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Expression of a *retinal homeobox (Rx)* gene during planarian regeneration

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ABSTRACT *Retinal homeobox (Rx)* genes, with representatives in vertebrates and invertebrates, encode fundamental regulators of early eye and brain formation. Here we describe the spatio-temporal expression profile of a candidate planarian orthologue of *Rx* during regeneration in *Dugesia japonica* and *Schmidtea mediterranea*. Although low levels of *Rx* transcripts were found throughout the body of intact planarians, high levels of *Rx* expression were specific to regenerating tissue in both head and tail fragments. We also observed that *Rx* was never expressed in the simple rhabdomeric planarian eyes, supporting the notion that only formation of eyes that use the ciliary type of photoreceptors requires *Rx* function.

KEY WORDS: *paired-class homeobox gene, Rx, planarian, regeneration, eye*

Rx paired-type homeobox transcription factors have been isolated in several vertebrate and invertebrate species (Arendt *et al.*, 2004; Bailey *et al.*, 2004 and references therein; D'Aniello *et al.*, 2006). A growing body of evidence suggests that *Rx* genes, early expressed in the anterior neural plate, are essential for the proliferation and specification of retina progenitor cells in chordates. In invertebrates, such as *Drosophila*, *Rx* does not seem to play a similar role. In fact, *Drosophila Rx* is not required for the formation of the visual system, but only for brain and clypeus development (Davis *et al.*, 2003). In addition, an *Rx* ortholog of the annelid *Platynereis* has been found expressed in the ciliary photoreceptor cells of the brain, but not in the rhabdomeric photoreceptors of the larval and differentiating adult eyes (Arendt *et al.*, 2004). Based on these observations, it has been proposed that an *Rx* ancestral function was related to the regulation of genes involved in brain development. During evolution, *Rx* genes became then essential for development of photoreceptors of ciliary type. No data are available on the function of *Rx* genes in a context different from the embryonic development, such as regeneration. Planarians (Platyhelminthes, Lophotrochozoa) offer a suitable model to investigate the role of *Rx* during regeneration. These organisms indeed can regenerate heads, including a complete brain, as well as other body parts in a short time, by virtue of the activity of adult stem cells, called neoblasts (Agata *et al.*, 2006; Saló 2006; Sánchez Alvarado, 2006). Planarian brain consists of two anterior cephalic ganglia connected to two ventral nerve cords that run along the body (Cebrià, 2007). A pair of simple rhabdomeric eyes is generally located on its dorsal side. Despite the planarian central nervous system (CNS) seems quite simple at the morphological level, it possesses a functionally and molecularly complex structure (Cebrià, 2007 and references therein).

We have cloned and sequenced the cDNA of a planarian *Rx* homolog in *Dugesia japonica* (GI strain) and *Schmidtea mediterranea* (asexual strain). Animals were maintained in autoclaved stream water at 18°C and starved for two weeks before being used in the experiments. Fragments regenerating a head (anterior or cephalic regeneration) or a tail (posterior regeneration) were obtained by transverse amputation at the pharynx level. In these experimental conditions, regeneration was completed in about 2 weeks. A partial sequence of *D. japonica Rx* (*DjRx)*, formerly named *DjRax* (accession number AB017633), was completed by SMART 5'RACE cDNA amplification kit (Clontech) and used for an *in silico* search in the *S. mediterranea* genome (Robb *et al.*, 2008) to amplify the ortholog, *SmedRx*. Based on *S. mediterranea* genome analysis, we hypothesize the existence of a single-copy *Rx* gene in planarians. The full-length sequence of *DjRx* and *SmedRx* contains an open reading frame

Abbreviations used in this paper: CNS, central nervous system; DjRx, *Dugesia japonica* retinal homeobox gene; SmedRx, *Schmidtea mediterranea* retinal homeobox gene; Rx, retinal homeobox.

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              Octa-                          Homeodomain                                  OAR
              peptide

                          1        10        20        30        40        50        60
                          |         |         |         |         |         |         |
HSRX         HSIEAILG     HRRNRTTFTTYQLHELERAFEKSHYPDVYSREELAGKVNLPEVRVQVWFQNRRAKWRRQE    ADPRNSSIAALRLKAKEHIQA
MMRX         ........     ............................................................    .Y...................
RNRX         ........     ............................................................    .Y...................
GGRX1        --------     ...................................M........................    V.Q.S....S..M.......T
GGRX2        ........     ...................................M........................    G....T...S..M.......S
XLRXA        ........     ...................................M........................    T....N...S..M.......F
XLRXB        ........     ...................................M........................    I....N...S..M.......S
OLRX2        --------     ...................................T........................    D.-.........M...----
DRRX1        ...DV...     ...................................M........................    V.Q.S.......M.......S
DRRX2        ...DV...     ...................................M........................    V.-.S.......M.......S
DRRX3        ....S...     ...................................L........................    .....T...S..M.......S
CIRX         YNE.VNNI     L.........F..........R...............I......................    NPD.SP.EKSFKRETA.EFRF
ODRX         --------     ..........F..................A.....T.IS.....................    ---------------------
SKRX         ...D....     ..........F........................L........................    N...S...VS..M......EN
DMRX         .T.D....     .........-.........................M........................    T.M.SN...T..I.....LDN
PDRX         --------     ...................................L.I......................    D...ST..VS..MR....MK.

DJRX         YN.DD...     ...................................M.IS.....................    SKDL..NSKTCSIRTADNLNT
SMEDRX       YN.DD...     ...................................M.IS.....................    SKDL..NNKTCSIRSSDN.NG
GTRX         --------     ----------.........................M.IS.............--------    ---------------------
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Fig. 1. Comparison of predicted amino acid sequences among the octapeptide, homeodomain and OAR domain of DjRx and SmedRx and other Rx proteins. *Identical amino acid residues are indicated by dots with respect to the HSRX:* Homo sapiens *RX (Q9Y2V3); incomplete sequences are indicated by hyphens. Sequences shown were obtained from the EMBL/ GenBank. CIRX:* Ciona intestinalis *RX (CAC34833); DRRX1:* Danio rerio *RX1 (O42356); DRRX2:* Danio rerio *RX2 (O42357); DRRX3:* Danio rerio *RX3 (O42358); DMRX:* Drosophila melanogaster *RX (CAA11241); DJRX:* Dugesia japonica *RX (AM942442); GGRX1:* Gallus gallus *RX1 (Q9PVY0); GGRX2:* Gallus gallus *RX2 (Q9PVX0); GTRX:* Girardia tigrina *RX (AY040121); MMRX:* Mus musculus *RX (NP038861); ODRX:* Oikopleura dioica *RX (AAW23065); OLRX2:* Oryzias latipes *RX2 (AJ007939); PDRX:* Platynereis dumerili *RX (AAU20320); RNRX:* Rattus norvegicus *RX (AAK07423); SKRX:* Saccoglossus kowalevskii *RX (AAP79282); SMEDRX:* Schimdtea mediterranea *RX (AM942443); XLRX-A:* Xenopus laevis *RX-A (O42201); XLRX-B:* Xenopus laevis *RX-B (O42567).*

(ORF) of 816 bp, encoding a protein of 272 amino acids with a paired (prd)-like homeodomain and an octapeptide motif (Fig. 1). DjRx and SmedRx appear almost identical at the amino acid level (90% identity) and contain typical Rx hallmarks in the homeodomain: i) a glutamine residue is present at position 50 – a site essential for the DNA-binding specificity – instead of the serine found in other members of the prd class, ii) they show methionine/glutamine exchange at position 36, a feature frequently found in vertebrate and invertebrate Rx (Fig. 1). The serine/asparagine replacement observed at position 39 of the planarian Rx homeodomain probably represents a peculiarity of these organisms, shared only by *Oikopleura dioica* Rx (Edvardsen *et al.*, 2005). Finally, planarian Rx do not show the 14 amino acid OAR domain identified in the C-terminal region of vertebrate and *Drosophila* Rx, and considered important for transactivation (Furukawa *et al.*, 1997).

Whole mount *in situ* hybridization was carried out to analyze the expression pattern of *Rx*. Although no specific hybridization signal could be detected in intact planarians even after long staining, real time RT-PCR in *D. japonica* revealed presence of *Rx* transcripts both in anterior and posterior regions of the planarian body (Fig. 2A). Considering the well-established localization of *Rx* transcripts to anterior domains in vertebrate and invertebrate species, it is striking that *Rx* can be expressed in a posterior part of the planarian body. The significance of this unusual expression is presently unclear, but it is tempting to speculate that distribution of *Rx* transcripts along the planarian body could be related to the amazing stem cell-based regenerative abilities of these animals, that can regenerate a complete individual from almost any body

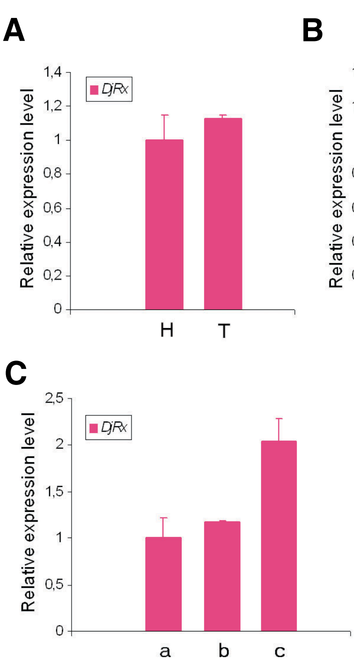

Fig. 2. *DjRx* real time RT-PCR analysis of fragments from intact planarians. (A) *Expression level of* DjRx *in anterior (H) and posterior (T) body fragments obtained from planarians transected at the pharynx level. Expression levels are indicated in relative units assuming as unity the value of the anterior region. Each value is the mean ± standard deviation of three independent samples, analyzed in duplicate.* **(B)** *Expression level of* DjRx*, 2 days after X-ray (30 Gy) irradiation. Expression levels are indicated in relative units, assuming the value of untreated specimens (c: control) as unity. Each value is the mean ± standard deviation of three independent samples, done in duplicate.* **(C)** *Expression level of* DjRx *in cell fractions obtained by filtration through nylon meshes of 50 μm (a), 20 μm (b) and 8 μm (c) pore size. Expression levels are indicated in relative units, assuming the value of 50 μm nylon meshes as unity. Each value is the mean ± standard deviation of three independent samples, analyzed in duplicate.*

fragment.

As in vertebrates Rx is required to control the proliferation of specific cell types (Bailey *et al.*, 2004), we asked whether the planarian gene might have a role in regulating neoblast proliferation or their correct specification toward a specific fate. To test this hypothesis, we performed real time RT-PCR in intact animals sacrificed 2 days after a lethal dose of X-rays, a treatment that destroys neoblasts (Hayashi *et al.*, 2006; Reddien *et al.*, 2005; Rossi *et al.*, 2007 and references therein; Salvetti *et al.*, 2000). As shown in Fig. 2B, we observed that the elimination of neoblasts in irradiated animals did not reduce *Rx* expression level with respect to the unirradiated controls, suggesting that *Rx* expression does not depend on the presence of proliferating cells. To extend our knowledge on the cells that express the planarian *Rx*, we carried out real time RT-PCR using RNA obtained from dissociated cell fractions, selected for size by progressive filtering (Rossi *et al.*, 2007; Salvetti *et al.*, 2005) and found that *Rx* expression was preferentially detected in the fraction enriched in small cells (Fig. 2C). These data suggest that *Rx* plays a role in the specification of small cells, that, based on the size (Baguñà and Romero, 1981), could be differentiating/differentiated nerve cells.

Further real time RT-PCR experiments, undertaken during regeneration, revealed that *Rx* expression was upregulated both in tails regenerating a head (Fig. 3) and in heads regenerating a tail (Fig. 4). During anterior regeneration an increase in *Rx* mRNA level was observed after 1 day of regeneration, reached a maximum around day 6, and reduced progressively as regeneration proceeded (Fig. 3A). Whole mount *in situ* hybridization experiments visualized an expression profile that confirmed the specific activation of *Rx* transcripts. *Rx* expression pattern appeared very similar in *D. japonica* and *S. mediterranea* (Fig. 3B-K). By day 3 of regeneration, *Rx* transcripts could be detected in the blastema. As regeneration proceeded, *Rx* hybridization signal was detected in the medial and lateral brain regions, while it was never found in the rhabdomeric eye cells that were visualized by immunostaining with VC-1 antibody (Sakai *et al.*, 2000) (Fig. 3L,M). This finding provides further confirmation that Rx function is required only for specification of photoreceptors of ciliary type (Saló *et al.*, 2002). By using real time RT-PCR we noticed that the *Rx* expression level observed during tail regeneration was lower and slightly delayed in time (day 6 to day 10, with a peak at day 8), with respect to that observed during head regeneration (Fig. 4A). At day 8, *Rx* transcripts could be visualized in some cells that appeared spread in the regenerating tissue (Fig. 4B). We knocked down the *Rx* gene both in *D. japonica* and in *S. mediterranea* by RNA interference (RNAi) and then investigated the effects at morphological and molecular level. Although small defects might be easily missed in this model, no significant alteration of the relative size of the blastema with respect to the water–injected controls was found by the analysis of the ratio between the blastema and the stump area using Nikon ACT-2U imaging software. Similarly, no defects could be visualized in the brain by whole mount *in situ* hybridization with the panneural marker *synaptotagmin*. To further identify possible effects of *Rx* RNAi treatment, we also checked *synaptotagmin* and *DjOtxA* (a marker specific for the brain region where the axons of photoreceptors project; Umesono *et al.*, 1999) mRNA level by real time RT-PCR. Unfortunately, our real time RT-PCR experiments did not reveal any significant variation of these transcripts after *Rx* RNAi (data not shown). As *Rx* RNAi did not

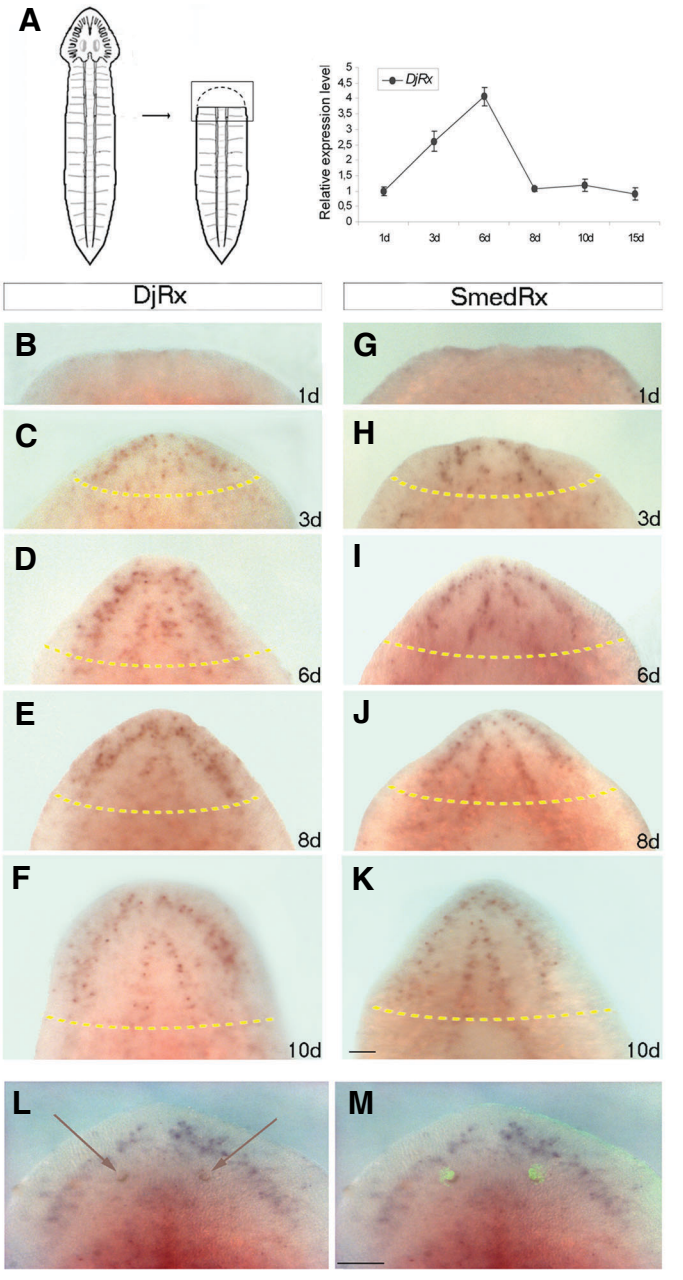

Fig. 3. *Rx* expression during planarian head regeneration. (A) DjRx *real time RT-PCR analysis. d, days of regeneration. cDNA samples derived from 4 small fragments including blastema and postblastema regions, boxed in the schematic drawing. Expression levels are indicated in relative units, assuming the value of one day-regenerating fragments as unity. Values are the mean of two independent experiments and error bars indicate standard deviation.* **(B-K)** *Ventral view of the regenerating fragments, as visualized by whole mount* in situ *hybridization with* DjRx **(B-F)** *and* SmedRx **(G-K)**. **(L)** *Dorsal view of regenerating* D. japonica, *as visualized by whole mount* in situ *hybridization with* DjRx *8 days after amputation. The pigment cells of two small regenerating eyes can be detected as brown spots (brown arrows).* **(M)** *Immunostaining of whole mount* in situ *hybridization depicted in (L) with the photoreceptor-specific antibody VC-1 allows visualization of rhabdomeric eye cells. d, days of regeneration. Anterior is to the top. The dashed yellow line indicates the border between the regenerating region and the stump in C-F, H-K. Scale bars, 50 μm.*

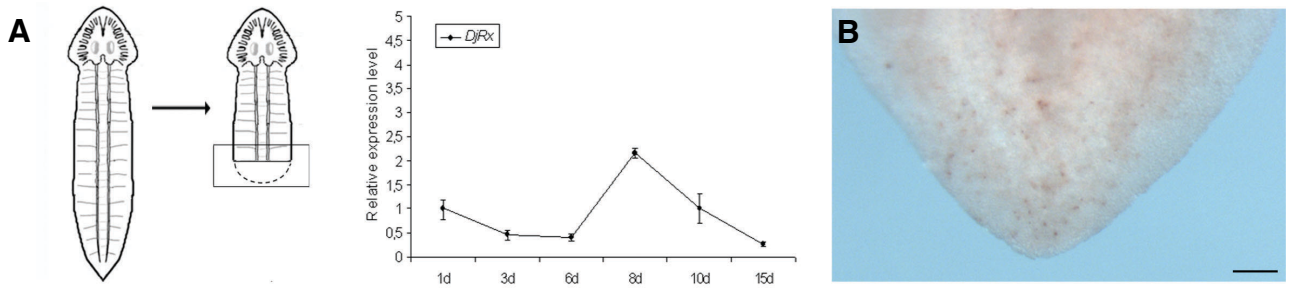

Fig. 4. *Retinal homeobox (Rx)* expression during planarian tail regeneration. (A) DjRx *real time RT-PCR analysis. d, days of regeneration. cDNA samples derived from 4 small fragments including blastema and postblastema regions, boxed in the schematic drawing. Expression levels are indicated in relative units, assuming the value of one day-regenerating fragments as unity. Values are the mean of two independent experiments and error bars indicate standard deviation.* **(B)** *Dorsal view of a regenerating tail as visualized by whole mount* in situ *hybridization with* DjRx *8 days after amputation. Scale bar, 50 μm.*

produce any relevant defects, it is possible that other genes may compensate for *Rx* in planaria and we can only speculate that the Rx activity can be of importance for the correct specification of nerve cells during cephalic regeneration. The possibility that *Rx* activation observed during tail regeneration may play a role in neoblasts committed to a neural lineage remains to be explored.

Experimental Procedures

Whole mount *in situ* hybridization experiments were performed according to (Umesono *et al.*, 1997; Umesono *et al.*, 1999) with minor modifications (Nogi and Levin, 2005). Color development of the alkaline phosphate-coniugated anti-DIG-antibody was carried out with a mixture of BCIP/NBT (Sigma). To detect *Rx* hybridization signal in regenerating planarians chromogenic exposure was extended for approximately 8 hours. In contrast, no specific signal was visualized in intact animals also after 24-36 hours of substrate incubation. After whole mount *in situ* hybridization, selected specimens were processed for immunostaining with the photoreceptor-specific antibody VC-1 (Sakai *et al.*, 2000). Total RNA was extracted with the NucleoSpin RNAII kit (Macherey-Nagel) and was reverse-transcribed using Superscript First Strand Synthesis System (Invitrogen) from intact planarians, regenerating fragments, dissociated cells samples or irradiated planarians exposed to a lethal X-ray dose. Quantitative real time RT-PCR amplification was carried out with the ABI Prism 7000 Sequence Detection System (Applied Biosystems). PCR reactions were carried out using 20 ng cDNA and TaqMan Universal PCR Master Mix (Applied Biosystems) following the manufacturer's protocol. *DjEF2* was used as a normalizing gene to eliminate variation in cDNA concentration between the samples (Rossi *et al.*, 2006; Rossi *et al.*, 2007). Reactions were run in duplicate and 2 independent samples per experimental condition were used. Relative quantification of gene expression was performed using the comparative CT method as described in the ABI Prism 7700 Sequence Detection System User Bulletin No. 2.

DjRx: probe 5'CAGCTTCACTGATTGCCG 3',
forward 5'GTCCAAAAGACAGTTCAGAACAATCAC 3',
reverse 5'TCCGGTGACGATTCTGTTTCG 3'.

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