MYOSIN HEAVY CHAIN GENE IN *DUGESIA (G.) TIGRINA*: A TOOL FOR STUDYING MUSCLE REGENERATION IN PLANARIANS

Francesc CEBRIÀ, Marcelo VISPO, David BUENO, Salvador CARRANZA, Phillip NEWMARK and Rafael ROMERO
Dept. Genètica. Fac.Biologia. U.Barcelona. Avda. Diagonal, 645, 08071 Barcelona, Catalonia (Spain)

In most organisms the main body axes are specified during embryonic development. However, in freshwater planarian *Dugesia (G.) tigrina* (Platyhelminthes, Turbellaria) the body axes must be continuously re-specified in the adult period (Bueno *et al.*, 1996a) due to the great morphological plasticity shown by these organisms. Planarians can grow or degrow in a continuous manner depending on food availability and temperature, and they can regenerate a new organism from a small piece of its body (Romero & Baguñà, 1988). During regeneration three basic processes must occur: a) a redefinition of the body pattern to establish the territories where the missing structures will appear; b) stem cells called neoblasts (Brøndsted, 1969) proliferate and differentiate into all the cellular types necessary to form the missing structures; and c) these "de novo" cells become properly arranged in time and space to restitute the original body pattern. One possible way to study how these processes take place is to consider the differentiation and restitution of the pattern of individual tissues and cell types and to examine possible relationships with the general mechanisms of regeneration.

One of the planarian cell types of particular interest is the muscle cells. These cells respond early in regeneration by closing the wound (contracting the body wall) in the zone where the regeneration blastema will appear, and together constitute what may be considered the skeleton that holds together all the other cell types. The complex and highly organized muscle net is arranged in several subepidermal layers of muscle fibers (Baguñà, 1973) and may be distinguished using fluorescein-labeled phalloidin (a phallotoxin that binds specifically to F-actin; Wulf, 1979) as a marker and visualization with conventional epifluorescence or confocal microscopy. There is an outer layer of circular fibers that runs below the basal lamina and over an intermediate layer of diagonal fibers. Below these diagonal fibers one may observe the longitudinal fibers. This pattern of fibers from the outer surface to the inside of the body is observed in both dorsal and ventral surfaces. These surfaces are themselves connected through dorsoventral fibers that show their highest densities in the head, tail and lateral regions of the body. Careful examination of dorsal longitudinal fibers shows that they seem to converge upon a zone near the anterior border of the organism. In contrast, ventral longitudinal fibers diverge in a fan-shaped pattern as they approach the anterior border.

During the regeneration process a regeneration blastema is formed in the wound region. Within this blastema, the new structures, including the new muscle fibers, will appear. Using fluorescein-labeled phalloidin one may distinguish the disorganized and thin fibers within the 3 day regeneration blastema. After 3 days of regeneration the muscle fibers become organized and by the fourth day of regeneration the re-establishment of the original muscle pattern can be observed. At 6 days of regeneration the muscle pattern is completely restored with the number of fibers increasing progressively in each of the following days. However, because phalloidin binds to differentiated muscle cells, we are unable to study the origin of these cells within the regeneration blastema.

To gain insight into the differentiation of the muscle cells within the blastema, we have used a monoclonal antibody (MAb) called TMUS-13 that immunoreacts not only with all the muscle fibers (fig.1) but also with cells that appear to be neoblasts (myoblasts) committed to a differentiation pathway towards muscle cell identity (Bueno *et al.*, 1996b). To characterise at the molecular level the antigen recognised by this MAb, a cDNA expression library constructed in Lambda Zap vector was screened with TMUS-13 MAb using standard procedures. We isolated several positive clones that, by PCR and digestion analysis, seemed to contain an equally sized cDNA insert. Sequence analysis (BLAST WWW server) demonstrates that the TMUS-13 antigen is a myosin II heavy chain (MHC) gene (fig.2), the first identified from planarians. This MHC shows a high degree of sequence similarity with the MHC genes from *Schistosoma mansoni* (Platyhelminthes, Trematoda) and other species throughout phylogeny, from *C.elegans* to human. Myosin II is a hexameric protein with two heavy and four light chains. Each heavy chain has an amino-terminal domain that folds into a globular head. The remainder of the myosin II heavy chain dimerizes to form an alpha-helical coiled coil tail (Kiehart, 1990). The isolated cDNA fragment corresponds to this last domain. To isolate the *tmus-13* gene, we have used the 1293 bp long cDNA to screen a *D.(G.)tigrina* genomic library and we are in the process of sequencing the complete gene.

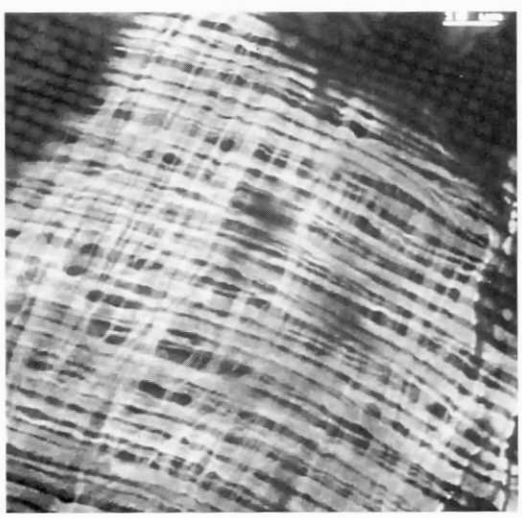

Figure 1. Muscle net of the body wall of *Dugesia (G) tigrina*. immunostained with TMUS-13. Note the circular (from left to right), longitudinal (from up to down) and diagonal muscle fibers.

By double labelling with phalloidin and TMUS-13 it should be possible to decide if all the muscle cells that appear in the blastema are of "new" origin or if they are a mixture of "old" fibers (the ones that contract the body wall during wound closure) and "new" fibers. Another question about the restitution of the musculature concerns the direction of differentiation of the muscle fibers within the blastema: is this differentiation a proximo-distal or disto-proximal event? The results obtained with phalloidin seem to support the idea that differentiation proceeds in a proximo-distal manner. At present, we are trying to answer these two questions from the study of regeneration with TMUS-13. From an evolutionary perspective, the study of muscle cells in *D. (G) tigrina* may be interesting because of the unstriated properties of the body wall and pharynx musculature in Platyhelminthes (found only in Platyhelminthes and Porifera). Ultrastructural examination led Sarnat (1984) to conclude that the muscle cells of *D. (G.) tigrina* appear more similar to striated than to smooth muscle in the organization of myofilaments and the presence of non-aligned fragmented Z-band material. On the other hand, physiological studies suggest a closer affinity to smooth than to striated muscle. These results are consistent with the idea that planarian musculature is composed of unspecialized myocytes with properties both of smooth and striated muscle cells.

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D.tigrina  KNFYRLGNTKIFFKGGTLADLEDMRDEKLSSLISLFQAQIRGYLMSFQYK 50
S.mansoni  **L *QC****V***A****H***L**D**NGI******E******RK***

D.tigrina  KLQDQRVALSIIQRNIRKYLXLRTWPWWKLYTKVKPLLNIARQEEEMKKA 100
S.mansoni  *********TLM********V**N****R*******M***********

D.tigrina  AEELAKLKEEFEKCEKFKKELEEQNVKLLEAKNDLFXQLQTEQDSLADAE 150
S.mansoni  **********Y**L**L*********TV*QQ*****L***********

D.tigrina  EKVSKLVLQKADMESRIKELEDHLLEEEDASAGLEETKKKMQGEIEELKK 200
S.mansoni  **I******RG***Q******ER*AD***QA*N*T*V****SA*******

D.tigrina  DVXDLESSLQKAEQDKAAKDQQIKSLQDQMARQEEEMNKMKKEKKAADEL 250
S.mansoni  **E**********E*QT**N**RT**SE**Q*D*MIG*LN*D**NLE*Q

D.tigrina  QKKTEESLLAEEEKVKNLNKVXAKLEQTIDEMEENLXREQKIRADVEKVK 300
S.mansoni  N*R*Q*A*Q***D**NH***LK****S*L******A******G****S*

D.tigrina  RKIEGELKQTPETVDDLERVKRELEEQLKRKEMELSSASSKIEDESGLVA 350
S.mansoni  **L**D**A*Q***************R***A*IGGL*G*F***Q****

D.tigrina  QLQKKIRELQARIQELEEDLEAERQARSKAEKSRHQLEAELEELSDRLEE 400
S.mansoni  ***R**K***T***********A*********Q***S****VV*****

D.tigrina  QGGATSAQMGLNKKREAELIELKRDLEEANVQHDQSVAQARKKQQDAVNE 450
S.mansoni  *D***A**SD*T*******MK******DTRL*NE*AI*TM****S**I**

D.tigrina  FSEQLDQLQKAKAKTEKEKNELKEDLNDFHGQLESLNKAKFNSDKRLKDL 500
S.mansoni  LAD****AN*****A***RSQF*AE*D*A*N*VD*IM***L**R*TV*A*

D.tigrina  EAQNSDLQGKLDETNRHLNDASNTSGKSQQLNAELQARLEEAEFQINQLN 550
S.mansoni  *S*LQEVSV****AT*N**EQAS*KAR*S*EVS***RQ*****S*LS***

D.tigrina  KVKQQMQAQLEEARQNLEDESRTKAKLSSDIRNLNADLDNFREALG     596
S.mansoni  *I***LS*******HS******M****NGEV***TS***SL**T*E
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Figure 2. Partial amino acid sequence of the MHC gene of *Dugesia (G.)tigrina* and *Schistosoma mansoni*. Asterisks denote identical amino acids. The percentage of amino acid identity is 68%.

Planarian muscle could resemble an ontogenetic stage preceding the divergence of muscle cells into striated and smooth cells within higher invertebrates and vertebrates. With the entire sequence of the MHC gene we will be able to compare this MHC gene with smooth and skeletal muscle myosins and see what kind of relationships àppear.

It would be also interesting to study the regulation of the expression of this MHC gene because it may provide insights into the mechanisms that control muscle differentiation and pattern restoration during regeneration. In the last years several transcription factors regulating muscle differentiation in higher invertebrates and vertebrates have been characterized. These transcription factors are divided into two groups: the MyoD family (myogenic bHLH factors) and the myocyte-specific enhancer factor 2 (MEF2) family (Olson et al. 1995). The members of the MEF2 family seem to regulate muscle gene expression in the three vertebrate myogenic lineages: smooth, skeletal and cardiac muscle. The members of the MyoD family seem to regulate only the expression of skeletal muscle genes. The relationships between members of both families are still not clear, although the analysis of some muscle-specific promoters suggests that both classes of transcription factors cooperate with one another to regulate muscle-specific gene expression in a combinatorial manner (Molkentin, 1996).

Finally we are also screening a cDNA expression library with the MAb TMUS-46, which recognizes only longitudinal muscle fibers. This MAb detects the first molecular difference among muscle fibers in planarians depending upon their position in the body wall. The characterization of the antigen recognized by TMUS-46 will allow us to begin a molecular dissection of the functional differences between different planarian muscle cells.

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