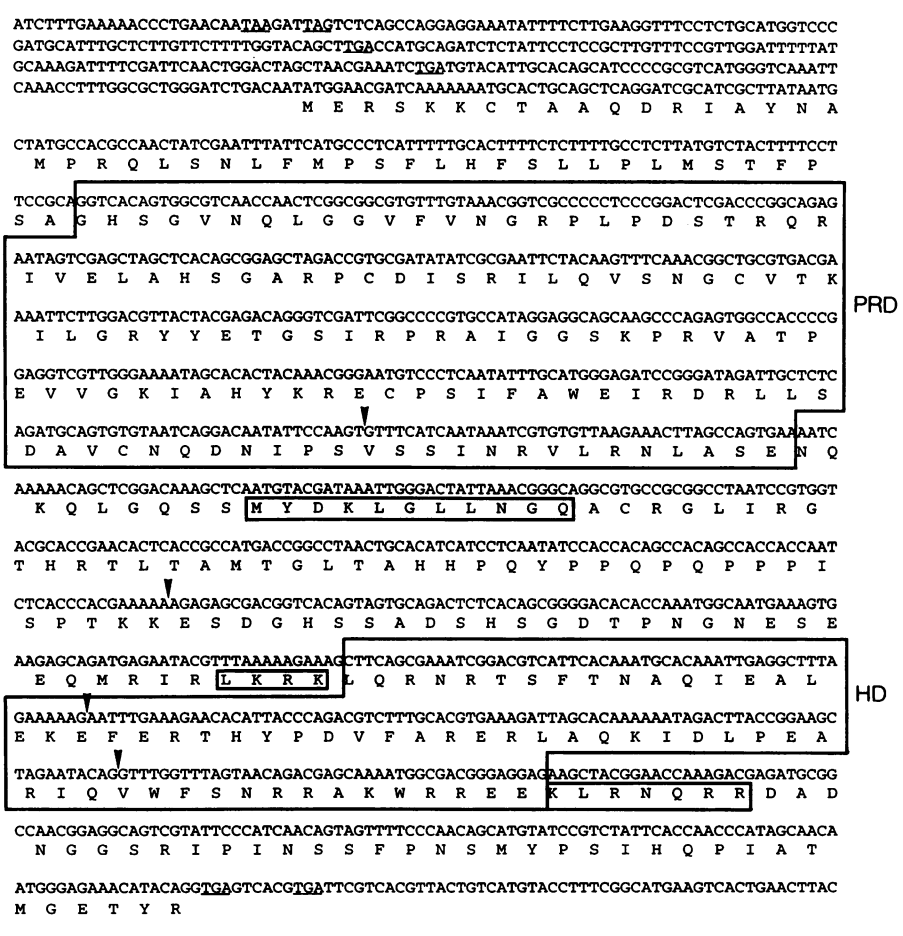

FIG. 2. Nucleotide and deduced amino acid sequence of *LsPax-6*. The paired domain (PRD) and the homeodomain (HD) are indicated with boxes. The conserved motif in the linker region and the conserved amino acids flanking the homeodomain are framed in. The splice sites are indicated by arrowheads. On the basis of sequence homology there may be an additional splice site in the first codon of the paired box. In-frame stop codons are underlined.

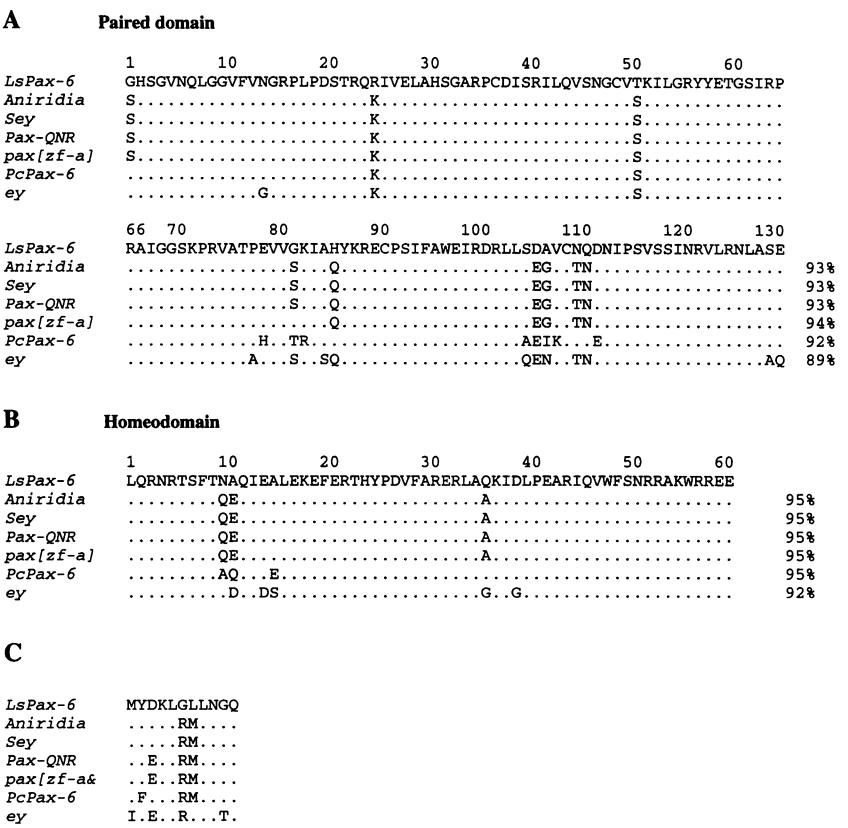

FIG. 3. Amino acid sequence comparison of the paired domain (*A*), the homeodomain (*B*), and the motif in the linker region (*C*) of *LsPax-6* to the *Pax-6* gene of humans (Aniridia), mouse (*Sey*), quail (*Pax QNR*), zebrafish (*pax[zf-a]*), sea urchin (*PcPax-6*), and *Drosophila* (*ey*). Identical amino acids are indicated by dots. The percentage of sequence identity to the respective *LsPax-6* amino acid sequence is indicated at the end of each line.

homeodomain of *LsPax-6*. This suggests that the identified open reading frame is not full length. Additional sequence homology is found in this carboxyl-terminal region of *LsPax-6* to the known vertebrate *Pax-6* genes. It shares 52% and 50% sequence identity with the quail, zebrafish, and the mammalian *Pax-6* genes, respectively, whereas the respective region of the sea urchin and *Drosophila Pax-6* genes are more diverged (26% and 21% sequence identity, respectively). The sequence comparison indicates that the cloned *L. sanguineus* gene is homologous to the known vertebrate and invertebrate *Pax-6* genes.

Three splice sites in the *LsPax-6* transcript are conserved. One splice site in the paired box is at the same position (codon 117) as in the human, mouse, quail, *Drosophila*, and *C. elegans Pax-6* genes. Both splice sites in the homeobox (codons 19 and 47) are also present in the human, quail, and *C. elegans Pax-6* genes, whereas the *Drosophila Pax-6* gene shares only the more 5′ splice site, in codon 19 of the homeodomain. Based on the sequence homology that includes intronic sequences we have identified a putative fourth conserved splice site in the first codon of the *LsPax-6* paired box. Eight out of 10 bp immediately 5′ of this putative splice site are identical to the conserved intron–exon boundaries present in the human and quail *Pax-6* genes. This splice site is also conserved in the *Pax-6* homologs of mouse, *Drosophila*, and squid (S. Tomarev and J. Piatigorsky, personal communication). As no full-length *LsPax-6* transcript was isolated, the 5′ end of exon one remains to be identified. The small exon present in the vertebrate *Pax-6* genes which gives rise to a 14-amino acid insertion by alternative splicing is absent in the *LsPax-6* gene. This exon is also missing in the *Drosophila* and sea urchin (28) *Pax-6* genes. The conserved gene structure indicates that the *LsPax-6* gene is orthologous to the vertebrate and the insect *Pax-6* genes.

***LsPax-6* Expression.** In the laboratory, *L. sanguineus* does not sexually reproduce. Therefore, it was not possible to examine *LsPax-6* expression during embryonic development. However, this nemertine has high capacities for regeneration and is capable of regenerating a complete head after experimental decapitation, including the brain and the anterior region with a variable number of simple eyes. Therefore we examined *LsPax-6* expression by *in situ* hybridization during head regeneration. Expression is first detected in the regenerating cerebral organs, which are two laterally located sense organs (Fig. 4 *B* and *C*) thought to have an olfactory role. At later stages, anterior to the cerebral organs, two expression domains are visible in the regenerating brain (Fig. 4 *B* and *C*). Subsequently, *LsPax-6* expression is detected in several small groups of cells below the dorsal epidermis, anterior to the brain (Fig. 4 *B* and *C*). The temporal occurrence and position of this dorsal *LsPax-6* expression correlates well with the temporal appearance and position of red pigmented spots in the regenerating head anterior to the brain, which constitute the earliest visible sign of the regenerating eyes (Fig. 4*A*). In several regenerating heads additional staining was observed in more ventrally located cells anterior to the brain (Fig. 4*C*). No expression was detected in fully regenerated heads.

To examine *Pax-6* expression during embryonic development we analyzed a closely related species, *L. viridis*, which reproduces sexually. With the same primers and conditions used to PCR-amplify clone 33-11 we isolated the corresponding *Pax-6* paired box region from the genome of *L. viridis*. Sequence analysis showed that the two *Lineus* Pax-6 genes are 98.5% identical at the nucleotide level in this region, coding for identical amino acid sequences. This indicates that the high degree of sequence conservation allows detection of *L. viridis Pax-6* expression with an *LsPax-6* probe under high-stringency

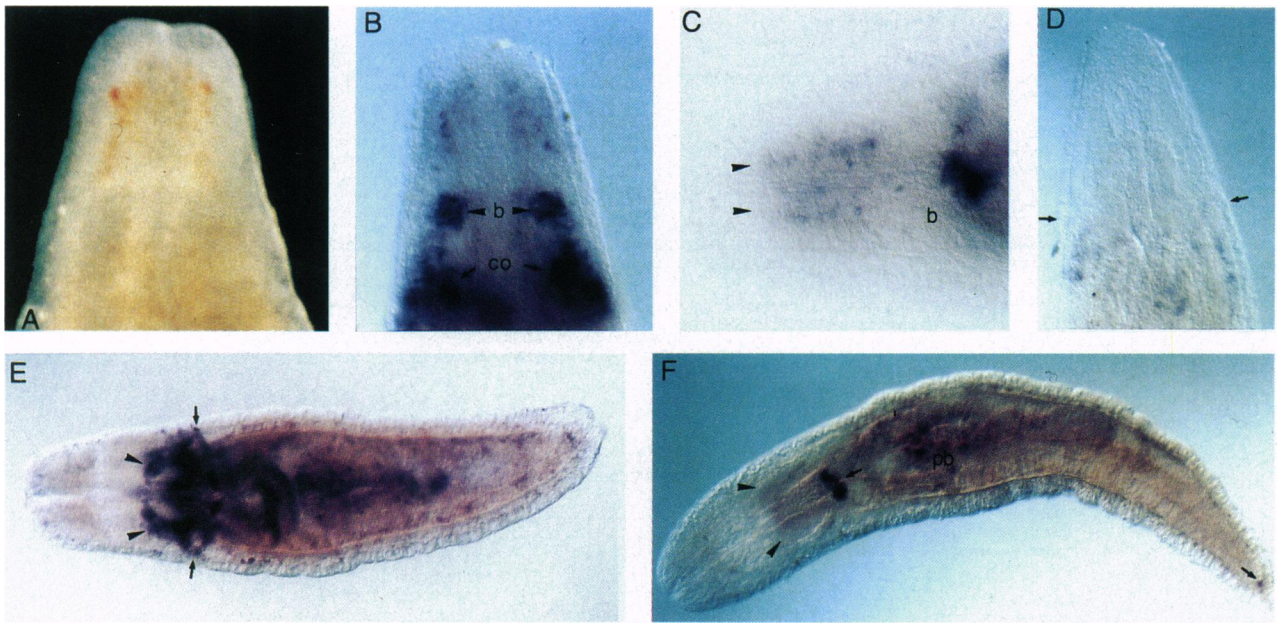

FIG. 4. *Pax-6* expression in regenerating heads of *L. sanguineus* (*B* and *C*) and in developing *L. viridis* worms (*E*). Whole-mount *in situ* hybridizations were done with digoxigenin-labeled RNA antisense (*B, C*, and *E*) and sense (*D, F*) probes. *A, B*, and *D–F* are dorsal views; anterior is on the top in *A, B*, and *D. C* shows a lateral view of the same head as in *A* and *B*; dorsal is at top. In *C, E*, and *F* anterior is at left. (*A*) Head after 14 days of regeneration. Position of regenerating eyes is marked by the red eye pigment. (*B*) *LsPax-6* expression in the same head as in *A*. Expression in the cerebral organs (co) and in the brain (b) is indicated. Position of the red eye pigment in *A* and the dorsal expression anterior to the brain in *B* correlate well. (*C*) Lateral view of same head as in *A* and *B* shows position of the dorsally and ventrally located *Pax-6*-expressing cells (arrowheads) anterior to the expression domain in the brain (b). (*D*) No staining is visible in the regenerating head (anterior to the arrows) with a sense control. (*E*) *Pax-6* expression in the brain (arrowheads) and cerebral organs (arrows) in developing *L. viridis* worms. (*F*) The brain (arrowheads) and cerebral organs show no staining with a sense control, whereas background staining is visible in the mouth region and posterior tip (arrows) and in some cells of the proboscis (pb).

conditions. *In situ* hybridization on developing *L. viridis* worms with an *LsPax-6* probe revealed strong *Pax-6* expression in the brain and cerebral organs (Fig. 4*E*). This shows that *Pax-6* expression in the central nervous system and cerebral organs is not restricted to regeneration, suggesting that also during *L. sanguineus* embryonic development *Pax-6* is expressed in the brain and these lateral sense organs. No reproducible staining was observed in the dorsal anterior region of the head in developing *L. viridis* worms, where the two eyes develop. It is possible that weak *Pax-6* expression in that region of the *L. viridis* embryo could not be detected with the *LsPax-6* probe under high stringency conditions. Alternatively, *Pax-6* could be expressed at a different stage or not at all in the region of the developing eyes in *L. viridis* embryos.

DISCUSSION

We have isolated a *Pax-6* gene from a primitive metazoan, the ribbonworm *L. sanguineus*. The paired domain and the homeodomain share a high degree of sequence identity with all known *Pax-6* genes. Additional sequence homology is found in the linker region between the two domains and in the carboxyl-terminal region. The conservation of at least one splice site in the paired box and two splice sites in the homeobox indicate that *LsPax-6*, the vertebrate, *Drosophila*, and *C. elegans Pax-6* genes are orthologs.

The highest overall sequence homology of *LsPax-6* is found in the squid *Loligo vulgaris Pax-6* gene (S. Tomarev and J. Piatigorsky, personal communication). Interestingly, 18S rRNA sequence comparison indicate a close evolutionary relationship of the mollusc, annelid, and nemertine phyla, suggesting that the nemertines are coelomate animals (29). This result contradicts the classical view that nemertines are closely related to the acoelomate flatworms (21). This conclusion is supported by the sequence comparison between *LsPax-6* and its homolog in the flatworm *Dugesia tigrina*, which reveals much less sequence identity (E. Salo, personal communication). The high sequence homology of the nemertine and the mollusc *Pax-6* genes supports a close phylogenetic relationship of these phyla. In addition, this correlation of sequence conservation in the *Pax-6* and the 18S rRNA genes suggests that comparison of the sequence and gene structure of *Pax-6* genes will prove useful in elucidating the phylogenetic relationship of different phyla.

The expression in the central nervous system and as suggested by the temporal and spatial occurrence in the eyes during head regeneration of *L. sanguineus* and in the developing central nervous system of *L. viridis* indicate a function of *LsPax-6* in the development of these organs, thus providing further hints at a conservation of the function of *Pax-6* in eye and central nervous system development. Extending this argument of functional conservation, it is interesting to note that the cerebral organ of the nemertines has been implicated, among other functions, with chemoreception. Considering the expression of *Pax-6* in the olfactory bulbs of zebrafish (7, 8) and the olfactory bulbs and nasal placodes of the mouse embryo (5), it is tempting to speculate that *Pax-6* expression in the cerebral organ of *L. sanguineus* and *L. viridis* indicates a conserved function of *Pax-6* in the chemosensory organs of these species. This raises the possibility of using *Pax-6* expression as a molecular marker to identify chemo- and photoreceptive organs in different species. Final proof for homologous function may eventually be obtained by the analysis of loss-of-function mutants in the respective species.

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1. Bopp, D., Burri, M., Baumgartner, S., Frigerio, G. & Noll, M. (1986) *Cell* **47,** 1033–1040.
2. Noll, M. (1993) *Curr. Opin. Genet. Dev.* **3,** 595–605.
3. Walther, C., Guenet, J.-L., Simon, D., Deutsch, U., Jostes, B., Goulding, M. D., Plachov, D., Balling, R. & Gruss, P. (1991) *Genomics* **11,** 424–434.
4. Gruss, P. & Walther, C. (1992) *Cell* **69,** 719–722.
5. Walther, C. & Gruss, P. (1991) *Development (Cambridge, U.K.)* **113,** 1435–1449.
6. Ton, C. C. T., Hirvonen, H., Miwa, H., Weil, M. M., Monaghan, P., Jordan, T., van Heyningen, V., Hastie, N. D., Meijers-Heijboer, H., Drechsler, M., Royer-Pokora, B., Collins, F., Swaroop, A., Strong, L. C. & Saunders, G. F. (1991) *Cell* **67,** 1059–1074.
7. Krauss, S., Johansen, T., Korzh, V., Moens, U., Ericson, J. U. & Fjose, A. (1991) *EMBO J.* **10,** 3609–3619.
8. Püschel, A. W., Gruss, P. & Westerfield, M. (1992) *Development (Cambridge, U.K.)* **114,** 643–651.
9. Quiring, R., Walldorf, U., Kloter, U. & Gehring, W. J. (1994) *Science* **265,** 785–789.
10. Chrisholm, A. D. & Horvitz, H. R. (1995) *Nature (London)* **377,** 52–55.
11. Glaser, T., Walton, D. S. & Maas, R. L. (1992) *Nat. Genet.* **2,** 232–238.
12. Dozier, C., Carrière, C., Grévin, D., Martin, P., Quatannens, B., Stéhelin, D. & Saule, S. (1993) *Cell Growth Differ.* **4,** 281–289.
13. Zhang, Y. & Emmons, S. W. (1995) *Nature (London)* **377,** 55–59.
14. Martin, P., Carrière, C., Dozier, C., Quatannens, B., Mirabel, M.-A., Vandenbunder, B., Stehelin, D. & Saule, S. (1992) *Oncogene* **7,** 1721–1728.
15. Li, H.-S., Yang, J.-M., Jacobson, R. D., Pasko, D. & Sundin, O. (1994) *Dev. Biol.* **162,** 181–194.
16. Hill, R. E., Favor, J., Hogan, B. L. M., Ton, C. C. T., Saunders, G. F., Hanson, I. M., Prosser, J., Jordan, T., Hastie, N. D. & van Heyningen, V. (1991) *Nature (London)* **354,** 522–525.
17. Matsuo, T., Osumi-Yamashita, N., Noji, S., Ohuchi, H., Koyama, E., Myokai, F., Matsuo, N., Toniguchi, S., Doi, H., Iseki, S., Ninomiya, Y., Fujiwara, M., Watanabe, T. & Eto, K. (1993) *Nat. Genet.* **3,** 299–304.
18. Hoge, M. A. (1915) *Am. Nat.* **49,** 47–49.
19. Lindsley, D. & Zimm, G. (1992) *The Genome of Drosophila melanogaster* (Carnegie Institution of Washington, Washington, DC).
20. Halder, G., Callaerts, P. & Gehring, W. J. (1995) *Science* **267,** 1788–1792.
21. Gibson, R. (1972) *Nemerteans* (Hutchinson, London).
22. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
23. Hempstead, P. G., Regular, S. C. & Ball, I. R. (1990) *DNA Cell Biol.* **9,** 57–61.
24. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
25. McGinnis, W., Levine, M. S., Hafen, E., Kuroiwa, A. & Gehring, W. J. (1984) *Nature (London)* **308,** 428–433.
26. Wittbrodt, J. & Rosa, F. M. (1994) *Genes Dev.* **8,** 1448–1462.
27. Hauptmann, G. & Gerster, T. (1994) *Trends Genet.* **10,** 266.
28. Czerny, T. & Busslinger, M. (1995) *Mol. Cell. Biol.* **15,** 2858–2871.
29. Turbeville, J. M., Field, K. G. & Raff, A. R. (1992) *Mol. Biol. Evol.* **9,** 235–249.