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The genetic network of prototypic planarian eye regeneration is Pax6 independent

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SUMMARY

We report the presence of two *Pax6*-related genes, *Pax6A* and *Pax6B*, which are highly conserved in two planarian species *Dugesia japonica* and *Girardia tigrina* (Platyhelminthes, Tricladida). Pax6A is more similar to other Pax6 proteins than Pax6B, which is the most divergent Pax6 described so far. The planarian *Pax6* homologs do not show any clear orthology to the *Drosophila* duplicated *Pax6* genes, *eyeless* and *twin of eyeless*, which suggests an independent *Pax6* duplication in a triclad or platyhelminth ancestor. *Pax6A* is expressed in the central nervous system of intact planarians, labeling a subset of cells of both cephalic ganglia and nerve cords, and is activated during cephalic regeneration. *Pax6B* follows a similar pattern, but shows a lower level of expression. *Pax6A* and *Pax6B* transcripts are detected in visual cells only at the ultrastructural level, probably because a limited amount of transcripts is present in these cells. Inactivation of both *Pax6A* and *Pax6B* by RNA-mediated gene interference (RNAi) inhibits neither eye regeneration nor eye maintenance, suggesting that the genetic network that controls this process is not triggered by Pax6 in planarians.

Key words: Planarians, Regeneration, Eye, Central nervous system, Paired domain, Pax6, RNAi, Ultrastructure, In situ hybridization

INTRODUCTION

An evolutionary conserved complex network of different signaling pathways and nuclear factors regulates brain and eye specification (Kurata et al., 2000; Callaerts et al., 2001; Kumar and Moses, 2001). One striking illustration of such genetic conservation is the *Pax6* gene, which is structurally and functionally characterized in a large number of triploblastic metazoans from planarians to humans (Quiring et al., 1994; Chisholm and Horvitz, 1995; Czerny and Busslinger, 1995; Zhang and Emmons, 1995; Loosli et al., 1996; Glardon et al., 1997; Tomarev et al., 1997; Glardon et al., 1998; Callaerts et al., 1999). Pax6 represents one of the nine members of the Pax class, characterized by the presence of two conserved DNA-binding domains, the paired domain and the homeodomain (Callaerts et al., 1997). The comparative molecular characterization of Pax6 in different animal phyla supports the hypothesis that this protein has a conserved function in a variety of developmental processes, ranging from regionalization to cell type specification, in eye and central nervous system development (Quiring et al., 1994; Kurusu et al., 2000; Pratt et al., 2000). In some vertebrates, Pax6 is also related to the development of head sensory organs or other structures such as pituitary or pancreas (Walther and Gruss, 1991; Turque et al., 1994). *Pax6* genes from various animal phyla are capable of inducing formation of ectopic eyes in *Drosophila* (Gehring and Ikeo, 1999). In frogs, Pax6 misexpression can also induce ectopic eyes (Chow et al., 1999). This finding indicates that *Pax6* is a master control gene for eye development, and also suggests a common genetic program for making eyes, despite the great variety of visual structures found in the animal kingdom. Two *Pax6* genes involved in eye development have been found in zebrafish (Nornes et al., 1998). The presence of a second *Pax6* gene arisen by gene duplication during insect evolution is also described in holometabolous insects, the two *Drosophila* genes *eyeless* (*ey*) and *twin of eyeless* (*toy*) being the best-studied examples. *ey* and *toy* share a similar pattern of expression in the developing visual system, and have non-redundant functions, indicating that they have been recruited with different roles into the eye genetic pathway (Czerny et al., 1999).

Planarians are flatworms well known for their exceptional regenerative capabilities. They are free-living members of the Platyhelminthes, considered a basal phylum of the Lophotrocozoa clade (Aguinaldo et al., 1997; Carranza et al., 1997; Bayascas et al., 1998). The study of the role of *Pax6* genes during eye and central nervous system (CNS) regeneration and maintenance will be of interest for

understanding the different ways that this genetic network evolved in the animal kingdom. Planarians are one of the simplest organisms with cephalization, defined by the presence of two dominant cephalic ganglia connected by commissural connections and followed by two ventral nerve cords. The nerve cords run the entire length of the body and are regularly connected by commissures producing a small concentration of neurons at the crossing points (Baguñá and Ballester, 1978; Rieger et al., 1991). Light perception by special photosensitive cells occurs in planarians. Photoreceptor structures, which are capable of detecting light and shadow, are grouped into eyespots defining a simple ancestral visual system that consists of bipolar retinal neurons whose dendrites project into a cup-shaped structure composed of pigment cells (Kishida, 1967; Sakai et al., 2000). Although the planarian eyes cannot focus the light pattern into images, as they have no focusing lens, they serve essentially the same function, receiving and transducing light into neuronal signals, as eyes do in all metazoans.

We report an extensive search for *Pax6* genes in Platyhelminthes that has led to the characterization of two related *Pax6* genes in the planarian species *Dugesia japonica* (*DjPax6A and DjPax6B*) and *Girardia tigrina* [*GtPax6A* and *GtPax6B*, the latter previously named *DtPax6* (Callaerts et al., 1999)]. The *Pax6A* and *Pax6B* sequences share high similarity and comparable expression patterns in both species, suggesting that they originated by duplication in a triclad or, possibly, in a platyhelminth ancestor. Both genes are detected in the adult CNS – a pattern of expression shared with all *Pax6* genes described so far – and are activated during regeneration, *Pax6A* being more expressed than *Pax6B*. Although no specific transcripts were detectable in the photoreceptors by whole-mount in situ hybridization, the presence of low levels of *Pax6A* and *Pax6B* mRNA could be demonstrated in the eye cells by electron microscope in situ hybridization. Inactivation of both *Pax6* genes by RNAi neither prevents eye formation during regeneration nor inhibits the eye expression pattern of the planarian genes *Gtsix-1* or *Gtops*, supporting the hypothesis that the function of *Pax6A* and *Pax6B* is not essential in planarian eye regeneration.

MATERIALS AND METHODS

Animals

Planarians used in this work belong to the species *Dugesia japonica*, clonal strain GI (Orii et al., 1993), and to an asexual race of *Girardia tigrina*, formerly classified as *Dugesia(G) tigrina*, collected in the Calders River (Barcelona, Spain). Animals were cultured as described previously (Salvetti et al., 1998; Saló and Baguñà, 1984) and starved for 2 weeks before being used in the experiments. Regenerating fragments were obtained by transverse amputation at the pre-pharyngeal or post-pharyngeal level. Anterior regeneration corresponds to a tail regenerating a head, while posterior regeneration corresponds to a head regenerating a tail. Finally, lateral regeneration was obtained by sagittal amputation.

Isolation and characterization of planarian genes related to *Pax6*

A *GtPax6A* PCR fragment of 111 bp was amplified with two degenerate primers. The primers correspond to the amino acid sequences LEKEFER (sense strand) and QVWFSNR (antisense strand) of the homeodomain. A λGt10 cDNA library from regenerating *G. tigrina* was screened according to Garcia-Fernandez et al. (Garcia-Fernandez et al., 1991), with the 111 bp PCR fragment as a probe. One positive clone was found corresponding to the 3′ *Pax6A* gene end (*Pax6A*3′). A 5′ RACE strategy was used to elongate the sequence at the 5′ end, obtaining a second clone (*GtPax6A790*) that contained 790 bp from the paired box to the homeobox of the *Pax6A* sequence. The *GtPax6A* sequence has been deposited to the EMBL/GenBank with the Accession Number AY028904. *DjPax6A* was directly amplified from the sequence deposited in the EMBL/Genbank (Accession Number, AB017632), as described by Rossi et al. (Rossi et al., 2001). A fragment (clone *DjPax6B*-520 bp) of the second *Pax6* gene, *DjPax6B*, was amplified in *D. japonica* (Rossi et al., 2001). A sense-strand degenerate primer was designed, taking advantage of the high sequence similarity in the paired domain between *DjPax6A* and the *Pax6* gene, here referred to as *GtPax6B*, previously characterized in *G. tigrina* (Callaerts et al., 1999). The antisense primer corresponded to the *GtPax6B* amino acid region TLFGYN. A 5′/3′RACE strategy was used to further characterize the *DjPax6B* sequence, deposited at the EMBL/Genbank with the Accession Number AJ311310. The sequence-specific antisense primer, corresponding to the amino acid region SKPRVATN was used to amplify selectively the 5′ region. The *DjPax6B* 3′ region was obtained with the sequence-specific sense primer, corresponding to INTWPPTS. The PCR products were TA-cloned using the pGEM-T easy vector (Promega). All clones were sequenced by automated fluorescent cycle sequencing (ABI).

Phylogenetic analysis of the Pax family homeodomains

The phylogenetic tree of the homeodomains and their flanking regions sequences was inferred using the CLUSTALX package. Sequences were aligned with CLUSTALX software. Evolutionary distances were calculated using Kimura's equation (Nei and Koehn, 1983), and used for phylogenetic tree construction by the Neighbor-joining method. Sequences were obtained from the EMBL/ GenBank.

In situ hybridization experiments

All sense and antisense digoxigenin-labeled RNA probes used for in situ hybridization experiments were made using the RNA in vitro labeling kit (Roche). Whole-mount in situ hybridization was carried out on intact and regenerating planarians according to Agata et al. (Agata et al., 1998). Cryosections after whole-mount in situ hybridization were performed as described by Pineda et al. (Pineda et al., 2000). In situ hybridization on sections was carried out as described by Kobayashi et al. (Kobayashi et al., 1998). Hybridization took place at 55ºC for 24-60 hours. The final probe concentration was 0.1 ng/μl. In some hybridized sections, 20 μg/ml DAPI was added to detect nuclei. Transmission electron microscope (TEM) in situ hybridization was performed on specimens of *D. japonica*, fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 hour at 4°C. After dehydration in a graded series of methanol (each step for 30 minutes at –20°C) specimens were embedded in Unicryl resin and polymerized for 72 hours at 4°C under u.v. light. Ultrathin sections, obtained with a diamond knife on Ultracut Reichert-Jung microtome, were placed on Formvar-carbon coated nickel grids and were incubated face down on a drop containing the hybridization buffer (50% formamide, 10% dextran sulphate, 4×saline sodium citrate, 400 μg/ml salmon testis DNA and 8 ng/μl of antisense or sense probes) for 4 hours at 37°C. After hybridization, ultrathin sections were washed in phosphate buffer, pre-incubated in 1% bovine serum albumin and then incubated in anti-digoxigenin antibody (1:40 in phosphate buffer) conjugated to gold particles of 10 nm in diameter, for 30 minutes. Grids were stained with uranyl acetate and lead citrate and observed with a Jeol 100 SX transmission electron microscope. Controls were performed using both sense probes and RNAse treatment (100 μg/ml of RNAse A for 90 minutes at 37°C) (Le Guellec et al., 1991) before the hybridization step.

The following clones were used as probes for in situ hybridization experiments.

D. japonica: *DjPax6*A, *DjPax6B* 520 bp, *Dj18S* (central region of 18S rDNA, about 1.1 kb) *Djops* 480 bp (Accession number, AJ421264) and *Djsyt* (Tazaki et al., 1999).

G. tigrina: *GtPax6A*3′ and *GtPax6A*790 were used in non-injected animals, *GtPax6A*790, *Gtsix*-1 *so*-5′ and *so*-3′.2 (Accession number, AJ251661), and *Gtops* p-250 (accession no. AJ251660) were used in dsRNA-injected animals.

Microinjection of double-strand RNA (dsRNA) and analysis of endogenous transcripts

Double-stranded RNA was synthesized as described by Sanchèz-Alvarado and Newmark (Sanchèz-Alvarado and Newmark, 1999). In *G. tigrina Pax6A3′* clone and a *Cla*I-*Hin*dIII fragment of 400 bp (*GtPax6B3′clone*) of *GtPax6B* were used for dsRNA synthesis. In *D. japonica* dsRNA was synthesized from *DjPax6A* 1600 bp and *DjPax6B* 520 bp clones. Planarians were injected with 10^{10}-10^{11} molecules of each dsRNA preparation or with an equimolar mixture of both *Pax6A* and *Pax6B* dsRNA. After the first injection, performed just after amputation, further injections were performed every 1 or 3 days, using a Drummond Scientific (Broomall, PA) Nanoject injector. Control injections were performed with water or β-Gal dsRNA. Injected planarians were kept at 17°C. Injected *G. tigrina* specimens were fixed at different regeneration times and processed for whole-mount in situ hybridization.

Total RNA was isolated from injected planarians after 1 to 3 days of regeneration and semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was performed according to Bayascas et al. (Bayascas et al., 1998). Control reactions were performed identically in the absence of reverse transcriptase. Specific oligonucleotides from *Pax6A* and *Pax6B* were used to ascertain the reduction of *Pax6A* and *Pax6B* endogenous transcripts. Each couple of primers was designed from two regions, one internal and the other external to the sequence used for dsRNA synthesis. As an internal control, the ubiquitous transcripts of the homeobox gene *Dth2* (Garcia-Fernandez et al., 1993) and the eye-specific *Gtsix1* and *Gtops* transcripts (Pineda et al., 2000; Pineda et al., 2001), were amplified in *G. tigrina*. Two specific primers for the constitutively transcribed elongation factor gene *DjEF2* were used for control amplifications in *D. japonica*.

Primer sequences used for PCR were as follows:
GtPax6A reverse, 5′-GAAGCTTCTGTTTCTGTTTTAGAG-3′;
GtPax6A forward, 5′-CGTACTTCGTTTTCGACAGATCAA-3′;
GtPax6B reverse, 5′-TCGCTTCTTTTGTTGTACAGTTTG-3′;
GtPax6B forward, 5′-CGGACTTCATTTACAAATGATCAG-3′;
Dth-2 reverse, 5′-TGGGAGACCGTTCTTTATCGTCAAC-3′;
Dth-2 forward, 5′-CCAATGCTAGTAATGATCCGCGTAT-3′;
Gtops reverse, 5′-GGACAGATACTTTGTTATCGCTCA-3′;
Gtops forward, 5′-TAACAAAATTCCCGATGTACATTC-3′;
Gtsix-1 reverse, 5′-AACGGCTCGGGATTTTTCTTTAAA-3′;
Gstix-1 forward, 5′-ATATGGTCTCTTCCACCTTGCCAA-3′;
DjPax6A forward, 5′-CCAAATCTTTCGCAATCTTC-3′;
DjPax6A reverse, 5′-CAATAAGTATCAAATACGTTACA-3′;
DjPax6B forward, 5′-CATCAATACATGGCCGCCTACAA-3′;
DjPax6B reverse, 5′CGTCTCCATTTTGCTCTGCGATT-3′;
DjEF2 forward, 5′-TTAATGATGGGAAGATATGTTG-3′; and
DjEF2 reverse 5′- GTACCATAGGATCTGATTTTGC-3′

For each PCR reaction the concentration of cDNA, primers and the number of cycles used were optimized with the aim of observing a quantifiable signal within the linear range of the amplification, according to both the putative abundance of each mRNA amplified and to the size of the corresponding PCR product.

Immunohistochemistry

Whole-mount immunohistochemistry of regenerating *G. tigrina* was carried out as described by Cebrià et al. (Cebrià et al., 1997). Rabbit anti-FMRFamide (Diasorin) was used as a primary antibody at a 1:100 dilution, and a fluorescein-conjugated goat anti-rabbit (IgG; Sigma) was used as a secondary antibody at a 1:50 dilution. The samples were examined with a epifluorescence microscope (Axiophot, Zeiss) and with a confocal laser scanning microscope (Leica Lasertechnik, Heidelberg).

RESULTS

Cloning of planarian *Pax6A* and *Pax6B* genes

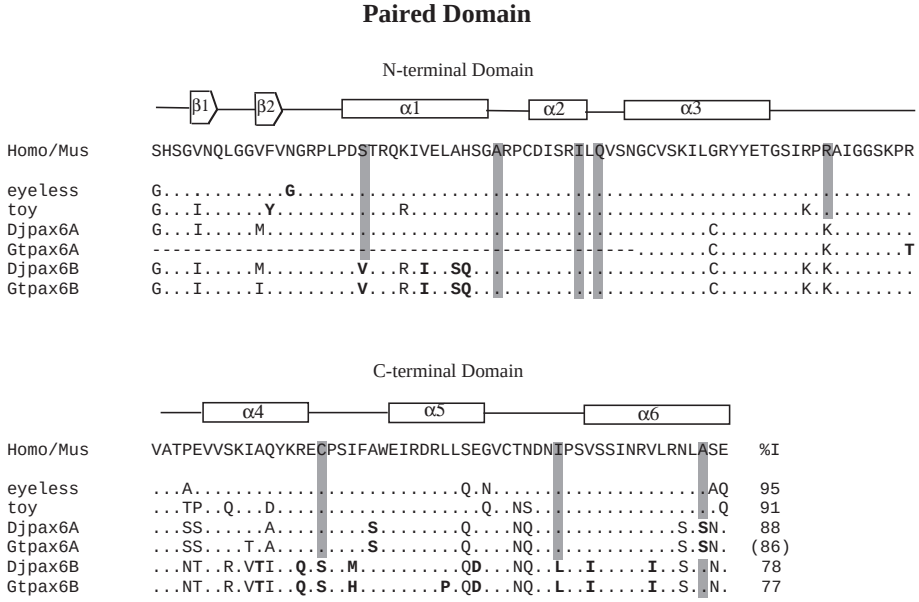
Using a PCR approach and first strand cDNA derived from planarian heads, two *Pax6* genes, *Pax6A* and *Pax6B*, were isolated in *G. tigrina* and *D. japonica*. Three types of sequences, which corresponded to three of the four families of *Pax* genes, *Pax2-5-8*, *Pax1-9* and *Pax4-6*, were found in *G. tigrina* using two different sets of degenerate primers directed against two regions of the paired box (GRPLP and WEIRD) and of the homeobox (LEKEFER and QVWFSNR). The subsequent screening of a cDNA library using *Pax4-6* fragment as a probe, allowed us to isolate a second *Pax6* gene, *GtPax6A*, in addition to the previously described *Pax6* gene (*DtPax*6) (Callaerts et al., 1999), here renamed *GtPax6B*. Two degenerate primers were selected on the basis of a comparative sequence analysis of *DtPax6* and *DjPax6A*, a *Pax6* clone previously isolated in *D. japonica* (Accession Number, AB017632). These primers allowed us to identify a second *Pax6* gene in *D. japonica, DjPax6B. DjPax6A* and *GtPax6A*, as well as *DjPax6B* and *GtPax6B* deduced proteins, show extensive sequence identity even outside the conserved DNA-binding boxes (Fig. 1A).

Characterization and phylogenetic relationships of Pax6A and Pax6B proteins

Both Pax6A and Pax6B show a significant similarity in the paired domain and homeodomain with vertebrate and invertebrate Pax6 proteins. Comparison of the paired domain of Pax6A with those of published Pax6 proteins shows that it is 88% identical, and maintains most (six out of eight) of the residues that typify the Pax6 family. In Pax6B paired domain the degree of sequence conservation is lower, 77-78%, and only four out of eight Pax6-specific amino acids are conserved (Fig. 1B). The homeodomain in Pax6A has a higher sequence identity to the corresponding region of vertebrate Pax6 (85%), maintaining nine out of 10 specific amino acids, while in Pax6B it shows a lower value (74%), where only five out of 10 conserved amino acids are present. Moreover, four conserved residues preceding the homeodomain, as well as five out of seven distinctive amino acids following the homeodomain, are conserved in Pax6A, but not in Pax6B (Fig. 1C). The length of the linker region between the paired domain and homeodomain is highly variable in different invertebrate as well as vertebrate Pax6 proteins. It spans 224 amino acids in *Drosophila ey* and 108 amino acids in *toy* (Quiring et al., 1994; Czerny et al., 1999), whereas it is only 72 amino acids in length in sea urchin Pax6 (Czerny and Busslinger, 1995). Planarian Pax6 proteins contain a long linker region. In fact, the linker sequence consists of 138 amino acids in Pax6A and it is 171 amino acids long in Pax6B. Moreover, as commonly found in Pax6 proteins of other organisms (Loosli et al., 1996), Pax6A linker sequence is typified by the presence of some conserved amino acids

A

B



C

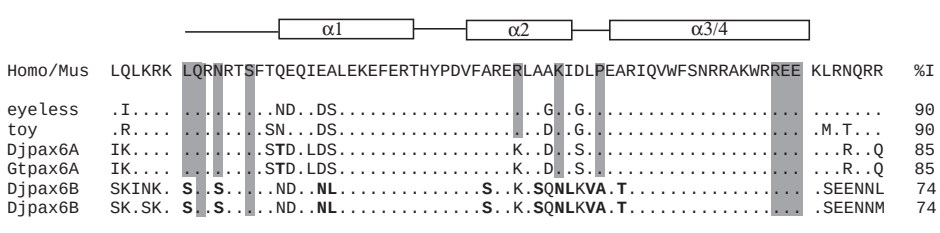


Fig. 1. Amino acid sequence comparison of Pax6A and Pax6B from *D. japonica* and *G. tigrina*. (A) Multiple alignment of DjPax6A, GtPax6A, and DjPax6B, GtPax6B sequences. Amino acids that are identical both in Pax6A and Pax6B are shown in blue; amino acids conserved between DjPax6A and GtPax6A are indicated in green; amino acids conserved between DjPax6B and GtPax6B are indicated in red; differing amino acids are shown in black. The paired domain, the conserved motif found in the linker region, and the homeodomain are boxed. The missing sequence in the 5′ end of GtPax6A is indicated by dots; the introduced gaps are indicated by dashes. (B,C) Sequence comparison of the paired domain (B), and the homeodomain (C) of the planarian Pax6 proteins to the Pax6 paired domain and homeodomain of different species. Only *Homo sapiens* and *Mus musculus* Pax6 are shown for vertebrates. *Drosophila* eyless and toy are included as invertebrate Pax6. The shaded bars indicate Pax6-specific amino acids. Percentages of sequence identity (%I), determined by comparison with *Homo sapiens* and *Mus musculus* Pax6, are indicated at the end of each line. The structure of paired domain and homeodomain are shown on the top in B and C, respectively. Identical amino acids are indicated by dots. The incomplete sequence of GtPax6A is indicated by a dashed line. Non conserved amino acid residues are indicated in bold. The homeodomain flanking regions are shown in C.

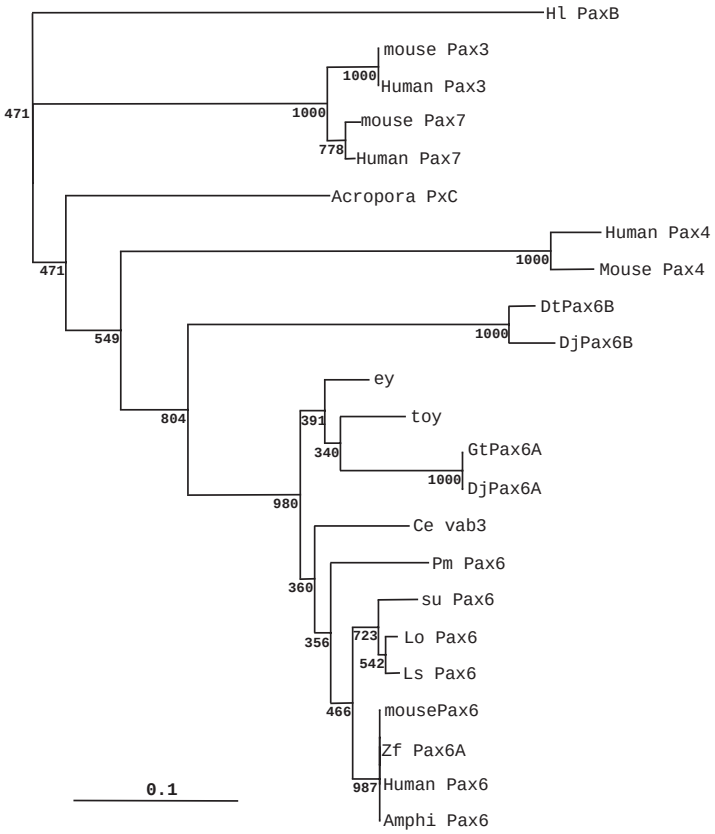


Fig. 2. Phylogenetic tree of Pax genes. The tree was derived from the homeodomain and flanking regions of representatives from different Pax classes (indicated on the right), using the Neighbor-joining method. Sequences reported in this paper are in bold. Bootstrap percentage values (1000 replicates) are shown over the corresponding nodes. All branch lengths are proportional to the distances between sequences. The tree was rooted using *Hydra* PaxB as an outgroup. The analysis includes: human Pax 3, 4, 6 (Aniridia) and 7; mouse Pax3, 4, 6 and 7; zebrafish Pax6; sea urchin suPax6; chordate PmPax6 and Amphi-Pax6; mollusc Lo-Pax6; *Drosophila* ey and toy; *C. elegans* Cevab-3; nemertine LsPax6; planarian GtPax6A and GtPax6B, DjPax6A and DjPax6B; and cnidarian PaxC and Hl PaxB.

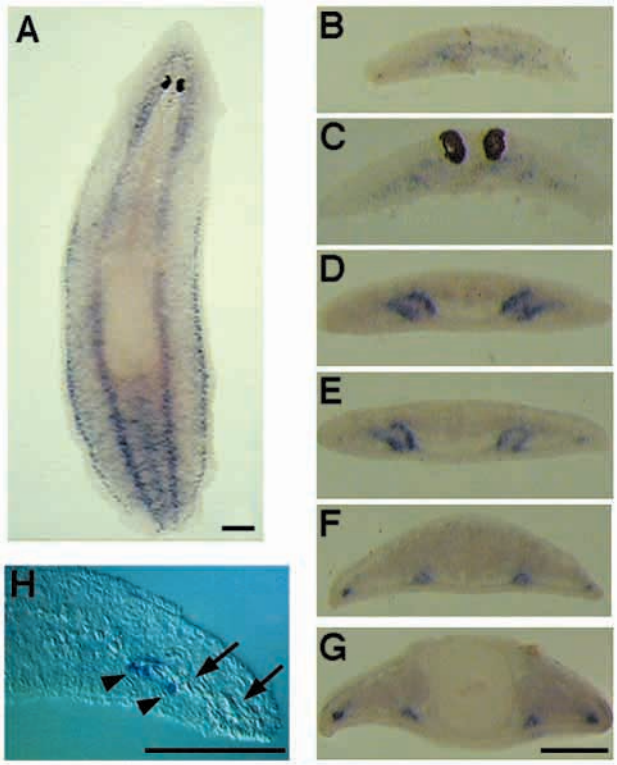


Fig. 3. Expression of *Pax6A* mRNA in an intact planarian, as detected by whole-mount in situ hybridization. (A) Dorsal view of *GtPax6A* expression in *G. tigrina*. (B-G) Anteroposterior sequence of some representative transverse cryosections of whole-mount depicted in A. *GtPax6A* is expressed in the cephalic ganglia and the nerve cords. Presence of *GtPax6A* transcripts can also be observed in the lateroposterior region, close to the dorsoventral border. (H) Nomarski view of a higher magnification of a transverse cryosection of the whole-mount in A, showing the localization of *GtPax6A*-labelled cells (arrowheads) in the lateroventral marginal region, close to the eosinophilic secretory cells (arrows). Scale bars: 0.5 mm.

(MYDKLSLLSGQ). A similar arrangement of amino acids has not been found in Pax6B.

The evolutionary relationships between the planarian Pax6A and Pax6B and Pax proteins of other organisms were inferred from the homeodomain and their flanking regions (Fig. 2). We can observe the closest similarity to Pax6 protostomate representatives, clustering the planarian Pax6A with *Drosophila* ey and toy, although with low bootstrap values. Pax6B shows the lowest similarity and is at the base of the Pax6 cluster. Such results indicate that Pax6A and Pax6B are *bona fide* Pax6 members.

Expression pattern of *Pax6A* and *Pax6B* in intact planarians

The expression pattern of *Pax6A* and *Pax6B* was analyzed by whole-mount in situ hybridization in intact adult specimens of both species. The presence of *Pax6A* transcripts was observed in the cephalic ganglia and in the nerve cords that cross the entire organism ventrally. (Fig. 3A-G). *Pax6B* showed a similar pattern, albeit with a lower level of expression (data not shown). In *G. tigrina*, the presence of additional *GtPax6A* transcripts was also detected in a non-cephalic parenchyma region close to the eosinophilic secretory cells, which give rise to the ventral marginal adhesive zone (Fig. 3A,F-H). This latter expression pattern suggests that *GtPax6A* has a derived role in the formation of this zone, an observation that deserves further investigation.

To investigate in detail the distribution of *Pax6*-expressing cells in the planarian CNS, we performed in situ hybridization directly on paraffin wax-embedded sections of intact *D. japonica* specimens (Fig. 4). The complete set of nerve cells at corresponding section levels was identified by the expression of the planarian neural marker synaptotagmin, *Djsyt* (Fig. 4C-H). Expression of *DjPax6A* was detected in a subset of cells of the CNS, but *Pax6* transcripts were not detected in the eye cells using this procedure (Fig. 4I-P). Conversely, electron microscope in situ hybridization showed expression of both *DjPax6A* and *DjPax6B* in the perikaryon, the dendrites and the

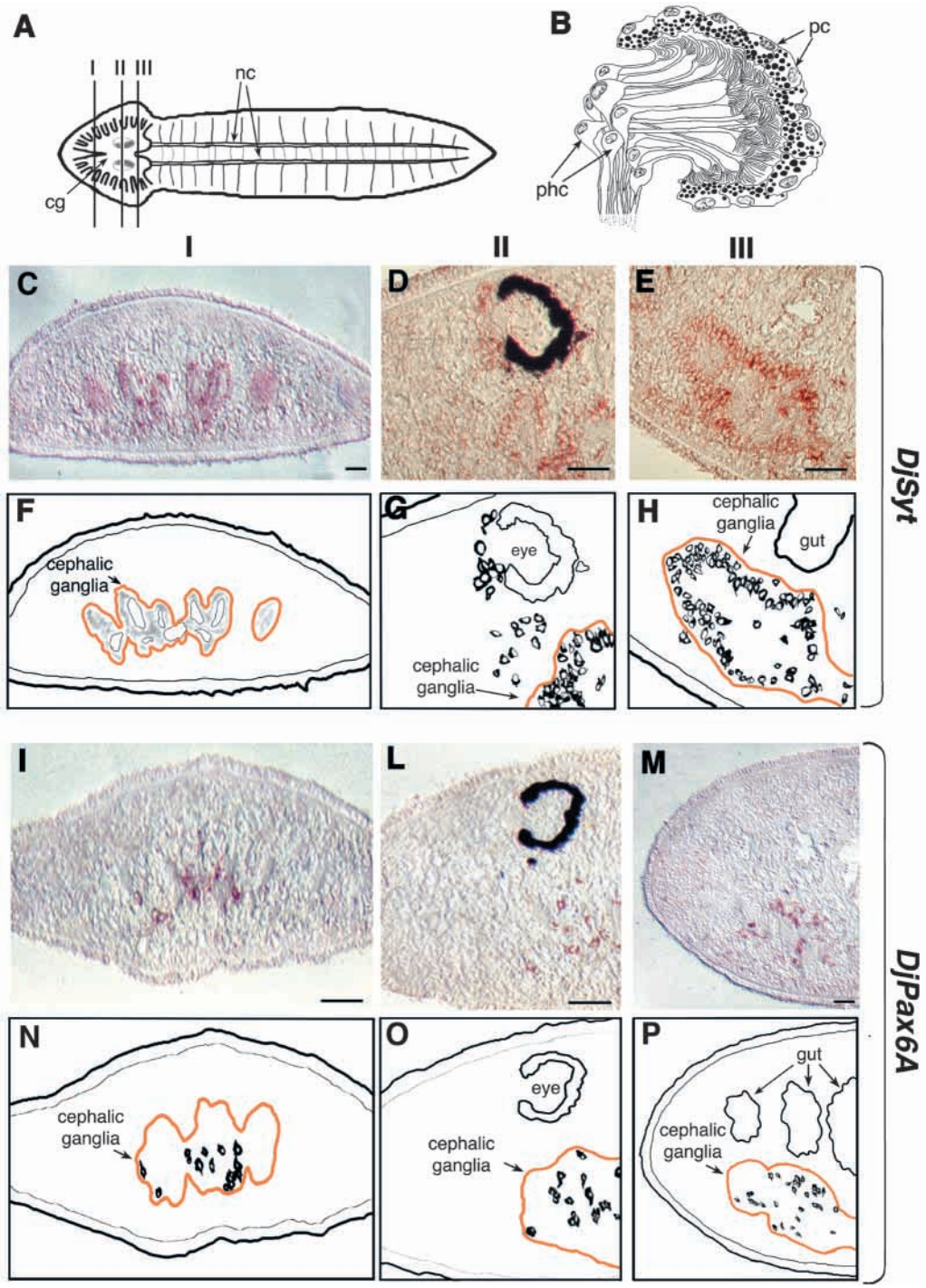


Fig. 4. In situ hybridization and camera lucida drawings of transverse paraffin sections from the cephalic region of *D. japonica*. (A) The CNS in an intact planarian, which is composed of a mass of nerve cells, the cephalic ganglia (cg) and a pair of ventral nerve cords (nc). I, II and III indicate paraffin section levels. (B) The planarian eye. pc, pigment cell; phc, photoreceptor cell. (C-E) Anteroposterior sequence of transverse paraffin sections, visualized after in situ hybridization with *Djsyt*. (F-H) Camera lucida drawings of sections in C-E, illustrating the various morphological structures. (I-M) Anteroposterior sequence of transverse paraffin sections, visualized after in situ hybridization with *DjPax6A*. (N-P) Camera lucida drawings of sections in I-M, illustrating the various morphological structures. The nerve cell marker *Djsyt* labels all nerve cells, including the photoreceptors, while *DjPax6A* is expressed only in a subset of nerve cells. No detectable *DjPax6A* expression is observed in visual cells. Scale bars: 0.05 mm.

rhabdomeric region of the photoreceptor cells (Fig. 5A-C). We also observed expression in the perinuclear cytoplasm of pigment cells (Fig. 5D). Positive controls with the ribosomal 18S riboprobe *Dj18S* showed that both the nucleolus and the endoplasmic reticulum of the cells were labeled (Fig. 5E). In addition, the *Dj18S* hybridization signal was seen in the rhabdomeric projections of photoreceptor cells, thus supporting the possibility of translational activity in this region (Fig. 5G). Transcripts from the planarian synaptotagmin, *DjSyt*, were specifically detected in the perikaryon of nerve cells (Fig. 5H). No signal was observed with *DjPax6A* or *DjPax6B* sense-strand probes (Fig. 5F), or with RNAse treatment followed by hybridization using *DjPax6A* and *DjPax6B* antisense-strand probes.

Expression pattern of *Pax6A* and *Pax6B* during regeneration

Planarians have a great regenerative plasticity. After experimental decapitation, a complete head including brain and eyespots is formed during anterior or cephalic regeneration. We examined the expression pattern of the planarian *Pax6*-related genes during regeneration by whole-mount in situ

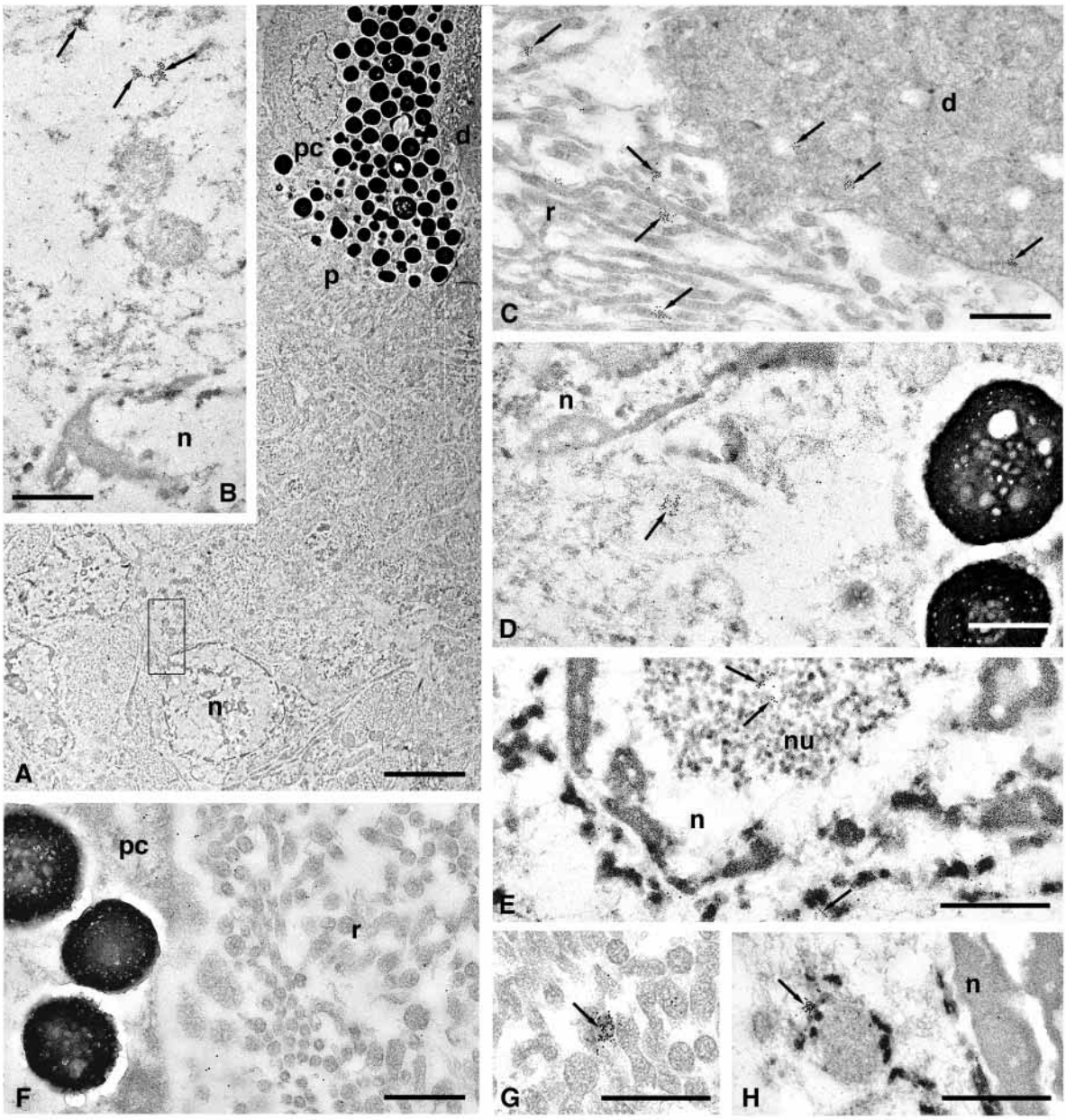


Fig. 5. TEM in situ hybridization on *D. japonica*. (A-D) *DjPax6A* antisense-strand RNA; (F) *DjPax6A* sense-strand RNA; (E,G) *Dj18S* antisense-strand RNA; (H) *DjSyt* antisense-strand RNA. (A) Low magnification of the pigment cup ocellus showing some nuclei of photosensitive cells. The box indicates the figure shown in B. (B) Enlargement of A with clusters of gold particles (arrows) on the cytoplasm. (C) Clusters of gold particles (arrows) on the dendrite and the rhabdomeric region of a photosensitive cell. (D) A cluster of gold particles on the perinuclear cytoplasm of a pigment cell. (E) Clusters of gold particles (arrows) on the nucleolus and the endoplasmic reticulum of a cell. (F) No cluster of gold particles is visible on the cytoplasm of a pigment cell or in the rhabdomeric region of a photoreceptor cell. (G) A large cluster of gold particles (arrows) on the rhabdomeric region of a photoreceptor cell. (H) A cluster of gold particles (arrow) on the perikaryon of a photoreceptor cell. d, dendritic region of the photosensitive cell; n, nucleus; nu, nucleolus; p, pigment cup; pc, pigment cell; r, rhabdomeres. Scale bars: 5 μm in A; 0.5 μm in B-H).

hybridization. After 1-2 days, regenerating animals showed two intense spots of *Pax6A* expression near the region where new fibers emerge from the amputated old nerve cords (Fig. 6A). After 3-6 days of regeneration, the hybridization signal was localized in an arch-shaped structure of the blastema, where neoblasts fated to become nerve cells intermingle with the bent fibers to regenerate the cephalic ganglia (Fig. 6B,C). The arch-shaped expression pattern was particularly clear in *G. tigrina*, probably owing to morphological differences in the ganglia of the two species. At later stages of regeneration – from 3 to 15 days – we observed a gradual spreading of the *Pax6A*-positive tissue consequent to the growth of the cephalic ganglia (Fig. 6D-F). After that, the expression decreased to the basal level observed in the adult CNS (Fig. 3). In posterior regeneration a slight increase of *Pax6A* transcripts was observed in the region where the new nerve cords would

differentiate from the edge of the old-sectioned nerve cords (Fig. 6G). Finally, after sagittal amputation, we observed *Pax6A* expression during lateral regeneration of the missing cephalic ganglion and nerve cord. Increased *Pax6A* expression was detected in the regenerating CNS area (Fig. 6H). This differential expression gradually decreased to the basal level as regeneration proceeded in a similar way to that observed during anterior regeneration (Fig. 6I). Owing to the weak *Pax6B* expression, almost undetectable under the experimental conditions used, there was no perceptible difference with *Pax6A* transcript distribution during regeneration.

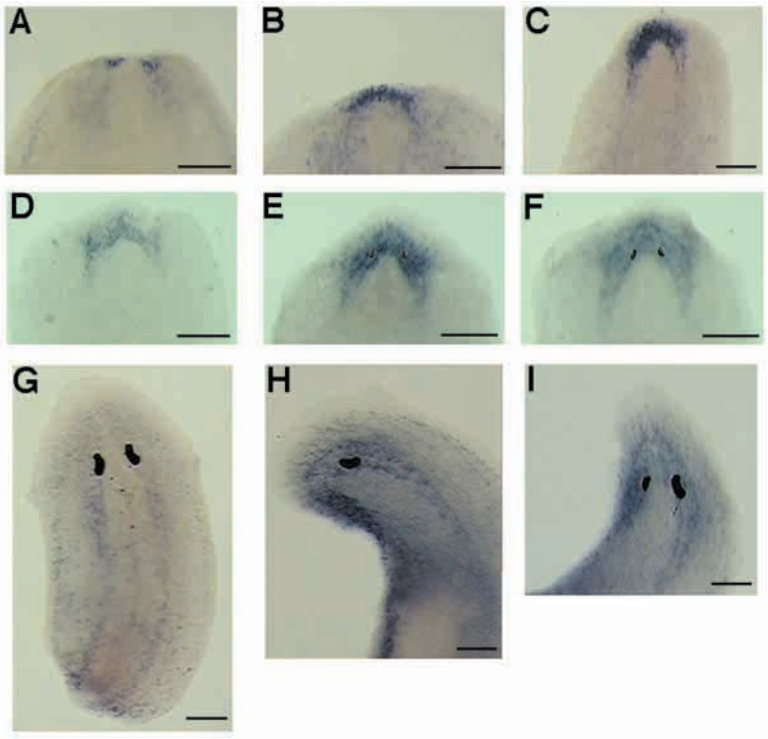


Fig. 6. Expression of *GtPax6A* mRNA in regenerating *G. tigrina*, as detected by whole-mount in situ hybridization. (A-F) Dorsal view of fragments regenerating a head. Anterior is towards the top. (A) Activation of *GtPax6A* after 2 days of regeneration is detected as two hybridization spots located in the region close to the sectioned old nerve cords, where new cephalic ganglia are forming. (B) After 3 days of regeneration, *GtPax6A*-positive spots merge and follow the fibers emerging from the old nerve cords. (C-F) After 6, 8, 11 and 15 days of regeneration, *GtPax6A* expression becomes broader and follows the regenerating cephalic ganglia. (G) Dorsal view of a posterior regeneration. Six days after cutting, two labeling spots in the area corresponding to the regenerating nerve cords are observed. (H,I) Dorsal view of a lateral regeneration. An increased level of *GtPax6A* expression is observed where a cephalic ganglion is regenerating. A basal expression is detected in the corresponding non-regenerating ganglion. Scale bars: 0.5 mm.

Reduction of the levels of endogenous *Pax6A* and *Pax6B* by RNA interference (RNAi)

As both planarian *Pax6* genes are expressed in the CNS and eye cells, we aimed to determine the function of *Pax6* in the maintenance or the regeneration pattern of CNS and eye. Consequently, we injected *Pax6A* and *Pax6B* dsRNA into the postblastema region in regenerating *D. japonica* and *G. tigrina*, as well as in the head region of intact animals. An equimolar mixture of dsRNA of *Pax6A/Pax6B* was also injected in another experiment, in order to prevent a possible gene redundancy effect. Injected adults did not reveal any gross morphological or behavioral abnormalities in any condition, and regeneration of the eyespots was also observed during cephalic blastema formation. Inspection of the photoreceptor cells by TEM showed a normal rhabdomeric organization of these structures. We also compared the number of photoreceptors in the eye of dsRNA-injected animals and water-injected controls of both species, after 15 days of regeneration. We counted about 15-20 cells in transverse adjacent sections at the eye level, hybridized with the photoreceptor-specific molecular marker opsin (*Djops* and *Gtops* clones), and also stained with DAPI to facilitate the quantification of photoreceptor cells. Although the number of photoreceptors depends on the species, planarian size and light exposition average, we did not detect any significant difference in the number of these cells between dsRNA-injected planarians and controls (Fig. 7A,B).

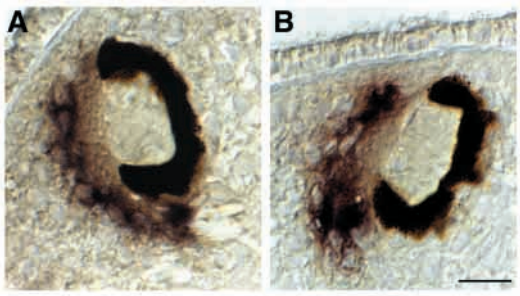


Fig. 7. Expression of *Djops* in regenerating *D. japonica* after injection with *Pax6A/Pax6B* dsRNA mixture, visualized by in situ hybridization on transverse paraffin sections at the eye level. (A,B) Nomarski images of *Djops*-expressing photoreceptors in a dsRNA-injected animal (A) and in a water-injected control (B), after 15 days of regeneration. Scale bar: 0.015 mm.

To assay for the reduction effects of dsRNA on the cognate mRNA, the level of *Pax6A* and *Pax6B* transcripts in injected animals was analyzed by comparative RT-PCR and whole-mount in situ hybridization. Total RNA was extracted from planarians injected with *Pax6A* and *Pax6B* dsRNA, as well as from animals injected with dsRNA *Pax6A/Pax6B* mixture. In both species, RT-PCR analysis showed a strong reduction in the level of expression of the corresponding endogenous mRNA, when compared with β-Gal- or water-injected controls (Fig. 8A). A similar strong reduction of endogenous RNA was observed after RNAi experiments with *Gtops* (Fig. 8A) and *Gtsix-1* genes (data not shown), which produce a loss-of-function phenotype (Pineda et al., 2000; Pineda et al., 2001).

Whole-mount in situ hybridization did not reveal *GtPax6A* mRNA in animals injected with dsRNA *Pax6A* or *Pax6A/Pax6B* mixture (Fig. 8D,E). By contrast, water- or *β-Gal*-injected head-regenerating fragments showed the typical pattern of *GtPax6A* mRNA expression (Fig. 6; Fig. 8B,C). These results demonstrate that RNAi interferes with the normal accumulation of endogenous transcripts of both *Pax6* genes in planarians. However, this elimination does not produce

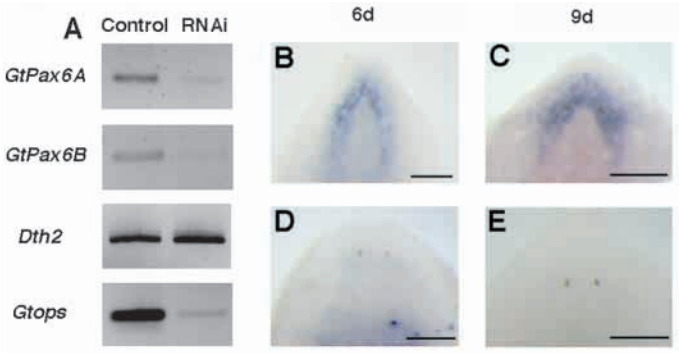


Fig. 8. Effects of *GtPax6A* and *GtPax6B* dsRNA injection in regenerating *G. tigrina*. (A) Visualization of comparative RT-PCR experiments. Relative levels of endogenous transcripts in water-injected controls and in *GtPax6A*, *GtPax6B* dsRNA-injected animals are shown. Reduction of the *Gtops* mRNA level after *Gtops* dsRNA injection is shown as a comparison. Expression of the homeobox gene *Dth2* is unaffected by *GtPax6A* and *GtPax6B* RNAi experiments. (B-E) Dorsal view of regenerating heads visualized by *GtPax6A* whole-mount in situ hybridization. (B-C) Control animals injected with water. The typical arch-shaped *GtPax6A* expression is observed in cephalic regeneration after 6 (B) or 9 (C) days from cutting. (D,E) After injection with *GtPax6A*/*GtPax6B* dsRNA mixture, no *GtPax6A* hybridization signal is detected in regenerating animals after 6 (D) and 9 (E) days from cutting. Regenerating eyespots can be observed both in the controls and in planarians injected with *GtPax6A*/*GtPax6B* dsRNA mixture. Scale bars: 0.5 mm.

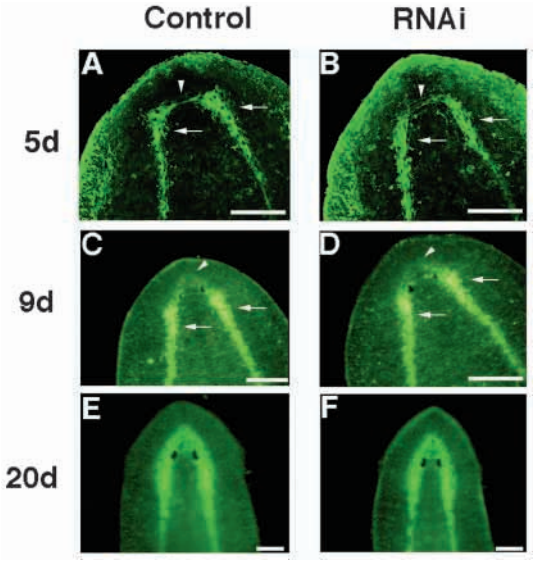


Fig. 9. Dorsal view of cephalic ganglia regeneration in *G. tigrina* after injection of *GtPax6A*/*GtPax6B* dsRNA mixture, visualized by whole-mount neuropeptide FMRFamide immunoreactivity pattern at different regenerative stages. (A,C) After 5 (A) and 9 days (C) of regeneration, new transversal commissures produced from the amputated old nerve cords and differentiating the proximal cephalic ganglia (arrows) can be observed in water-injected controls. The distal part of the new cephalic ganglia is organizing an arch-shaped structure that connects the nerve cords (arrowhead). (B,D) No abnormal cephalic ganglia regeneration is apparent in corresponding head-regenerating fragments subsequent to injection of *GtPax6A*/*GtPax6B* dsRNA mixture. Regenerating eyes appear as small black spots of similar size in both C and D. (E,F) After 20 days from cutting, a complete regeneration of the cephalic ganglia can be observed. No differences in the CNS pattern can be seen between a water-injected control (E) and an dsRNA-injected planarian (F). Scale bars: 0.5 mm.

morphological defects in the planarian eye phenotype. No gross neuroanatomical alterations were observed during cephalic ganglia regeneration by immunohistochemical staining with a general nerve cell marker, the anti-FMRFamide neuropeptide (Fig. 9A-F). However, the lack of specific neuronal cell markers prevented us from detecting neuronal cell fate changes due to the Pax6 loss of function._

To determine the possible effects of exogenous *Pax6A* and *Pax6B* dsRNA on the activation of other genes expressed in the planarian eye, we performed whole-mount in situ hybridization on injected animals using *Gtsix-1* and *Gtops* (Pineda et al., 2000; Pineda et al., 2001) as probes. No appreciable delay or reduction in the level of *Gtsix-1* mRNA expression was observed in the cephalic blastema during eye determination or differentiation, with respect to water-injected animals (Fig. 10A-F). Similar experiments performed with the planarian opsin gene *Gtops* again did not reveal any spatio-temporal change of the expression level during eye regeneration (Fig. 10G-I).

DISCUSSION

Planarians have two *Pax6* genes

We report that the genome of two planarian species belonging to different genera (*D. japonica* and *G. tigrina)* has two genes, *Pax6A* and *Pax6B*, that encode distinct paired domain- and homeodomain-containing proteins. The DNA-binding domain sequence analysis of both Pax6A and Pax6B unambiguously identifies them as members of the Pax6 family. While Pax6A appears more closely related to Pax6 proteins of other organisms, Pax6B can be considered the most diverged Pax6 characterized so far (Callaerts et al., 1997; Callaerts et al., 1999). In fact it shares only 77-78% identity in the paired domain and 74% identity in the homeodomain of the vertebrate/mammalian Pax6 sequence, while in Pax6A the identities are 88% and 85%, respectively. Pax6A also possesses a conserved Pax6-specific motif of eleven amino acids (Loosli et al., 1996) in the linker region that is not present in Pax6B. With the exception of the *Pax6* homologs *ey* and *toy*, which are found in *Drosophila* and other holometabolous insects (Czerny et al., 1999), and a *Pax6* duplication reported in zebrafish by Nornes et al. (Nornes et al., 1998), no duplicated *Pax6* genes have been described in other organisms so far. The phylogenetic analysis of the homeodomain and flanking regions with representative *Pax* genes supports a weak but closer clustering of planarian Pax6A to the duplicated *Drosophila* ey and toy, compared with the Lophotrocozoa clade representatives such as the nemertine LsPax6 or the mollusc LoPax6. Such weak clustering could be produced by a long branch attraction effect between *Drosophila* and planarians. According to its structural divergence, Pax6B is located outside the main Pax6 clustering in the tree. Although the amino acids that determine DNA-binding specificity are well conserved in Pax6B, half (nine out of 18) of the invariant residues present in the paired and homeodomain are not

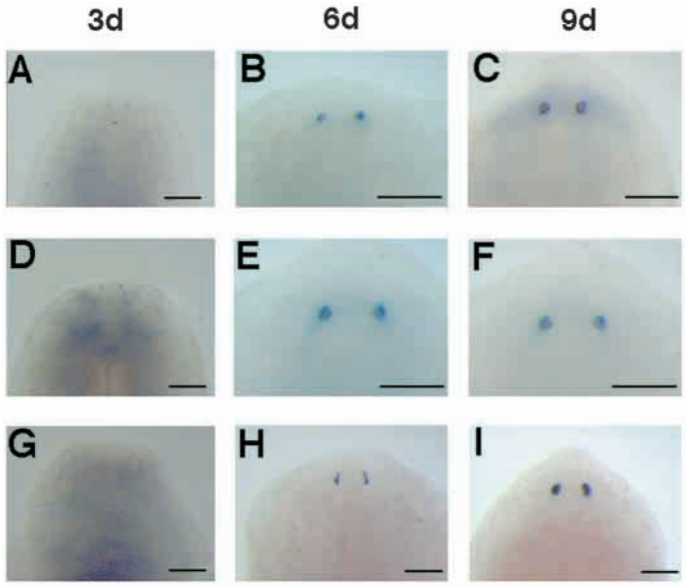


Fig. 10. Expression of *GtSix-1* and *Gtops* in regenerating *G. tigrina* after injection with *Pax6A/Pax6B* dsRNA mixture, visualized by whole-mount in situ hybridization. Dorsal view of head regenerating fragments after 3, 6 and 9 days from cutting. (A-C) Water-injected controls hybridized with *Gtsix-1*. (D-F) *GtPax6A*/*GtPax6B* dsRNA mixture-injected organisms hybridized with *GtSix1*. (G-I) *GtPax6A*/*GtPax6B* dsRNA mixture-injected organisms hybridized with *Gtops*. (A,D) A faint *GtSix1* hybridization signal is visible after 3 days of regeneration both in the controls and in injected animals. Later on, *GtSix1* mRNA is clearly visualized at the eye level. No differences are detected between controls (B,C) and injected animals (E,F). (G-I) A normal expression pattern of *Gtops* mRNA can also be detected in animals injected with *GtPax6A*/*GtPax6B* dsRNA. Scale bars: 0.5 mm.

conserved, thus representing a notable exception to the notion that Pax6 is a highly conserved transcription factor. Ectopic expression of this gene in *Drosophila* fails to produce eyes (Callaerts et al., 1999). From an evolutionary point of view, we can hypothesize that gene duplication produced two *Pax6* paralogs in a triclad or platyhelminth ancestor. After that, the two genes evolved with different selective pressures, probably resulting from different functional constraints. Nowadays Pax6A would conserve most of the structural characteristics of the ancestral Pax6, and consequently, higher similarity to Pax6 proteins of other organisms. In evolutionary times, mutations accumulating in the duplicated gene generated the structural differences present in Pax6B.

Pax6A and *Pax6B* are expressed in intact planarians and activated during regeneration

Both *Pax6* planarian genes are expressed in the CNS of adult organisms, *Pax6A* being more strongly expressed than *Pax6B*. The presence of *Pax6B* transcripts along the anteroposterior planarian axis, which was barely detected by in situ hybridization, was confirmed by RT-PCR experiments (Callaerts et al., 1999). Both genes are expressed in a subset of cells located along the entire CNS. The time course of *Pax6A* expression in cephalic regeneration clearly demonstrated activation of this gene during cephalic ganglia formation. Similarly, increased production of *Pax6B* transcripts during regeneration was demonstrated in *G. tigrina* (Callaerts et al., 1999). Regeneration of the new cephalic ganglia requires the presence of the old nerve cords. This process has been followed by immunoreactivity to the molluscan cardioactive peptide (FMRFamide). New neural fibers emerge very early from the sectioned old nerve cords and reach the blastema, then bend transversally and fuse, producing a commissure, where cephalic ganglia will differentiate (Reuter et al., 1996) (Fig. 9A). Early activation of *Pax6* at the level of cells located near the old nerve cords that reach the blastema suggests pivotal functions of these genes in the formation of neural structures in these organisms. A role for *Drosophila ey* in axon pathway selection during embryogenesis has recently been proposed by Noveen et al. (Noveen et al., 2000). In addition, it has been reported that severe defects in adult brain structures that are essential for vision, olfaction and the coordination of locomotion, are detectable in *eyeless* mutant *Drosophila* (Callaerts et al., 2001). As planarians are considered to be close relatives of primitive animals that acquired the CNS, further study of the role of *Pax6* genes during CNS regeneration will be of interest for understanding the evolution of the genetic program which triggers brain formation in higher organisms.

Owing to the low expression level, *Pax6A* and *Pax6B* transcripts were not detected in the eye cells in either intact or regenerating planarians by conventional in situ hybridization on paraffin sections with digoxigenin-labeled riboprobes. However, *DtPax6B* expression in the eye cells was detected with a more sensitive in situ hybridization method using radioactive riboprobes (Callaerts et al., 1999). TEM in situ hybridization also revealed *Pax6A* and *Pax6B* mRNA in eye cells (Fig. 5) (Callaerts et al., 1999). These transcripts were distributed both in pigment eye cells and in different subcellular compartments of photoreceptors, i.e. throughout the perikaryon, and in the rhabdomeres. The presence of Pax6 transcripts has recently been monitored by competitive RT-PCR in adult human lens epithelium, cornea and monkey retina (Zhang et al., 2001). Moreover, Pax6 expression persists in amacrine and ganglion cells of the mature retina (Ashery-Padan and Gruss 2001; Marquardt et al., 2001). On the whole, these results support a role of Pax6 in the maintenance of eye cells.

Reduction of *Pax6A* and *Pax6B* endogenous transcripts by RNAi indicates that both genes are functionally dispensable in eye regeneration

In many organisms, the *Pax6* transcription factor is critical for eye formation, as well as in patterning the CNS (Quiring et al., 1994; Kurusu et al., 2000; Pratt et al., 2000). As the basic functioning of the eyes in capturing photons and transmitting the information to the brain is similar in all animals, the presence of *Pax6* transcripts in light-sensitive cells and pigment cells of planarian eye strongly suggested a conserved role of both *Pax6* genes in this structure. *Pax6* is considered very ancient and it has been indicated that the ancestral role of this gene was to construct a light-sensitive unit by direct regulation of opsin expression (Sheng et al., 1997; Pichaud et al., 2001). The primitive eye of basal metazoans such as planarians is the most suitable model system for studying *Pax6* ancient function(s) in visual structures (Gehring and Ikeo, 1999).

The use of dsRNA to disrupt gene expression is a powerful method of achieving RNA interference in planarians (Sanchèz-

Alvarado and Newmark, 1999). Thus, complete loss of eye has been obtained after *Gtsix-1* dsRNA injection in planarians regenerating a head (Pineda et al., 2000). Moreover RNAi-mediated depletion of opsin mRNA also leads to the loss of phototactic behavior in these animals (Pineda et al., 2001).

Our experiments using dsRNA synthesized by *Pax6A* and *Pax6B* provide strong evidence that RNAi acts by decreasing endogenous cognate mRNA levels. The reduction of these gene products was comparable with that obtained for the corresponding RNAs in *Gtsix-1* or *Gtops* RNAi experiments, which give rise to abnormal eye phenotypes. Despite the drastic RNAi-induced reduction in *Pax6A* and *Pax6B* transcripts, we did not observe any gross morphological alterations of the CNS in intact planarians or during regeneration. Moreover, eyes formed without any sign of defects during head regeneration and contained several photoreceptors comparable with those found in the eye of water-injected controls. A simple interpretation of these results is that the genetic network that controls eye formation in planarians is not triggered by *Pax6* genes. The low level of Pax6 expression in the eye cells opens an alternative hypothesis, that both planarian *Pax6* genes control eye cell fate decisions by a dose-independent mechanism, insensitive to the RNAi-induced transcript reduction. We favor the former possibility, as gene dose appears to be a fundamental requirement for the activity of Pax transcription factors, *Pax6* being one of the best documented examples (Schedl et al., 1996; Van Raamsdonk and Tilghman, 2000). An exhaustive search for other *Pax6* did not yield any additional *Pax6* gene in either planarian species, making an eye-specific Pax6 highly improbable. If *Pax6A* and *Pax6B* genes are not crucial for eye induction during regeneration in planarians, we can speculate that other genes play a related role and substitute or compensate *Pax6* action in the regulatory eye network. In this respect, the planarian *Gtsix-1* gene, which is essential for eye regeneration (Pineda et al., 2000), could represent a putative candidate. The finding that *Pax6* is not expressed during ganglionic photoreceptors (Joseph cells and organs of Hesse) development in *Amphioxu*s (Glardon et al., 1998), the demonstration that Rx, but not Pax6, is essential for the formation of retinal progenitor cells in mice (Zhang et al., 2000), and our data on a Pax6-independent eye regeneration process in two species of planarians support the hypothesis that more than one molecular pathway can generate functional visual cells. Moreover, the recent hypothesis that other factors acting in parallel to Pax6 during retinal development can compensate Pax6 function (Ashery-Padan and Gruss, 2001) supports the possibility that several alternative combinations could give rise to the same phenotypic structure. The selection of an alternative combination could be promoted by a peculiar developmental scenario, i.e. blastemal regeneration. In this context, the Pax6-independent eye regeneration can be considered a remarkable example of such flexibility. Further analysis of the role of Pax6 during planarian eye development could contribute to establishing similarities between the processes of eye development and eye regeneration.

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