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Maintenance of A/P body regions in planarians by *tcen49*, a putative cystine-knot neurotrophin

David Bueno [1], Marcelo Vispo [1], Vanessa Sancho and Rafael Romero

Departament de Genètica, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 645, ES-08028 Barcelona, Catalonia (Spain)
[1] These authors have contributed equally to this work

ABSTRACT. In freshwater planarians, the protein TCEN49 has been linked to the regional specification of the central body region, which includes the pharynx. Here we present the genomic and deduced amino acid sequence of the *tcen49*, show the expression of *tcen49* mRNA and compare the location of its protein in intact and regenerating organisms. The open reading frame encodes a secreted protein of 70 amino acids that shows no similarity to any other known protein, although it displays a cysteine pattern found in some members of the neurotrophic family. In intact adult planarians, transcripts are detected specifically in secretory cells in the central body region, whereas the protein is secreted from them to all the tissues in this region. Neither mRNA nor protein is detected in the anterior or posterior regions. During regeneration, the timing and dynamics of *tcen49* expression and TCEN49 location are always detected prior to any morphological evidence of the formation of the new central region, although the protein is not secreted until the pharynx starts its maturation. During anterior regeneration, *tcen49* is detected as early as day 1 of regeneration throughout the regenerate, which is different from posterior regenerates. Our results suggest (1) the presence of inhibitory mechanisms that regulate *tcen49* expression as well as the post-transcriptional regulation of its RNA and (2) that TCEN49 is not necessary to start regeneration but is involved in regional pattern maintenance.

KEY WORDS: Platyhelminthes, planarian, region-specific molecule, antero-posterior patterning, regionalisation, pattern maintenance, regeneration.

INTRODUCTION

For many organisms, including invertebrates and vertebrates, it has been shown that the establishment of the antero-posterior (A/P) polarity is preceded by the expression of different molecular regions, controlled by a number of genes that sequentially restrict different body regions. Once established, these regions have to be maintained. This becomes crucial in organisms that exhibit great morphological plasticity, as freshwater planarians do. The discovery and examination of an increasing number of genes involved in these phenomena help to give an integrated view of the mechanisms that govern the formation and maintenance of a defined body plan.

Freshwater planarians (Platyhelminthes, Tricladida) have attractive features that make them interesting for the analysis of the pattern-forming and -maintaining processes. These organisms are able to regenerate a whole organism from a small piece of the body, consistent with its original polarity (BRØNDSTED, 1969, for a historical review). Furthermore, planarians have the ability to grow and degrow depending on environmental conditions (food availability and temperature) (for a general review on regeneration, growth and degrowth, see BAGUÑÀ et al., 1990).

These features make the mechanisms used for the establishment, maintenance and re-specification of A/P polarity during regeneration especially intriguing. Although some *Hox* genes have already been identified in planarians (GARCIA-FERNÀNDEZ et al., 1993; ORII et al., 1995; BAYASCAS et al., 1997; ORII et al., 1999), the cellular and molecular mechanisms underlying the patterning of cells for a specific region along the A/P axis remain obscure. Particularly pertinent to these mechanisms is the protein TCEN49 from the planarian *Girardia tigrina*.

Corresponding author: D. Bueno, e-mail: bueno@porthos.bio.ub.es

TCEN49 is a region-specific secreted molecule present solely in the central body region. Its pattern of location and its dynamics during regeneration clearly suggest a role in the mechanisms that specify and/or maintain A/P body regions (BUENO et al., 1996).

In this paper we report the complete nucleotide and amino acid sequence of *tcen49*, a novel gene with a cysteine distribution resembling that of a cysteine-rich neurotrophic factor. The location of TCEN49 and the expression of its mRNA in intact and regenerating organisms suggest that TCEN49 is involved in the maintenance of A/P planarian body regions.

MATERIAL AND METHODS

Species, culture conditions and nomenclature

The freshwater planarians used belong to an asexual race of the species *Girardia tigrina* collected near the city of Barcelona. They were maintained in spring water in the dark at 4-6 ˚C and fed once a month with beef liver. The planarians chosen for the experiments were starved for at least 15 days before use. Organisms of 7-10 mm in length were cut at the levels described in BUENO et al. (1996) (see Figs 3 and 4) and the temperature was kept at 17±1˚C.We refer to the monoclonal antibody (MAb) as TCEN-49; to the protein as TCEN49; and to the gene as *tcen49*.

Isolation of *tcen49*

The MAb TCEN-49 (ROMERO et al., 1991) was used as a probe to screen a *G. tigrina* cDNA Lambda Uni-ZAP library (Stratagene) kindly provided by Dr. E. Castillo. The isolated *tcen49* cDNA (315 bp) was labelled by random-primer with [a^{32}P]dCTP (3000 Ci/mmol) (Amersham), and was then used as a probe to screen a *G. tigrina* genomic Lambda FIX II (Stratagene) library. Sequence analysis was carried out by using the BCM Search Launcher software. Southern blot analysis was performed by using 10 µg of *G. tigrina* DNA (GARCIA-FERNÀNDEZ et al., 1993) digested with *Eco*RI or *Hin*dIII (Promega) and hybridised with the same *tcen49* cDNA probe used in the genomic screening.

Generation of fusion protein, polyclonal antibodies, and western blot analysis

tcen49 was subcloned into the pGEX4T-3 vector (Pharmacia). The resulting glutathione S-transferase (GST) fusion protein (GST-TCEN49) was purified by using a Bulk GST Purification Module (Pharmacia), and thrombin digestion was done following Pharmacia´s instructions. Western-blot analysis of total protein extracts and the resulting thrombin-digested GST-TCEN49 was done under renaturing conditions as described in Bueno et al. (1996). Polyclonal antibodies to thrombin-digested GST-TCEN49 were obtained by sequential intraperitoneal injections in Balb/C mice according to standard procedures.

RNA in situ hybridisation and immunohistochemistry

Northern blot analysis was performed by using 15 µg of total RNA isolated from *G. tigrina* by the guanidinium thiocyanate method (Chirgwin et al., 1979). The filter was hybridised with the *tcen49* cDNA probe.

Whole-mount planarians were hybridised as described by Bueno et al. (1997a), using a 348 bp long-*tcen49* digoxygenin (DIG) labelled antisense riboprobe. Images were obtained by using a Zeiss Stemi SV 6 dissecting stereomicroscope (dark field) connected by a Sony video camera to a Macintosh Centris computer running Adobe Photoshop software. Cryosections were obtained in a *Clinicut Bright* cryostat and were hybridised at 60°C with the DIG labelled riboprobe, following standard procedures. The hybridisation was detected by using an alkaline phosphatase anti-DIG antibody and was developed with NBT/BCIP (Boheringer Manheim).

Immunostained paraffin sections were obtained as described in Bueno et al. (1996), using the Avidin-Biotin Complex method (ABC, peroxidase conjugated, Vector) to detect the primary antibody (TCEN-49).

RESULTS

Nucleotide and amino acid sequences

The *tcen49* genomic sequence was organised into two putative exons (9 and 201 nucleotides in length) and one intron (53 nucleotides in length), located at positions 504-556 (Figs 1A and 1B). Its nucleotide sequence revealed a short open reading frame encoding a deduced protein of 70 amino acids. This sequence revealed no significant similarity to any other reported protein. The VON HEIJNE method (1983) suggested a cleavage site between Ser 20 and Leu 21 (Figs 1A and 1B). The predicted mature polypeptide would have a *M*r of 5.3 kDa, in agreement with Western-blot analysis of the native secreted protein (BUENO et al., 1996). The distribution of the cysteine residues followed the pattern C-X(6)-C-X(3)-C-X(6)-C-X(3)-C-X(6)-C, where C is cysteine and X is any other amino acid, as for some members of the neurotrophic family (Fig. 1C). Moreover, these cysteines formed three disulphide bonds, as detected by cysteine methylation and mass spectrometry (work in progress).

To elucidate the genomic organisation of *tcen49,* we performed a Southern blot hybridisation analysis. As shown in Fig. 2A, three and five hybridisation bands were detected in the *Eco*RI and the *Hin*dIII digestions respectively. These results could be due to the polymorphism found in natural populations (Carranza, 1997), although we cannot discard the possibility that *tcen49* is present in

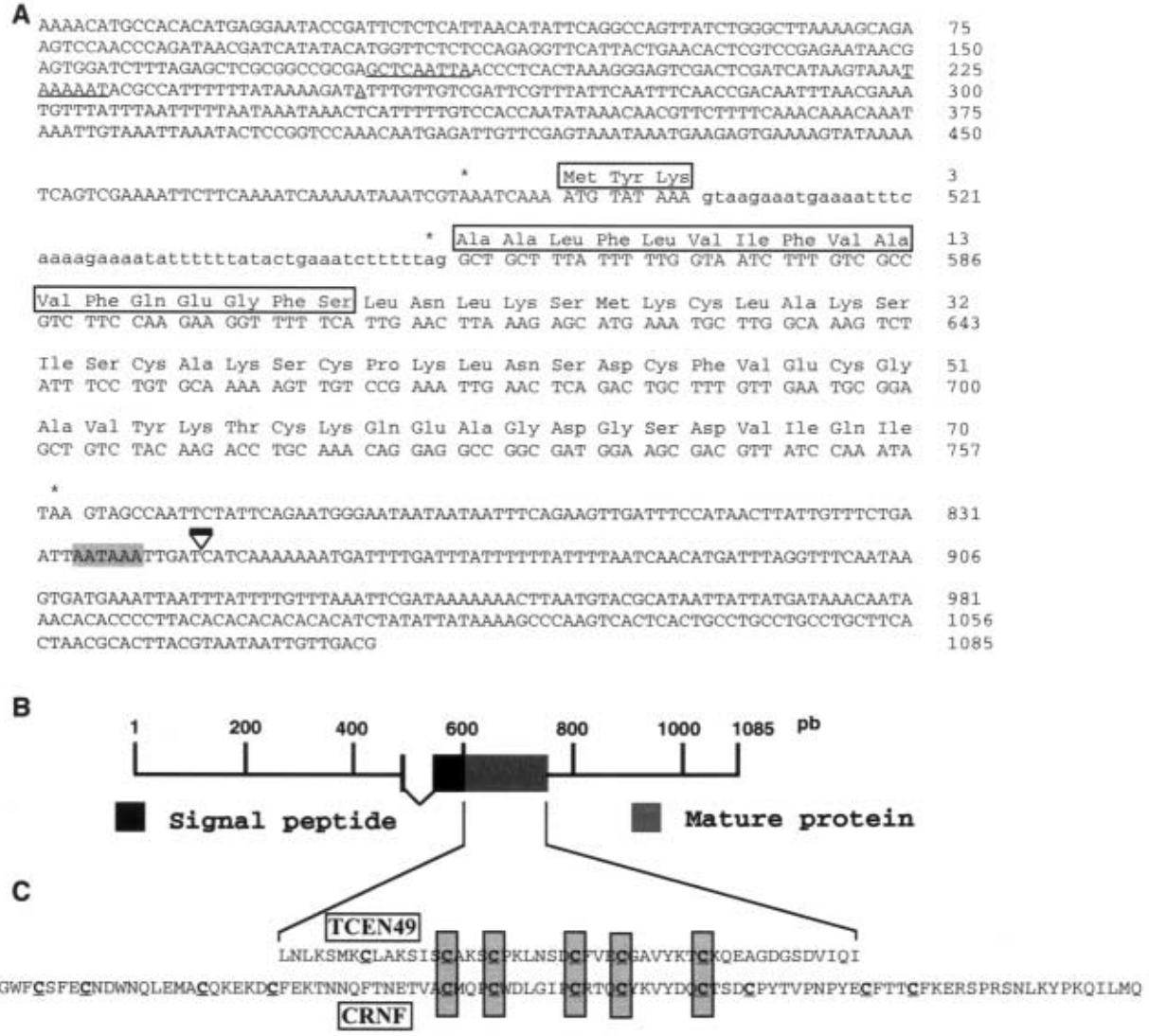

Fig. 1. – (A) DNA and deduced protein sequences of *tcen49*. The predicted signal peptide is shown in a box. Putative CAAT box, TATA box and transcription start site are underlined. A shaded box indicates the poly(A) signal sequence. The nucleotide and protein sequences are available in the GenBank Nucleotide Sequence Databases under the access number AF006956. (B) Diagrammatic structure of *tcen49*. The signal peptide is represented by black boxes; the mature protein by a grey box. Numbers at the top refer to the starting nucleotide of the sequence. (C) Comparison between TCEN49 and CRNF cysteine distribution.

more than one copy in the genome of *G.tigrina*. On Northern blots, the initially isolated 315 bp long cDNA detected a unique species of *tcen49* mRNA of approximately 550 nucleotides (Fig. 2B), which is consistent with the predicted size of the transcript (Fig. 1A).

To confirm that the sequence obtained corresponds to the planarian antigen recognised by the MAb TCEN-49, we performed a Western blot analysis of the fusion peptide generated from the *tcen49* cDNA with the MAb TCEN-49. As shown in Fig. 2C, TCEN-49 was able to recognise both the TCEN49 fusion protein and the thrombin-cleaved TCEN49 mature protein. We also produced a polyclonal antibody to the thrombin-cleaved TCEN49 mature protein, which exhibited the same pattern of immunostaining as the MAb TCEN-49 (Fig. 2D). From these experiments we can conclude that the *tcen49* gene of *G.tigrina* encodes the epitope recognised by the MAb TCEN-49.

Expression of *tcen49* and location of TCEN49

The results described below were obtained by analysing at least 10 organisms at each stage.

Intact adult planarians

tcen49 transcripts were detected in cells located within the parenchyma of the central-body region (Fig. 3A), except within the pharynx; no signal was observed in anterior and posterior regions, or using the *tcen49* sense riboprobe (negative control; Fig. 3B). In order to identify more accurately which cells express *tcen49* we performed RNA *in situ* hybridisation on planarian cryosections. *tcen49* expression was detected exclusively in cyanophilic-secretory cells of the central region of the body (Figs 3C and 3D). The area of immunochemical

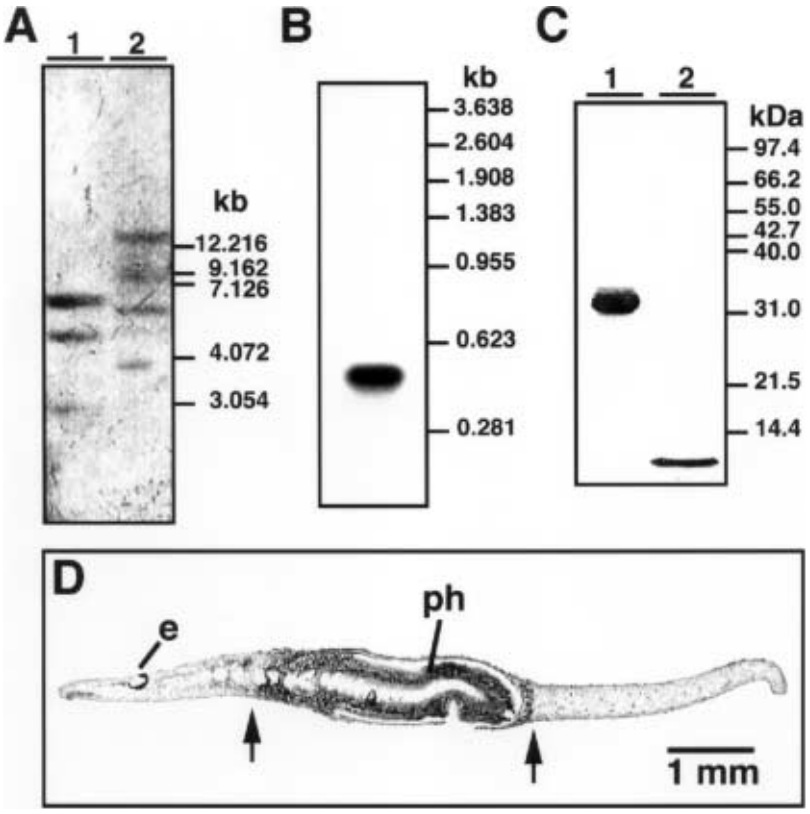

Fig. 2. – (A) Southern blot analysis of *tcen49*. Lane 1: EcoRI digestion; lane 2 HindIII digestion. (B) Northern blot analysis of *tcen49* mRNA. (C) Western blot immunodetection of the *in vivo* synthesised TCEN49 mature protein detected with the MAb TCEN-49. Lane 1: GST-TCEN49 fusion protein; lane 2: TCEN49 mature protein after thrombin digestion. (D) Sagittal section immunostained with the polyclonal antibody generated against the thrombin-digested GST-TCEN49 protein. Anterior is to the left, and dorsal to the top. Arrows indicate TCEN49 location boundaries. Abbreviations: e, eye; ph, pharynx.

location of the TCEN49 protein in intact adult organisms coincides with the area where *tcen49*-expressing cells are distributed and has been described elsewhere (Bueno et al., 1996; see Fig. 4 for a summary).

Regenerating organisms

During posterior regeneration in organisms cut at level A (levels of cutting are shown in Figs 3 & 4), the first hybridisation signal was observed at 6-7 days of regeneration. The signal was restricted to a condensed group of cells within the parenchyma, localised in the postblastema, in the area where the pharynx would form (Fig. 3F). The number of cells expressing *tcen49* mRNA increased as regeneration proceeded (not shown), forming a new central region with a new pharynx within it (the pharynx bud was detected at 7-8 days of regeneration). In organisms cut at level C (not shown in Fig. 3, but see Fig. 4 for a summarising scheme), *tcen49*-expressing cells were restricted to a group of cells in the postblastema as early as day 1 of regeneration, and were not detected in the growing blastema. From day 3-4, the number of *tcen49*-expressing cells increased, reestablishing a new central region.

During anterior regeneration in planarians cut at level C (Figs 3G, 3H and 3I), level D (Fig. 3J, 3K and 3L), and level E (not shown in Fig. 3, but see Fig. 4 for a summary), we detected *tcen49*-expressing cells as early as day 1 of regeneration throughout the regenerate, within and outside the region that usually expresses this gene in adult organisms (Figs 3G and 3J). From day 2-3 of regeneration, cells expressing *tcen49* became restricted again in the area where the pharynx was present (level C; Fig. 3H), or in the area where the pharynx would form (levels D and E; Fig. 3K) prior to any morphological evidence of its formation. *tcen49* expression was never detected within the blastema. From day 3, the number of positive cells increased and were sited exclusively around the old (level C; Fig. 3I) or new (level D and E; Fig. 3L) pharynx. Subsequently, each of these regenerates restored the adult proportions (Fig. 4) through an epimorphic-morphallactic process (Saló & Baguñà, 1984).

The area of immunochemical location of the TCEN49 protein coincides with the area of *tcen49*-expressing cells except during the transient stage of generalised transcription in anterior regenerates, and has been described elsewhere (Bueno et al., 1996; see Fig. 4 for a summary).

DISCUSSION

TCEN49, a molecule with no similarity to any reported protein

The data presented in this paper reveal that TCEN49 shows no significant similarity to any other known protein. However, the cysteine residues (see Fig. 1C) are distributed in a pattern also found in some neurotrophins, i.e. CRNF (cystein-rich neurotrophic factor) from the mollusc *Lymnaea stagnalis* (Fainzilber et al., 1996). Molecules belonging to this family have very diverse sequences but very similar structures, including a cystine-knot bonding in which these cysteines form three disulphide bonds. It is important to note that the cysteines from TCEN49 form three disulphide bonds, which is consistent with a cystine-knot bonding for TCEN49. TCEN49 crystallographic analysis (work in progress) could well confirm the extent of this.

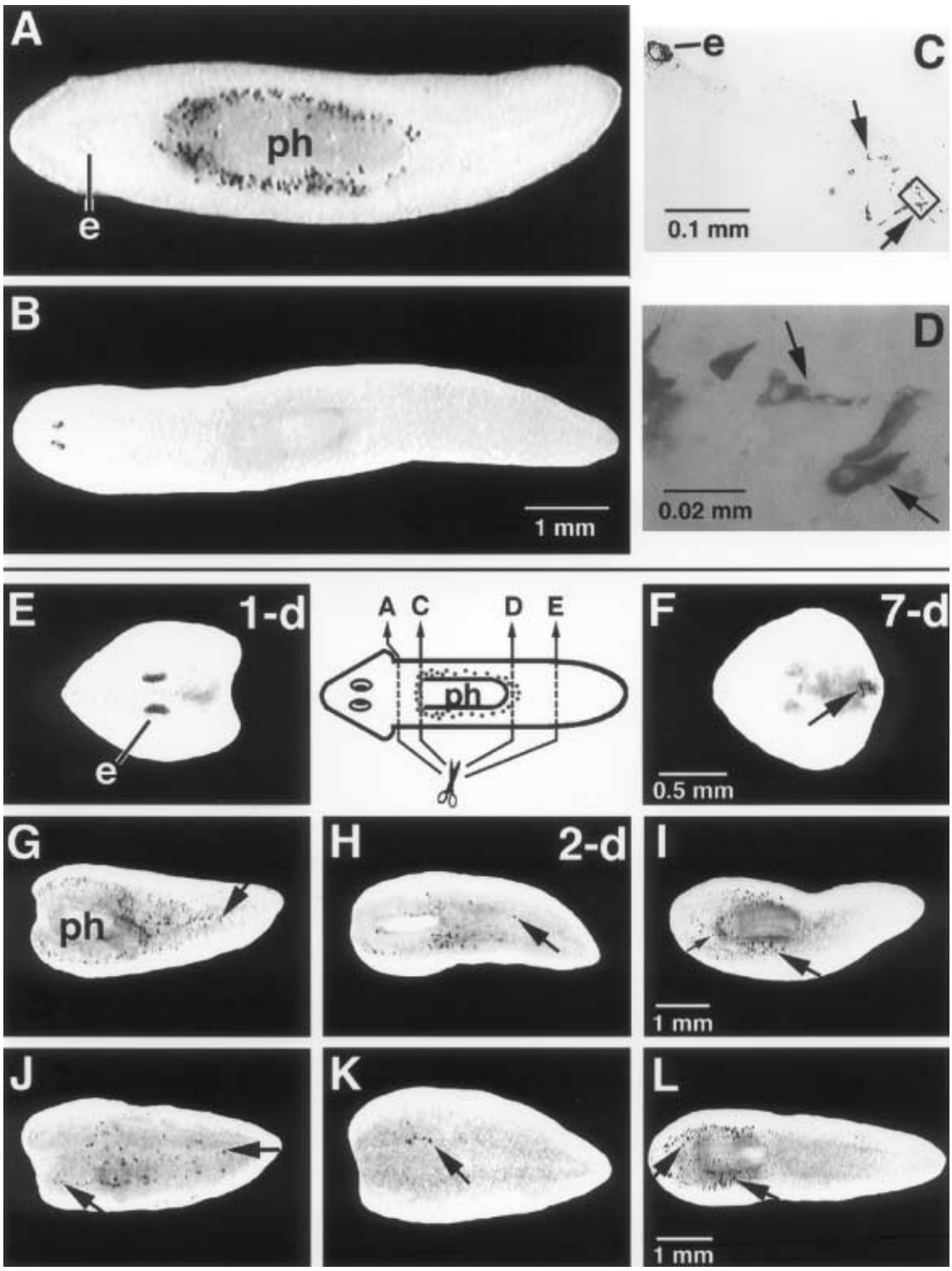

Fig. 3. – Expression of *tcen49* in intact adult planarians (A-D) and in regenerating organisms (E-L). Anterior is to the left. The ventral surface is shown (A, B). Whole-mount RNA in situ hybridisation with *tcen49* antisense riboprobe (A) or *tcen49* sense riboprobe (negative control; B). (C, D) Sagittal cryosections of intact adult planarians hybridised with *tcen49* antisense riboprobe. Arrows indicate some *tcen49*-expressing cells. (D) is the enlargement of the boxed region in (C). (E, F) organisms cut at level A; (G, H, I) organisms cut at level C; (J, K, L) organisms cut at level D. Days of regeneration indicated in the top-right corner apply for each column. Arrows indicate some of the parenchyma cells expressing *tcen49* mRNA. The diagrammatic scheme between (E) and (F) indicates the levels of regeneration analysed in this study. Spots indicate *tcen49*-expressing cells in an intact adult organism (compare with picture A). Abbreviations as in Fig. 2.

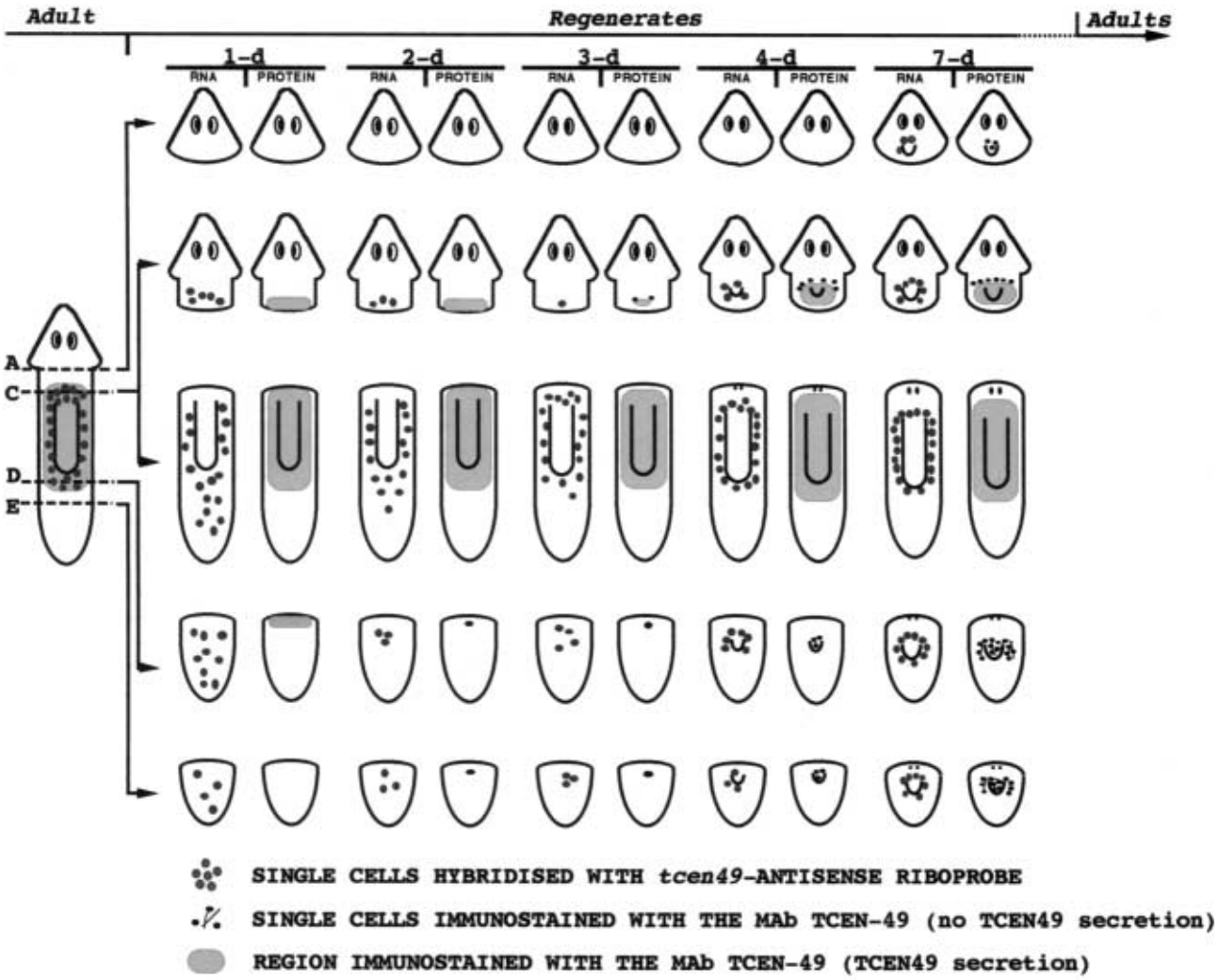

Fig. 4. – Summarising scheme of *tcen49* expression and TCEN49 localisation in adult and regenerating organisms.

tcen49 is differentially expressed in anterior *versus* posterior regeneration

Changes in *tcen49* mRNA expression in regenerates parallel changes reported for TCEN49 protein location (Bueno et al., 1996; see Fig. 4 for a summary) for both timing and dynamics. There is one interesting exception: during anterior regeneration in planarians cut at level C, D and E, *tcen49*-expression is transiently detected at day 1 of regeneration all along the regenerates. This contrasts with TCEN49 protein location at the same stages, which is never located outside the former central region or the new central-forming region. It also contrasts with posterior regenerates (levels A and C), which never undergo this stage of generalised *tcen49* mRNA expression. These results indicate that (1) *tcen49* behaves differentially in anterior *versus* posterior regeneration, and (2) during anterior regeneration there is a post-transcriptional regulation of *tcen49*.

Taken together, the results on *tcen49* expression lead to the clear differentiation of three molecular regions in planarians: (1) the anterior region, where *tcen49*/TCEN49 are never detected; (2) the central region, where *tcen49*/TCEN49 are always detected; and (3) the posterior region, where *tcen49*/TCEN49 are detected depending on the physiological conditions of the organism.

As the class II cyanophilic-secretory cells (the only cells that express *tcen49*) are present throughout the organism, several explanations may account for the head/tail differences in *tcen49* expression. (1) Differences in cell composition. Only a subtype of these cells is able to express *tcen49*, and it is present only in the central and posterior regions. This alternative implies the inhibition of *tcen49* expression in the posterior region of the adult. (2) No differences in cell composition. All class II cyanophilic-secretory cells are able to express *tcen49*, but *tcen49* expression is inhibited in adult organisms in the head and in the tail. This alternative implies a differential release of the inhibitory condition in the head from in the tail.

For both alternatives, generalised expression of untranslated *tcen49* mRNA at day 1 of anterior regeneration (lev-

els C, D and E) may be due to initial induction mechanisms initiated in response to the traumatic cutting (for general reviews on induction mechanisms, see BAGUÑÀ et al., 1990, and BAGUÑÀ, 1998), transiently releasing the inhibition of *tcen49* expression in the posterior region. Then, from day 2, *tcen49* expression would be inhibited in the newly forming anterior and posterior regions.

TCEN49 may be involved in A/P body region maintenance

It has been reported that the structures to be formed by the regenerative blastema and the postblastema close to the blastema are determined at 3-24 hours of regeneration, and that the determination of pharyngeal structures (such as the structure representing the central region) occurs at 12-36 hours of regeneration (for a general review, see BAGUÑÀ et al., 1994). Although the first TCEN49 location during regeneration occurs in the areas where the new central region will form between day 1 and 2 of regeneration before any morphological evidence of pharyngeal structures, it is not secreted until day 7-9 of regeneration. For this reason it may not function in the central region until the pharynx is completely regenerated and begins its maturation to become functional (BUENO et al., 1997b). All these data suggest that TCEN49 is not necessary for the initiation of regeneration, but rather is involved in the maturation and maintenance of this region. This is supported by preliminary RNA inhibition (RNAi) experiments performed on regenerating tails (level E) by injecting *tcen49* dsRNA, in which the organisms regenerate a new central region with a complete pharynx, but lyse at 9-12 days of regeneration.

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