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Molecular aspects of cell proliferation and neurogenesis in planarians

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ABSTRACT : An *MCM2* gene (*DjMCM2*) which represents a suitable molecular marker for detecting proliferating neoblasts was isolated in planarians. Neoblasts are the only self-renewing cells in these organisms and may be able to differentiate into all cell types lost to physiological turnover or injury. To understand the molecular basis leading to neoblast commitment to a differentiative fate, some regulatory genes were cloned. Our interest was focused on the process of nerve cell differentiation. *DjXnp*, a planarian gene coding for a protein similar to Xnp/ATRX, a DNA helicase involved in mammalian brain development via chromatin structure modification, was obtained by RT-PCR. A similar strategy was also used to clone two different *Pax-6*-related planarian genes. Identification of factors involved at different levels in the control of gene expression during nerve cell differentiation could be of importance to understand the regulatory programs, which operate during neoblast differentiation in planarians.

KEY WORDS : Platyhelminthes., *Dugesia japonica*, neoblasts, cell proliferation, differentiation, MCM, Pax-6, XNP.

INTRODUCTION

A stable population of stem cells referred to as neoblasts is responsible for the renewal of all differentiated cell types in planarians. The presence of these cells is also crucial for the regenerative ability of these organisms. During regeneration, neoblasts increase in number by active cell proliferation and begin to accumulate beneath the wound epithelium giving rise to the regeneration blastema. Then, these cells differentiate into the various specialised cell types, and replace any missing structures by morphogenesis (BRØNDSTED, 1969 ; GREMIGNI, 1981 ; BAGUÑÀ, 1998).

Despite the obvious interest for the study of neoblasts, these cells are largely unexplored at the molecular level. Only recently the thymidine analogue 5'-bromo-2'-deoxyuridine (BrdU) has been successfully utilised to label stem cells in planarians (NEWMARK & SANCHEZ ALVARADO, 2000). At the same time, our analyses have demonstrated that the expression of *DjMCM2*, a member of the MCM2-7 family of DNA replication factors, can be used to specifically detect proliferating neoblasts (SALVETTI et al., 2000). These results provide new potential tools to improve our understanding of the cellular renewal system in these organisms.

The molecular mechanisms that regulate the differentiation program, by which neoblasts acquire distinct identities and specialised functions, are completely unknown. The central feature of cell differentiation is a change in gene expression. Three different regulation levels can be hypothesised for this process. As a first stage, an initial decondensation of the chromatin structure may be required in preparation for transcription. Subsequently, transcription factors can bind to specific sites on the chromatin to induce RNA synthesis. Finally, post-transcriptional regulatory mechanisms can be crucial for the modulation of the right amount of protein to be produced.

Besides a refinement of previous findings concerning the distribution of *DjMCM2*-expressing neoblasts (SALVETTI et al., 2000), the identification of some genes potentially involved in different processes of regulation during nerve cell differentiation is here reported : *DjXNP*, coding a putative protein similar to the mammalian Xnp/ATRX DNA helicase, and two *Pax-6*-related genes, *DjPax-6A* and *DjPax-6B*.

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MATERIAL AND METHODS

Animals

Planarians used in this work belong to the asexual strain GI of *Dugesia japonica* (Platyhelminthes, Tricladida) (ORII et al., 1993). Intact worms and regenerating fragments were kept in autoclaved stream water and maintained at 18°C in aquaria. Planarians were fed weekly with chicken liver and starved for one week before being used in experiments. Some specimens were amputated pre- and post-pharyngeally in order to induce bi-directional regeneration. Posterior regeneration was obtained by pre-pharyngeal amputation.

cDNA cloning and sequence analysis

DjMCM2 was cloned as described by SALVETTI et al. (2000).

A partial *DjXNP* cDNA fragment was amplified by RT-PCR using two degenerate primers corresponding to two conserved regions (RRIILTG and PPKHEYV) between human and mouse XNP/ATRX proteins. The sequences used for the comparison correspond to the EMBL/Genbank entries U72938 and AF026032. A similar procedure was utilised to isolate *DjPax-6B* (see Results). *DjPax-6A* was directly obtained from the sequence deposited in the EMBL/Genbank, using two specific primers.

Amplification products were cloned in pGEM-T Easy Vector (Promega). All clones were sequenced by automated fluorescent cycle sequencing (ABI). Similarity searches were performed using BLASTX; sequences were aligned with CLUSTAL W.

In situ hybridization experiments

Whole mount in situ hybridizations were carried out in intact and regenerating planarians according to the method described by AGATA et al., (1998). In situ hybridization on sections was performed as indicated by KOBAYASHI et al., (1998).

Synthesis of the digoxigenin-labelled sense and antisense RNA probes was performed according to standard protocols (Boehringer).

RESULTS AND DISCUSSION

DjMCM2

DjMCM2 is a putative member of a hexameric complex of proteins, MCM2-7, which are essential components of the prereplication chromatin, (for a recent review on MCM proteins, see TYE, 1999). We have isolated this gene in *D. japonica,* and demonstrated that it can be used as a molecular marker for specifically detecting proliferating neoblasts (SALVETTI et al., 2000). Proliferating neoblasts appear to be distributed in a non-uniform manner along the cephalo-caudal and dorso-ventral axes, being preferentially accumulated in dorso-lateral peripheral areas, with a minimum distribution at the cephalic level. This uneven distribution of the proliferative compartment in intact planarians has been further confirmed by using the in situ procedure directly on sections. This highly sensitive method has allowed us to investigate the distribution of *DjMCM2*-expressing cells in detail (Fig. 1).

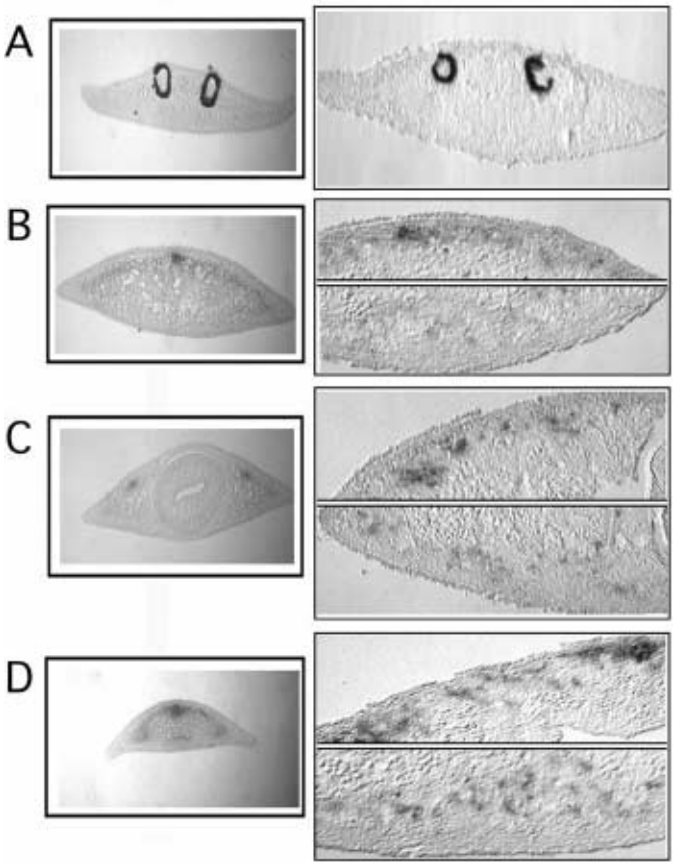

Fig. 1. – Expression of *DjMCM2* mRNA in an intact *Dugesia japonica*. (A-D) antero-posterior sequence of transverse sections. Left: wax sections after whole mount *in situ* hybridization. Right: *in situ* hybridized sections. In B-D the upper part is a magnification of the dorsal side, and the lower part is a magnification of the ventral side.

The analysis of *DjMCM2* expression pattern shows that a spatially regulated proliferative activity also characterises regeneration in planarians. In particular we were able to observe the presence of spatio-temporal changes in the *DjMCM2* RNA expression pattern in the stump, coordinated with the orientation of the cut. Moreover, during blastema growth, intensive cell proliferation was detected at the postblastema level, whereas no dividing cells were found in the blastema area (Fig. 2), where neoblasts are known to give rise to differentiated cells.

These observations support the hypothesis that a variety of intrinsic and/or extrinsic positional signals constitute a sort of cell niche (WATT & HOGAN, 2000), signalling

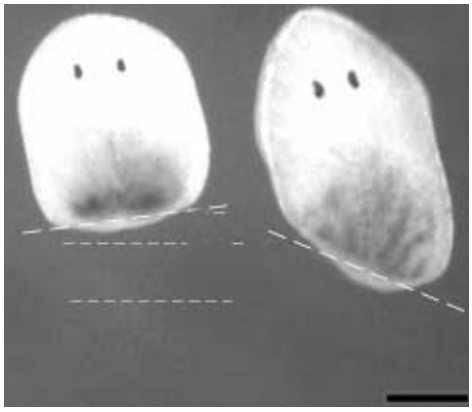

Fig. 2. – Expression of *DjMCM2* mRNA in posterior regeneration of *D. japonica*, visualized by whole mount *in situ* hybridization at 3 days after cutting. Blastema regions are devoid of hybridization signal. Dashed white lines indicate the limits of the blastema area. Scale bar = 500 μm.

neoblasts to divide or to undergo differentiation (AGATA & WATANABE, 1999). Identification of these factors will be an important step towards an understanding of the molecular mechanisms regulating neoblast behaviour.

DjXNP

Transition from inactive to active chromatin at specific chromosomal domains is probably one of the first steps required for cell differentiation. Regulatory factors and mechanisms involved in changes in the chromatin structure are until now, almost completely unknown. Recent reports indicate that putative DNA helicases, the XNP/ATRX proteins, appear to be involved in chromatin remodelling during nerve cell differentiation in mammals (GECZ et al., 1994; CARDOSO et al., 1998). These findings encouraged us to investigate the presence of similar molecules in planarians. We succeeded in isolating a cDNA fragment (*DjXNP*; Fig. 3A) which shows some similarity to the human and murine XNP/ATR-X genes (69%) (GECZ et al., 1994; STAYTON et al., 1994) and to the closest known non-mammalian relative, the nematode *xnp-1* gene (56%) (VILLARD et al., 1999).

We localised *DjXNP* transcripts in planarians by whole mount *in situ* hybridization. The hybridization signal had a widespread distribution throughout the parenchyma, and a significant amount of transcripts were found in proximity of the cerebral ganglia (Fig. 3B), which were identified by expression of the planarian synaptogmin gene (*DjSyt*: TAZAKI et al., 1999) (Fig. 3C).

During early regeneration, a preferential accumulation of *DjXNP* transcripts was observed in the parenchyma area, beneath the wound surface (Fig. 3D). Later on, these transcripts appeared preferentially localised in the nervous system presumptive territories (Fig. 3E).

The *DjXNP* expression pattern found in planarians essentially resembles that observed for *XNP/ATRX* transcripts in humans and rodents. In these organisms XNP/ATR-X transcripts are present in a variety of tissues,

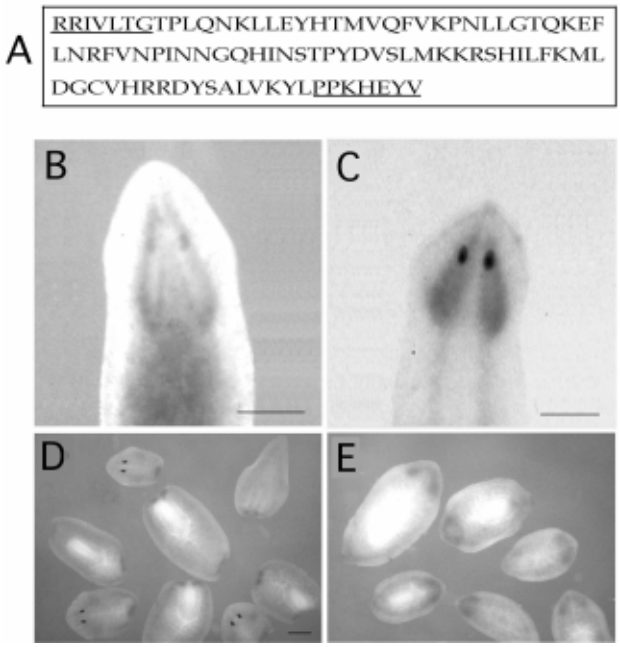

Fig. 3. – *DjXnp* characterization.(A) Deduced amino acid sequence of *D. japonica DjXnp* cDNA fragment. The regions corresponding to the primers are underlined. (B-E) Whole mount *in situ* hybridization. (B) Expression of *DjXnp* mRNA in an intact *D. japonica*. (C) Expression of *DjSyt* mRNA in an intact *D. japonica*. (D-E) Expression of *DjXnp* mRNA in regenerating *D. japonica*, at 3 days (D) and 7 days (E) after cutting. Scale bars = 500 μm.

and a preferential accumulation is found in developing brain (GECZ et al., 1994).

Although the identity of the *DjXNP*-expressing cells was not determined, these findings suggest that DjXNP may have a conserved function at the cellular level, playing a role *via* chromatin structure modification in differentiating neoblasts. In this case DjXNP could potentially regulate a subset of target genes specifically activated during early neuronal differentiation. The use of dsRNA-mediated genetic interference (SANCHEZ ALVARADO & NEWMARK, 1999) represents an essential tool for investigating this possibility.

DjPax-6A and *DjPax-6B*

A wide range of regulatory factors binding specific modules along active chromatin is known to play a key role in the control of gene expression in eukaryotes.

Pax-6-related genes could be used as informative molecules for an understanding of the regulatory cascade of the nervous and visual system, at the transcriptional level, during planarian regeneration. These molecules are sequence-specific transcription factors related to eye morphogenesis in a variety of animal phyla and are highly conserved during evolution. For example, the murine and human Pax-6 proteins are identical to each other and show extensive sequence similarity to the *Drosophila* Pax-6-related gene, *eyeless* (QUIRING et al., 1994; GEHRING & IKEO, 1999).

Fig. 4. – Comparison of the deduced amino acid sequence of *DjPax-6B* with those of *G. tigrina* (*GtPax-6*) and *D. japonica* (*DjPax-6A*). The amino acid sequences corresponding to the primers used, are boxed.

Up to now, two *Pax-6*-related genes have been identified in planarians: *GtPax-6* from *Girardia tigrina* (CALLAERTS et al., 1999) and *DjPax-6* from *Dugesia japonica* (Accession No. AB017632). These molecules are very similar in the two DNA-binding domains that typify the *Pax-6* gene class. However, we have observed that they share a much lower overall sequence identity than expected on the basis of the high conservation of *Pax-6* homologs. Accordingly, a homology search showed that *DjPax-6* had the highest overall sequence similarity ($p = e^{-107}$) with *twin of eyeless*, a second *Pax-6* gene, recently identified in *Drosophila* (CZERNY et al., 1999).

With the aim of contributing to the characterization of *Pax-6*-related genes in planarians, we looked for the presence of a second *Pax-6* gene in *D. japonica*. Two degenerate primers were used. The sense primer was designed taking advantage of the high sequence conservation between *DjPax-6* (here called *DjPax-6A*) and *GtPax-6* in the amino acid region QVSNGCV. On the other hand, the antisense primer corresponded to the *GtPax-6*-specific amino acid region TLFGYN (Fig. 4).

One of the cDNA fragments that we isolated, called *DjPax-6B*, was 520 bp long and contained an uninterrupted open reading frame, with 86% sequence identity to the *GtPax-6* corresponding region (Fig. 4).

Analysis of the expression pattern of *DjPax-6A* and *DjPax-6B,* and their functional characterisation, will contribute to an understanding of their role during nervous system regeneration. In particular, it will be possible to verify whether, similarly to the ones found in *Drosophila*, they act as key regulators in the genetic hierarchy controlling eye formation in planarians.

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