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Evolutionary conservation of the initial eye genetic pathway in planarians

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ABSTRACT. Eyes of all organisms share a common function, visual perception. In addition, the different types of eyes (camera-, mirror-, and compound) are present in different phyla and share the same visual pigment, rhodopsin, and the same initial genetic pathway triggered by the master control gene Pax-6. Although the developmental mechanisms are quite diverse, all data suggest that the different eye types found in metazoans derive from a common prototype and evolved in the different phyla by parallelism, intercalating new genes independently. In this manuscript, we describe the isolation and characterization of several genes that constitute the eye gene regulatory network in the planarian *Girardia tigrina* (Platyhelminthes; Turbellaria; Tricladida). Two Pax-6 genes, *GtPax6A* and *GtPax6B*, do not show an obvious correspondence to the two Pax-6 of Drosophila *ey* and *toy*. Two sine oculis genes *Gtsix-1* and *Gtsix-3* are closely related to the Six 1-2 and Six-3 families respectively. Furthermore, we demonstrate that the opsin gene *Gtops* shows greater similarity to mollusc opsins. *GtPax-6B* is expressed in both cell types of the planarian eye spots: the photoreceptor cells and the pigmented cells. In addition, *Gtsix-1* and the opsin gene *Gtops* are expressed in the photoreceptor cells. This expression pattern is present throughout the whole eye regeneration process and maintained in adults. *Gtops* double strand RNA injection does not inhibit eye regeneration but produces light insensitive eyes due to the absence of photopigment. The loss of function of *Gtsix-1* by dsRNA injection produces a non-eye phenotype in head regenerating blastemas, while the injected intact adult heads show a loss of the differentiated state of the photoreceptor cells through inhibition of opsin expression and the production of a blind phenotype. Our results on the prototypic eye spots of Platyhelminthes provide further important support for the idea of a universally conserved early eye genetic cascade in the Metazoa.

KEY WORDS: Platyhelminthes, planarian, homeobox, opsin, eye evolution, regeneration.

INTRODUCTION

Several studies indicate that the genetic program regulating eye development has been conserved in evolution. The gene regulatory network that controls the development of the *Drosophila* visual system is composed of several transcription factors and other nuclear proteins required for the specification of early eye morphogenesis (QUIRING et al., 1994; HALDER et al., 1995; SHEN & MARDON, 1997; PIGNONI et al., 1997). Pax-6 genes encode transcription factors that contain a paired domain and a homeodomain. Two duplicated Pax-6 genes have been described in *Drosophila*, *eyeless (ey*) and *twin of eyeless (toy*). Both genes and their homologs from other metazoans are capable of inducing ectopic eye development in *Drosophila*. Mutations in *eyeless* lead to defects in eye formation, suggesting that Pax-6 is the universal master control gene for eye morphogenesis (QUIRING et al., 1994). The study of other genes in the genetic cascade and their genetic interactions resulted in the identification of three genes in *Drosophila*, *sine oculis* (*so*), *eyes absent* (*eya*), and *dachshund* (*dac*) that act downstream of *ey* (QUIRING et al., 1994; SHEN & MARDON, 1997). The *so* homologs, called *Six* genes, share a diverged homeodomain and N-terminal to the homeodomain, and another conserved region, the Six domain, which contributes to DNA-binding specificity (SERIAKU & O'TOUSA, 1994; CHEYETTE et al., 1994; OLIVER et al., 1995a; 1995b; KAWAKAMI et al., 1996). The Six genes are subdivided in three different families: Six1-2, Six3 and Six4. *eya* encodes a novel nuclear protein (BONINI et al., 1993) and shares a region of homology with the Eya vertebrate proteins, the Eya domain (XU et al., 1997; ZIMMERMAN et al., 1997). Eya and Six proteins are expressed in overlapping patterns including the eye primordia of vertebrates,

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whereas *eya* and *so* do so in *Drosophila* (OLIVER et al., 1995b; XU et al., 1997; SEIMIYA & GEHRING, 2000). These factors appear to act in a hierarchy in which *so* is directly regulated by *eyeless* (HALDER et al., 1998; NIIMI et al., 1999), the master control function. In turn, *so* requires *eyes absent* (*eya*) to induce ectopic eyes (PIGNONI et al., 1997). This genetic pathway has been established in *Drosophila* (reviewed in GEHRING & IKEO, 1999). Homologous proteins also regulate eye development in vertebrates, suggesting that this regulatory network is old, conserved in evolution, and has been adapted to the control of development of different visual systems found in both Protostomia and Deuterostomia clades (TREISSMAN, 1999).

Charles Darwin in "The Origin of species" discussed the question of eye evolution, and reasoned that such complex and perfect organs should have evolved from a simple prototypic eye; those primitive eyes can be found in planarians. The planarian eye spots consist of two cell types: a bipolar nerve cell with a rhabdomere as a photoreceptive structure and a cup-shaped structure composed of pigment cells (KISHIDA, 1967). During head regeneration, new eye spots are formed from precursor cells that probably differentiate into both cell types in a restricted area of the newly regenerated tissue or blastema.

Here we address the hypothesis that the eye genetic network is conserved in evolution, and, as a consequence, that *Girardia tigrina* eye development requires *Pax-6* and *sine oculis* homologs. We report the identification of two Pax-6 genes not closely related to *Drosophila ey* and *toy;* two *so* genes closely related to the *Drosophila so* and *optix* genes, which belong to *six 1-2* and *six 3* families respectively; and an *opsin* gene with high identity to that found in the Lophotrochozoa clade. Some of these genes are expressed in the eye primordia during regeneration and in the differentiated adult eyes. RNA interference (RNAi) experiments provide functional evidence that *Gtsix-1* is essential for maintenance of the differentiated state of photoreceptor cells, for opsin expression and for eye regeneration. Such results support the conservation of the early genetic pathway in the different eyes of metazoans.

MATERIAL AND METHODS

Gene isolation

A *GtPax6A* fragment was amplified by PCR from planarian cDNA (Smart PCR cDNA synthesis Kit, Clontech). The sense primer used (Px9), consisting of a degenerate sequence corresponding to amino acid sequence LEKEFER and the antisense primer used (Px10) consisting of a degenerated sequence corresponding to amino acid sequence QVWFSNR. The cycling program consisted of 35 cycles (94ºC, 30 sec; 45ºC, 30 sec; 72ºC, 30 sec). The identity of *GtPax6A* fragment was confirmed by sequencing. A lambda gt10 amplified cDNA library was screened with the 110 bp amplified fragment of *GtPax6A* according to GARCIA-FERNANDEZ et al., 1993. One phage was isolated containing an insert of 645 bp that spans from the homeodomain to the 3' end of the *GtPax6A* cDNA. This fragment was cloned in pBluescript (Stratagene) and the sequence was determined with Thermosequenase II dye terminator cycle sequencing Kit (Amersham). A partial fragment of 380 bp of *Gtops* was amplified using two specific primers based on *Schmidtea mediterranea* opsin partial fragment kindly provided by A. Sanchez and P. Newmark (Carnegie Institution, Baltimore). The amino acid sequence of the upstream primer used (op1) and the downstream primer used (op2), GFIGGLG and ELEMLK respectively.

Phylogenetic analysis of the Gtops opsin gene

The phylogenetic trees of opsin genes using the sequence between the 3rd and the 5th transmembrane domains were inferred by using the CLUSTALX package. Sequences were aligned with the software CLUSTALX, and refined alignment was done manually. The neighbor-joining method was used for phylogenetic tree construction. Sequences were obtained from the Swissprot and EMBL GenBank.

Whole-mount *in situ* hybridization

Intact animals and animals at different regenerative stages were used for whole-mount *in situ* hybridization according to UMESONO et al.,1997. The opsin clone op-170 corresponding to the last 170 bp of the *Gtops* fragment (GenBank accession no. AJ251660) was used to synthesize the DIG-labelled antisense probes (Boehringer Manheim).

Double-strand RNA (dsRNA) synthesis and microinjection

The Gtsix-1 clones so-5' and so-3'-2 (GenBank accession number AJ251661O), were used for dsRNA synthesis as described in SANCHEZ & NEWMARK 1999. Planarians were injected as described in PINEDA et al., 2000. At different stages of regeneration the injected animals were photographed, fixed and whole-mount *in situ* hybridizations for *Gtopsin* were performed.

RESULTS AND DISCUSSION

Isolation of eye developmental genes in Girardia tigrina

A large number of eye developmental genes have been isolated in *Girardia tigrina*. Initially, CALLAERTS et al., 1999 isolated the first Pax-6 homolog, which we now refer to as *GtPax-6B* since it shows the lowest similarity to the Pax-6 genes described in other Metazoa (Table 1).

TABLE 1

The number of different planarian eye network genes, together with the type of clones isolated and the level of amino acid identity to the most similar homeodomain and proteins. All sequences for comparison are taken from the EMBL and Swiss Prot databases. The function of some genes is also shown.

Planarian eye genes					
Class	**Pax-6**		**sine oculis**		**opsin**
	DtPax-6	*GtPax-6*	*Gtso*	*Gtsix-3*	*Gtops*
New proposed name	*GtPax-6B*	*GtPax-6A*	*Gtsix-1*	*Gtsix-3*	*Gtops*
Type of clone isolated	-PCR -cDNA -genomic	-PCR -cDNA	-PCR -cDNA -genomic	-PCR -cDNA	-PCR -cDNA
Identity	72% and 78% to *Drosophila* toy and eye homeodomains	92% and 90% to *Drosophila* toy and eye homeodomains	93% and 95% to mouse six1 and six-2 homeodomains	88% and 92% to *Drosophila* optix and mouse six3 homeodomains	66% to mollusc opsin protein
Expression and Function	photoreceptor and pigmented eye spot cells	Central Nervous System	Photoreceptor cells. It is essential for eye determination and differentiation	?	Photoreceptor cells. It is essential for eye light sensitivity

Studies by in situ hybridization on paraffin sections and electron microscopy of ultrathin sections revealed expression in the perinuclear area of both eye spot cell types, the photoreceptor cell and the pigmented cells. dsRNA injection of *GtPax6B* does not produce any clear eye phenotype. One explanation for these unexpected results could be the occurrence of gene redundancy. It was therefore of interest that more recently we were able to isolate a second Pax-6 homolog, which we name *GtPax-6A* as it shows a higher similarity to the Pax-6 family homeodomain (Table 1). No expression pattern nor functional data are yet available, but preliminary whole-mount in situ hybridization showed expression in the central nervous system. We anticipate that *GtPax6A* and *GtPax6B* are at least partially redundant in the determination of both eye cell types, and that, as a consequence, the production of any phenotype when the *GtPax6B* function has been disrupted, is prevented. Injection of dsRNA of both GtPax-6 in head regenerating organisms will clarify this point (work in progress).

A second type of eye genetic network genes isolated in planarians was the *sine oculis* genes. The first *so* planarian gene isolated was originally named *Gtso*. However, molecular comparative phylogenetic analysis places it in the Six-1, -2 family. Therefore, we changed the name from *Gtso* to *Gtsix-1* (Table 1). *Gtsix-1* is closely related to *Drosophila sine oculis* and C. elegans *Ceh-33* and *Ceh-34* and clusters in the family group with another branch in which the vertebrate representatives of Six-1 and Six-2 are situated. Sequence comparison of a second *sine oculis* gene from *Girardia tigrina* supports its orthology to the Six-3 family and for that reason we call it *Gtsix-3*. It is closely related to *Drosophila optix* and *C. elegans Ceh-32*. So far, no other Lophotrochozoa *sine oculis* genes have been isolated. *Gtsix-1* whole-mount *in situ* hybridization shows that it is expressed continuously in the rhabdomeric photoreceptor cells of the adult differentiated eye spots and during the different stages of eye regeneration (Pineda et al. 2000). Loss of function experiments by RNA double strand injections in regenerating animals completely inhibit eye regeneration, producing a non-eye phenotype (Fig. 1). RNA interference in adult heads leads to the gradual loss of the photoreceptor differentiated state, producing a blind phenotype at one week post-injection. These injected planarians do not show any phototropism, while the non-injected controls have a negative phototropism. The change in the differentiated state can be observed by analyzing the alterations in opsin

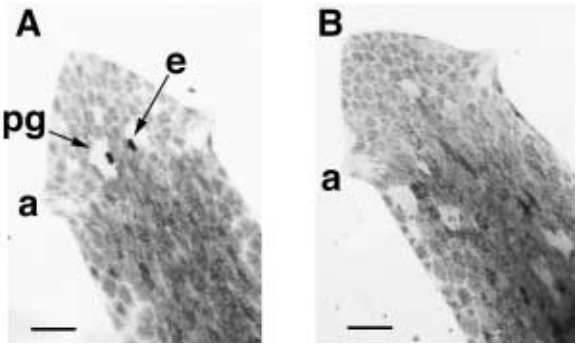

Fig. 1. – Inhibition of eye regenerative capacity by Gtsix-1 dsRNA. Three weeks head regenerating organisms viewed dorsally. (A) Bright field image shows the differentiated eyes in the regenerated head of a control organism. (B) Bright field image shows the absence of eye differentiation and periglobular unpigmented area in dsRNA Gtsix-1 injected at postblastema level after 3 weeks of regeneration. a, auricle; e, eye spot; pg, periglobular unpigmented area. (Bars= 400 μm).

expression by whole-mount *in situ* hybridization at different times post-injection and by studying eye morphology. Opsin expression decreases gradually during the first seven days post-injection (Fig. 2). A decrease of expression to zero can be explained as the result of a disruption of the eye gene regulatory network where, according to SHEN et al., 1997 and PIGNONI et al., 1997, sine oculis is located in the early eye genetic cascade. The gradual loss of opsin expression by Gtsix-1 RNAi could thus be due to an indirect effect. However, since Gtsix-1 is also expressed in the differentiated photoreceptor cells, it may also directly control opsin gene expression. Further analysis of the cis-regulatory region of the opsin gene will confirm if it is recognized by sine oculis, or by Pax-6 proteins, or both. We also checked Gtsix-1 function in photoreceptor cell maintenance by the histological analysis of *Gtsix-1* dsRNA injected organisms in comparison with controls. While the control eyes show a high density of photoreceptor cells with their rhabdomeric structures inside the eye cavity, the injected ones at 7d and 14d post-injection have a lower density of differentiated photoreceptor cells (work in progress).

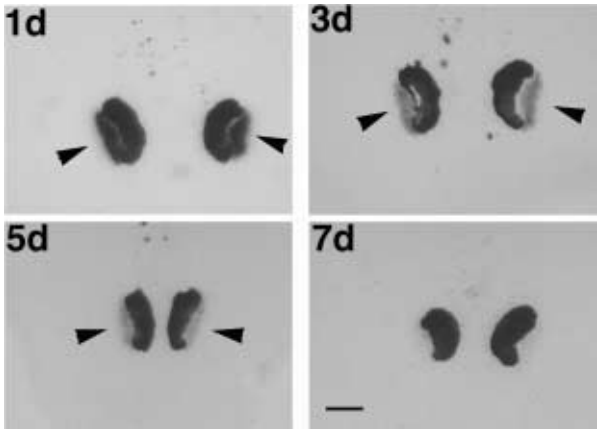

Fig. 2. – Dorsal view of Gtops gradually declining expression by whole mount *in situ* hybridization at different days post-injection of dsRNA of Gtsix-1. Internal black dots correspond to the pigmented cup shape of the eyes, while the external weak signal (arrow heads) corresponds to the blue signal from the whole mount in situ hybridization of opsin gene. Anterior at the top and upper left edge, the days after injection are indicated. (Bars: 200μm).

Another key gene shared by photoreceptors of Metazoa is the opsin gene, encoding the photoreceptor pigment present in all visual systems. Rhodopsin sequences analysed so far in vertebrates and invertebrates show a high degree of conservation, and all belong to the same family (for review see GEHRING & IKEO, 1999). Opsins are also present in bacteria, and have sensory functions. Despite a low overall sequence conservation, they show conserved structural functions like the seven transmembrane domains. The unicellular green algae *Chlamydomonas* develop at the base of the flagella a light sensitive organelle that contains a type of photopigment with limited sequence homology to invertebrate rhodopsins (DEININGER et al., 1995). The similarities observed in the photopigments have been used as another indication for the common origin of the visual system through a prototypic eye. We have isolated a *Girardia tigrina* opsin gene *Gtops* . Its amino acid sequence was compared and phylogenetic trees were constructed with opsin protein sequences of bacteria, algae, yeast and Metazoa. The neighbor-joining method was used for tree construction. We can observe the clustering of the two planarian opsin genes with the mollusc sequences, one of their Lophotrochozoa counterparts (Fig. 3). Such phylogenetic results are in agreement with other studies using different molecules such as ribosomal 18S and Hox genes

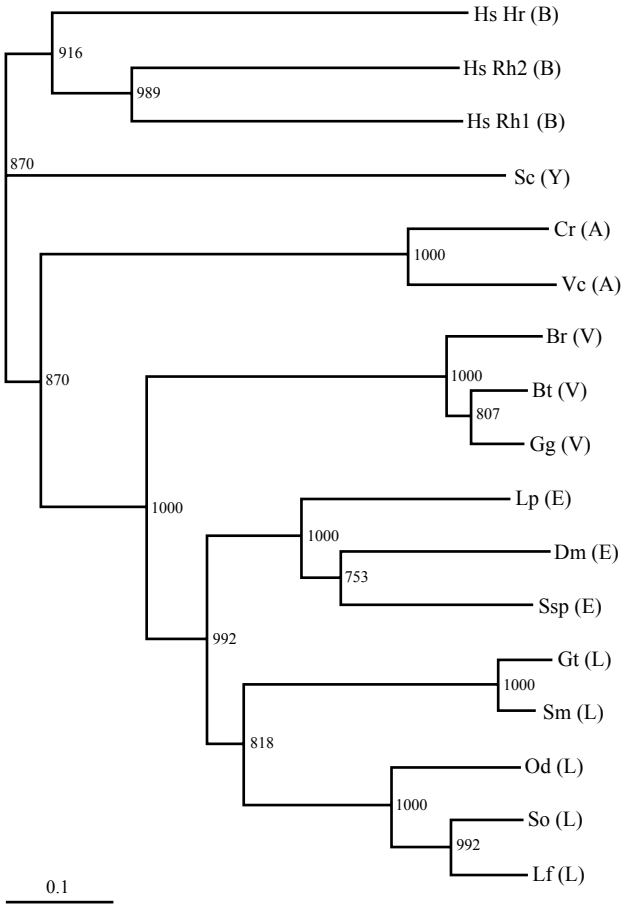

Fig. 3. – Phylogenetic unrooted tree of opsin proteins. Bootstrap values of 1000 runs are indicated as percentages at the nodes. The planarian Gtops protein clearly clusters with the other opsin proteins from the Lophotrochozoa clade. (B): Bacteria. Hs: *Halobacterium salinarium*. Hr: halorhodopsin. Rh1 and Rh2: sensory rhodopsin I and II. (Y): Yeast. Sc: *Saccharomyces cerevisiae*. (A): Algae. Cr: *Chlamydomonas reinhadtii* chlamyopsin. Vc: *Volvox carteri* volvoxopsin. (V): Vertebrate. Br: *Brachydario rerio* rhodopsin. Bt: *Bos taurus* rhodopsin. Gg: *Gallus gallus* rhodopsin. (E): Ecdysozoa. Dm: *Drosophila melanogaster* opsin1. Lp: *Limulus polyphemus* ocellar opsin2. Ssp: *Sphodromantis sp*. rhodopsin. (L): Lophotrochozoa. Gt: *Girardia tigrina* opsin. Lf: *Loligo forbesi* rhodopsin. Od: *Octopus dofleini* rhodopsin. Sm: *Schmidtea mediterranea* opsin. So; *Sepia officinalis*. Scale bar, genetic distance.

(CARRANZA et al., 1997; BAYASCAS et al., 1998). *Gtops* spatial expression was determined by whole-mount *in situ* hybridization of intact adults and regenerating pieces. In adults, *Gtops* was expressed continuously and uniformly in the photoreceptor cell bodies, whereas the rhabdomeric region of the photoreceptor cells was negative. A similar pattern of expression can be observed with *Gtsix-1*, but with a lower expression level. During the early stages of head regeneration, *Gtops* expression was detected in a group of differentiated photoreceptor cells close to the dorsal epidermis. This expression was maintained throughout regeneration (PINEDA et al., 2000). Opsin dsRNA injection induces a fast depletion of endogenous gene expression in the photoreceptor cells 24 hours post-injection, which eventually leads to the loss of phototactic behavior in the animal (SANCHEZ & NEWMARK, 1999; PINEDA et al., 2000).

Eye evolution: a common origin from a prototypic eye and an independent evolution by parallelism

The comparative embryological and morphological studies of metazoan eyes show different developing mechanisms and different morphologies suggesting an independent evolution of the different types of eyes (SALVINI-PLAWEN & MAYR 1961). However, molecular studies in the last decade have revealed the universality of rhodopsin as the visual pigment and the conservation in all studied Metazoa, including Platyhelminthes, of the early genetic cascade initiated by the gene Pax-6. Such molecular results suggest that all different eye types observed in Metazoa derive from a common prototypic eye and as a consequence have a monophyletic origin. Such prototypic eye can be found in some Platyhelminthes. The current work suggests that the development of the prototypic eye is controlled by a similar early genetic cascade. In molluscs we can observe a great variety of eyes in the mantle edge of Bivalvia (compound eyes, closed lens eyes with inverted retinal cells, reflecting mirror eye). Another eye type, the cephalopod eye, is similar in design to the vertebrate eye camera, but large embryological differences can be observed between the two (GEHRING 1996; HARRIS, 1997). The similarities in eye design in molluscs compared with the other metazoan eyes can be interpreted as evidence for a phenomenon of parallelism in the mechanisms by which the different Metazoa evolved their eyes from a common prototypic eye, using initially the same genetic network. The recruitment of different genes by intercalary evolution (GEHRING & IKEO, 1999) in the eye gene networks of the various evolutionary lines can lead to eyes with dramatically similar designs as a consequence of comparable developmental constraints, or, of course, to radically different final structures.

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