

REGIONALISATION ALONG THE ANTEROPOSTERIOR AXIS OF THE FRESHWATER PLANARIAN
Dugesia(Girardia)tigrina BY TCEN49 PROTEIN

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Freshwater planarians (phylum Platyhelminthes) are unsegmented and bilaterally symmetrical organisms which branched from the rest of metazoans at an early phylogenetic stage. These simple triploblastic organism have been used as a model system to study pattern formation mainly due to their simple structure, biological plasticity and capability of regeneration. They can grow or degrow continuously depending on food availability and temperature (Romero, 1987) showing a continuous state of cell renewal from small, undifferentiated, multipotent, self-renewing stem-cells called neoblasts. The neoblast give rise to 12-15 non-proliferating differentiated cell types that are continuously replaced during the life-time of the animal. In addition, a whole organism can be regenerated from a small, excised piece of the body. During these events, planarians maintain and re-establish the original polarity of the body.

Although planarian regeneration is fairly well understood at the tissue and cell levels, few is known about the "factors" which are needed to define and maintain positional value along the anteroposterior axis. Here, we have undertaken the study of *Dugesia(G.)tigrina* regeneration through the search for genes involved in regulation of the anteroposterior body axis. To obtain molecular markers relevant to these phenomena in planarians, a hybridoma library producing cell-, tissue- and position-specific monoclonal antibodies against *D. tigrina* was generated (Bueno *et al*, 1996b). One of the position-specific monoclonal antibodies, TCEN49, recognizes a ~5 kDa glycoprotein present in almost all cell types within the central body region, including the pharynx (Fig. 1) (Bueno *et al*, 1996a). The boundaries between labelled and unlabelled regions are very sharp and labelled cells are not related by lineage but by position. Closer examination of the stained region revealed that cells of mesodermal (mesenchyme, secretory, nerve and muscle) and endodermal (digestive and globet) origins were labelled, while epidermal cells and some cells at the lateral edge of the mesenchyme were unlabelled. Semithin and ultrathin immunogold sections have shown that TCEN49 antigen (TCEN49Ag) is strongly located within the large secretory granules of the cyanophilic secretory cells (class II, Bagnà, 1973). In cells other than cyanophilic secretory cells, labelling is found within heterochromatin blocks close to the nuclear membrane, in non-electrondense cytoplasmic vesicles, and in the cellular membrane (Bueno *et al*, 1996a). The TCEN49Ag expression domain is not correlated with any morphological structure, but serves to clearly define the central, as opposed to anterior or posterior, body region, suggesting that TCEN49 protein is involved somehow in the regionalization along the anteroposterior body axis. In regenerating planarians the TCEN49Ag expression domain is dynamic, preceding the formation of a new central body region, suggesting that it could be involved in the expression of central-body positional identity (Bueno *et al*, 1996a). Thus, TCEN49 protein seems to play a key role during regionalisation and regeneration in *D. tigrina*.

With the aim of identifying sequences encoding the TCEN49Ag, we have used the TCEN49 monoclonal antibody to screen a *D. tigrina* expression cDNA library constructed in Lambda ZAP. We isolated several positive clones from a screening of 3×10^6 phage clones. All of them contained the same DNA insert as shown by PCR analysis. After *in vivo* excision, a partial TCEN49 cDNA of 315 bp containing an open reading frame of 67-amino acids with an N-terminal signal sequence typical of secreted proteins was sequenced. The finding of this signal sequence is in agreement with previous immunolocalization studies and supports the idea that TCEN49Ag is a secreted glycoprotein (Bueno *et al*, 1996a). In order to analyze the genomic organization of TCEN49 gene we performed Southern Blot hybridization using a ^{32}P -labelled synthetic oligodeoxynucleotide probe made according to the nucleotide sequence predicted from the TCEN49 partial cDNA clone. Under conditions of high stringency, the TCEN49 cDNA hybridizes to four *Eco* RI fragments and five *Hind* III fragments of *D. tigrina* genomic DNA. These results permit us to speculate that this flatworm might have *tcen49*-like genes and/or more than one copy of *tcen49* gene. On the other hand, the bands observed could also be considered as the result of genome partial digestion and/or polymorphic sites. Using the same conditions of hybridization at high stringency and the same cDNA probe, TCEN-specific clones were isolated from a *D. tigrina* genomic library. All of them contained the same DNA insert as shown by restriction analysis. This insert contained a non-continuous open reading frame which included the complete sequence of TCEN49 protein (Fig. 2). The *tcen49* gene encodes a 70 amino acid protein whose coding sequence is interrupted by a 52 bp intron. The predicted mature TCEN49 protein is also in agreement with the apparent relative molecular mass of TCEN49Ag shown by previous western immunoblot studies (Bueno *et al*, 1996a). From sequence data, it is worth mentioning that TCEN49 protein does not show strong similarity to any described protein, but has the same interesting aspects. First, TCEN49 protein shows similarity to a region of the CRUMBS protein of *Drosophila melanogaster*. CRUMBS is a developmentally regulated protein that exhibits a great similarity to Epidermal Growth Factor-like proteins (EGF-like proteins) and participates in the process of establishing and/or maintaining epithelial cell polarity (Tepass *et al*, 1990). CRUMBS is characterized by the presence of thirty repeat units, called EGF-like repeats, each with similarity to EGF. It has been postulated that one or several of these EGF-like repeats, are clipped off and exert their function(s) as diffusible molecules (in a manner similar to EGF itself). TCEN49 protein shows similarity (66%) to one of these EGF-like repeats of the CRUMBS. Second, TCEN49 sequence contains cysteine residues arranged in a manner similar to that commonly seen in members of the Epidermal Growth Factor Receptor family (EGFRs) (Katz *et al*, 1996).

Our findings clearly indicate that TCEN49 encodes for a small secreted protein (~5 kDa), as suggested by previous work (Bueno *et al*, 1996a), with no known counterpart among those described previously in vertebrates and invertebrates. However, sequence comparisons show a poor similarity between TCEN49 protein and those involved in different developmental processes like cell proliferation and determination (CRUMBS and EGFRs). TCEN49 protein is the first region-specific marker identified in *D. tigrina*. The cloning of its regulatory sequences as well as the study of its dynamic expression during embryogenesis in sexual races may give us insight into the mechanisms of establishment and/or maintenance of the regionalisation along the anteroposterior axis. It remains to be seen the biological effect of TCEN49 protein, promoting central-region formation or causing, by its absence, head and/or tail region formation.

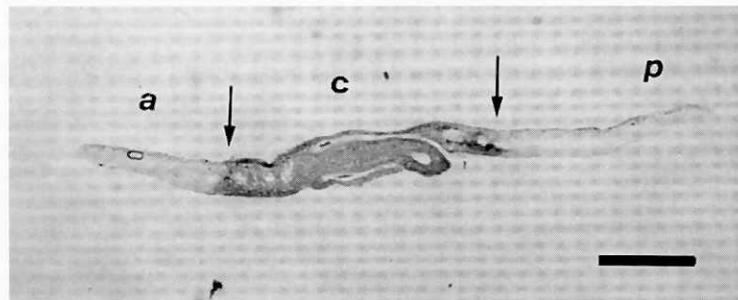


Figure 1: *D. tigrina* immunostained with the monoclonal antibody TCEN49. Saggital section. ABC method. a, anterior region; c, central region; p, posterior region; arrows, boundaries between regions. Scale bar= 1 mm.

Met	Tyr	Lys	Ala	Ala	Leu	Phe	Leu	Val	Ile	10
Phe	Val	Ala	Val	Phe	Gln	Glu	Gly	Phe	Ser	20
Leu	Asn	Leu	Lys	Ser	Met	Lys	Cys	Leu	Ala	30
Lys	Ser	Ile	Ser	Cys	Ala	Lys	Ser	Cys	Pro	40
Lys	Leu	Asn	Ser	Asp	Cys	Phe	Val	Glu	Cys	50
Gly	Ala	Val	Tyr	Lys	Thr	Cys	Lys	Gln	Glu	60
Ala	Gly	Asp	Val	Ser	Asp	Val	Ile	Gln	Ile	70

Figure 2: Complete amino acid sequence of *D. tigrina* TCEN49 protein. Amino acids (in one-letter code and in bold) are numbered at right. In the amino-terminal region the protein has a stretch of 13-20 amino acids which are rather hydrophobic and closely resemble a signal peptide. Val-14, Phe-19 and Leu-21 represent possible candidates for the amino-terminal residue of TCEN49 protein.

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