Short Review ISSN- 0102-9010

EXTRACELLULAR MATRIX IN FIN REGENERATION IN TELEOSTS

Manuel Marí-Beffa, Jesús Alberto Santamaría, Josefa Ruiz-Sánchez, Leonor Santos-Ruiz and José Becerra

Department of Cell Biology, Genetics and Physiology, Faculty of Sciences, University of Málaga, 29071-Málaga, Spain

ABSTRACT

Since 1982, our group has collaborated with Prof. Gregorio S. Montes in analyzing the composition and function of the extracellular matrix during fin regeneration in teleosts. The structure and ultrastructure of fully formed and regenerating fins of various teleostean species have been studied. The dermal skeleton of fins consists of rays formed by lepidotrichia and which is surrounded by loose connective tissue and a multistratified epidermis. Distally, there are hyperpolymerized macrofibrils of elastoidin, a collagen-like protein, named actinotrichia. Both lepidotrichia and actinotrichia are formed during regeneration. However, whereas the lepidotrichia form distally by the addition of new material, the actinotrichia are synthesized very early at proximal sites and then maintained by a continuous turnover at the distal margin of the regenerating ray blastema, as shown by radioactive pulse-chase experiments. Using the picrosirius-polarization method, as well as immunocytochemistry for various extracellular matrix components and enzymatic digestion, we have established correlations between the various extracellular matrix components; glycosaminoglycans and collagen in the mature adult structure. During regeneration and after wound healing a blastema is formed. Histological analysis of the extracellular matrix has indicated that the blastema consists of the blastema proper, which is rich in hyalunorate and the actinotrichial blastemic region, which is rich in glycosaminoglycan sulphates and collagen. The inhibition of collagen synthesis by several specific drugs during fin regeneration attenuated blastema formation, regenerative outgrowth and extracellular matrix formation. These findings suggest an interesting morphogenetic and regenerative function for collagen which could provide an interesting field for future research.

Key words: Collagen, extracellular matrix, fin, regeneration, teleosts

INTRODUCTION

The study of fish fin regeneration and development is an expanding field of research in developmental biology [1]. Modern techniques such as transplantation [17,18], gene over-expression [21,24], and the analysis of mutant genes [7,8,20] provide molecular tools for understanding this process better. The analysis of fin regeneration started in the early 1900s with Thomas Hunt Morgan, the father of *Drosophila melanogaster* (the fruit fly) genetics [12-15]. Thereafter the subject was almost forgotten, except for a few comparative and experimental analyses by Professor Jacqueline Géraudie in Paris [5,6]. Professors Montes and Junqueira revived the study of this subject, with two papers on the morphological and histochemical analyses of teleostean fins [3,11]. These studies, initiated in collaboration with our group in Málaga at the beginning of the 1980s, were the basis for our research during the next two decades. These two papers have since become classics in the field because of the very useful morphological foundations they have provided for the "explosion" in fin regeneration studies during the present decade. Prof. Gregorio S. Montes' stimulating hypothesis of fin regeneration as a model system for studying cartilage regeneration [11] provides an appropriate conceptual basis for comparative analysis of the tissue origin of dermal bone [16]. Prof. Montes also analyzed the function of the extracellular matrix (ECM) during fin regeneration, and was the first to propose the involvement of collagen in fin blastema formation and in fin morphogenesis during regeneration outgrowth [4].

In this paper, we will try to convey something of Prof. Montes' inquisitive spirit and will review his ideas and studies on fin morphology and regeneration in order to show how these have influenced current studies of fin regeneration as well as the careers of many scientists, including ourselves.

HISTOLOGICAL STRUCTURE OF THE FIN

Fins are appendages of the fishes that are crucial for swimming, but may be used in additional co-opted functions such as reproduction or defence [19]. The actinopterygii have ray-fins with basal endoskeletal bones and exoskeletal dermal bones that form the rays. The first systematic histological study of the structure

Correspondence to: Dr. José Becerra
Departamento de Biología Celular, Genética y Fisiología, Facultad de Ciencias, Universidad de Málaga, 29071 Málaga, Spain. Tel: 952131966, Fax: 952132000, E-mail: becerra@uma.es

of actinopterygian fins was done by Prof. Montes in collaboration with our group [3]. The basic method used was the innovative picrosirius-polarization technique which allowed the easy discrimination of collagen types using a simple staining protocol and provided information about fiber and fibril orientation. This staining protocol had been established by Prof. L. C. U. Junqueira before Prof. Montes joined his laboratory [9]. The various collagen-containing structures appear bright in different colours when stained with picrosirius and observed under polarized light. Prof. Montes used this procedure to study a variety of tissues, including the fins of eight species of teleosts. The three dimensional drawing which summarized the morphological features observed appeared in reference [3] (Fig.1) and was the results of various contributions by the authors, as understood and interpreted by Tigr Orlow, a Russian painter who collaborated with Prof. Junqueira. In this work, the specific orientation of collagen fibrils in the ray lepidotrichia matrix was described for the first time. In cross-section, the dermal ray is formed by a parenthesis-like structure of two apposed hemirays. Each hemiray consists of two bands, one external and

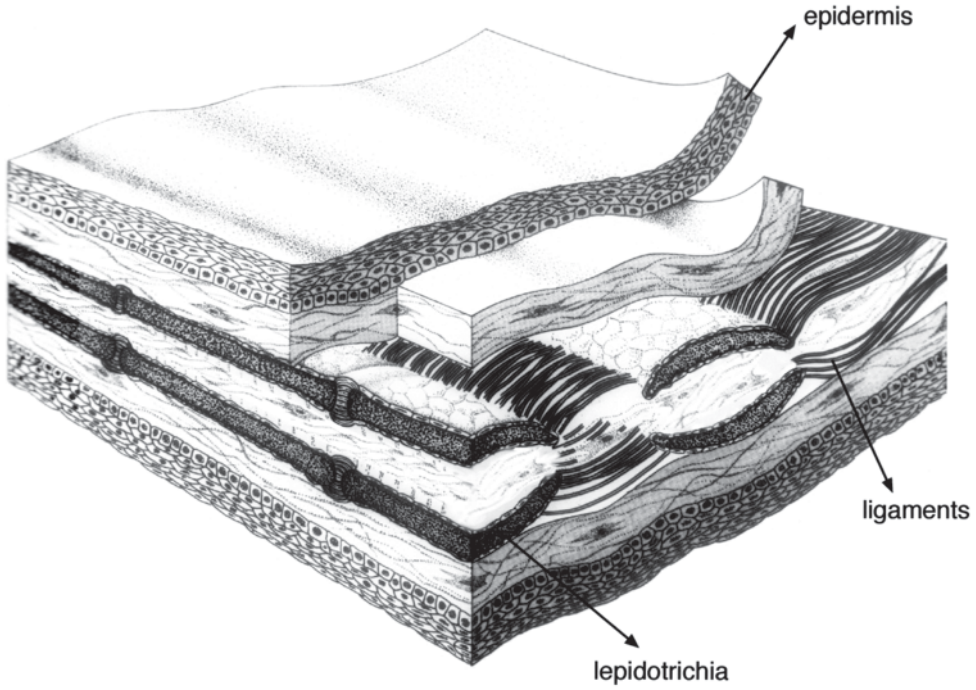
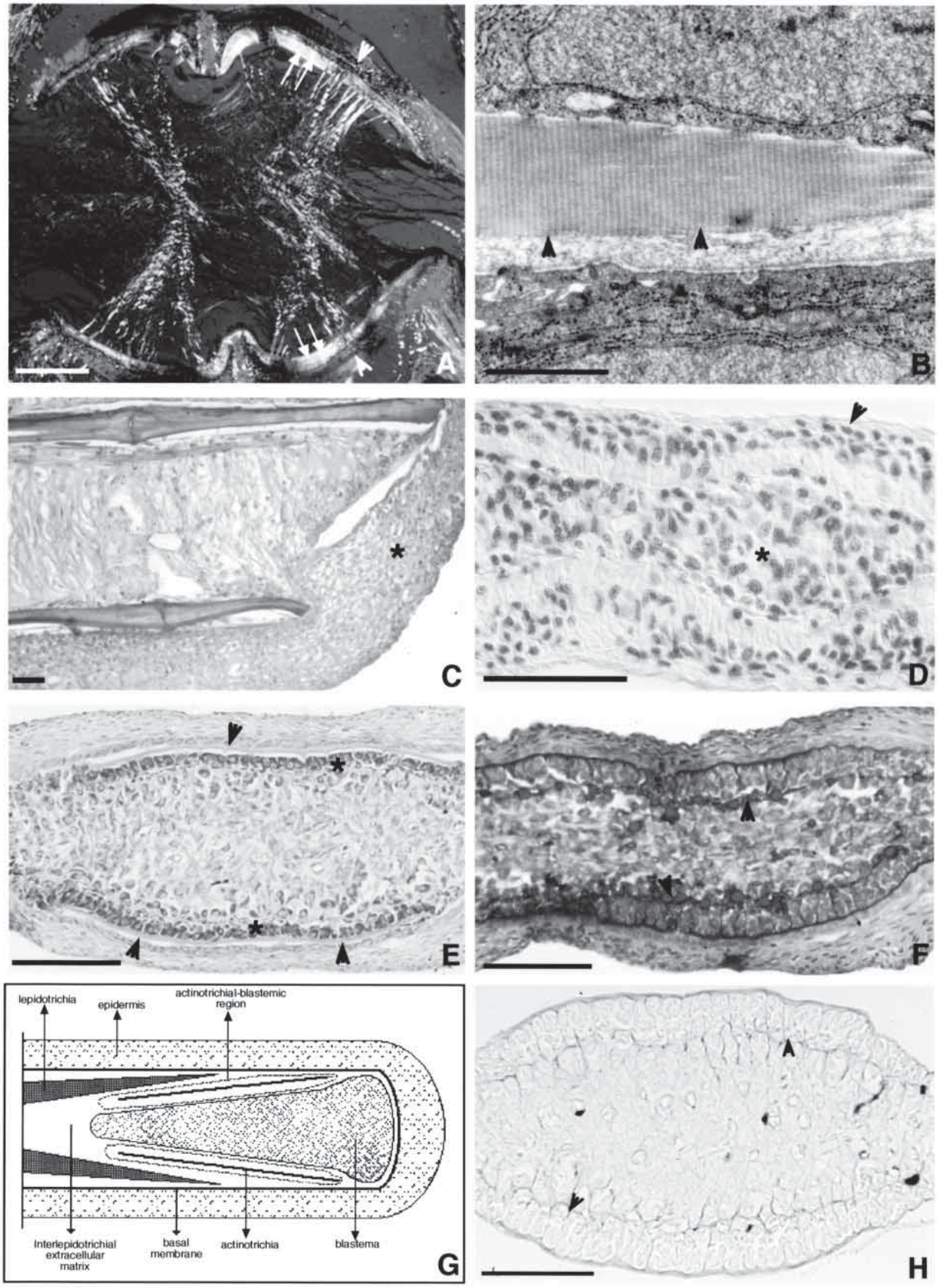


Figure 1. Schematic three-dimensional drawing of a section of a characteristic adult fin. Note that the two apposed, segmented hemirays are joined by ligaments. Each hemiray consists of skeletal material known as lepidotrichia. The whole structure is surrounded by a multilayered epidermis. Adapted from Becerra *et al.* (1983) [3].

Figure 2.A. Transversal section of a ray of *Geofagus* stained with Sirius Red. Note that the external (**arrowheads**) and internal (**double arrows**) regions of the lepidotrichia hemisegment show different collagen fibril orientation, based on the brightness observed under polarized light. Bar = 100 μm. **B**. Longitudinal ultrastructural section of the distal area of a regenerating ray with an actinotrichia (**arrowheads**). Note the transversal banded pattern characteristic of collagen fibrils. However, the width (5 μm) exceeds that of regular collagen fibrils (30 nm), suggesting a special type of hyperpolymerization. Bar = 1 μm. (Adapted from Becerra *et al.* (1996) [2]). **C**. Longitudinal section in the distal region of a regenerating ray from the tail fin of *Carassius auratus* (the goldfish), 24 h after ablation. Note that the wound has healed with a thick epithelium (**asterisk**). Bar = 50 μm. **D**. Transversal section of a regenerating ray from the tail fin of *Carassius auratus*, labeled with BrdU, 4 days after ablation. Note that both the blastema (asterisk) and the surrounding epidermis (**arrowhead**) are labeled. Bar = 100 μm. **E**. Transversal section of a regenerating ray of *Carassius auratus* stained with toluidine blue, 12 days after ablation. Note the new lepidotrichia being formed (**arrowheads**) by differentiating scleroblasts which adjoin the basal lamina (**asterisk**) on both sides of the ray blastema. Bar = 50 μm. **F**. Transversal section of a regenerating ray 12 days after ablation at a more distal region than in **E** and stained with Sirius Red and hematoxylin. Note that the actinotrichia now form a conspicuous line (**arrowheads**). Scleroblasts occupy the space between the actinotrichia and the incipient lepidotrichia. Bar = 50 μm. **G**. Scheme of a distal blastema in longitudinal section. Note the arrangement of the actinotrichial blastemic region and the blastema proper (blastema). The different histological regions are indicated in the figure. Adapted from Santamaría and Becerra (1991) [22]. **H**. Transvesal section of the distal blastema of a regenerating ray from a tail fin 12 d after ablation (staining with alcian blue). Note the positive reaction in the actinotrichial blastemic region (arrowheads). Bar = 50 μm.


A
B
C
D
E
F
lepidotrichia
epidermis
actinotrichial-blastemic region
Interlepidotrichial extracellular matrix
basal membrane
actinotrichia
blastema
G
H

one internal, with collagen fibrils arranged in different orientations. When observed under polarized light, these two regions appear bright at different angles of polarization (Fig. 2A). These lepidotrichia are surrounded by a loose connective tissue and joined by ligaments to contralateral hemiray lepidotrichia (Fig. 2A). The complete structure is surrounded by a multistratified epidermis.

Distal to the lepidotrichia there are two bundles of hyperpolymerized fibrils of elastoidin, a collagen-like protein. Each actinotrichia, as each macrofibril is known, has characteristic collagen-like transversal bands when observed in transmission electron microscopy (Fig. 2B, adapted from Becerra *et al.* (1996) [2]).

EXPERIMENTAL ANALYSIS OF FIN REGENERATION

Wound closure occurs within 24 h after fin ablation (Fig. 2C). Cell dedifferentiation and migration from the stump to distal regions is initiated and a proliferating blastema is formed, as indicated by the incorporation of the marker bromodeoxyuridine (BrdU) during the S phase of cell proliferation (Fig. 2D). The blastema is formed of undifferentiated cells which proliferate to restore the missing part of the fin [23]. The blastemic cells adjoining the basal lamina in proximal blastemic regions differentiate into scleroblasts or lepidotrichia-forming cells [2] (Fig. 2E). These scleroblasts form new lepidotrichia from the old stump. Five days after ablation and after the new lepidotrichia has been initiated, two lateral palisades of actinotrichia are formed (Fig. 2F). Pulse-chase experiments with radioactive tracers have indicated a dynamic turnover in the maintenance of the actinotrichia during fin regeneration after ablation [10]. This cellular process seems to occur after epimorphic regeneration [13] in which cell proliferation (Fig. 2D) is crucial for restoration of the ablated structure.

EXTRACELLULAR MATRIX (ECM) COMPOSITION OF THE RAY BLASTEMA

During fin regeneration, an ECM is formed that serves as an inducer of morphogenetic processes and as a structural scaffold for newly formed scleroblasts. The biochemical composition of this ECM has been analyzed using histochemical techniques and partial enzymatic digestions [22], the latter technique having been well developed and extensively used by Prof. G. S. Montes throughout his career. Hyaluronate was found to be an important component of the blastemic ECM whereas sulphated glycosaminoglycans (GAGs) were the main components of the actinotrichial blastemic region and of the ECM interface joining the actinotrichia in each hemiblastema (the blastema regenerating a hemiray) [22] (Fig. 2G).

These conclusions were based on alcian blue staining. The ECM of the blastema proper is highly positive at 0.2 M $MgCl_2$ (which suggest the presence of acidic GAGs), whereas the actinotrichial blastemic region is positive at 0.9 M $MgCl_2$ (Fig. 2H) (indicating sulphated GAGs). These preliminary results were further supported by enzymatic digestions with hyaluronidase and papain. The ECM of the blastema proper showed a significant decrease in staining after treatment with hyaluronidase, indicating that hyaluronate is an important component of this ECM. The actinotrichial blastemic region also showed a significant decrease in alcian blue staining after papain digestion, which degrades proteoglycan core proteins, but not collagen. These results are consistent with sulphated GAGs being an important element of the ECM in the actinotrichial blastemic region.

CONCLUSIONS AND PERSPECTIVE

Prof. G. S. Montes was a dedicated student of the ECM who used histochemical techniques to study a large number of different tissues and organisms. In this paper, we have described studies inspired by Prof. G. S. Montes in which the composition and dynamics of the ECM during the regeneration of adult tail fin were investigated histochemically. The lepidotrichia, the main structural dermal element of the fin, gradually regenerates by the distal addition of new material. The actinotrichia, an important skeletal element of the distal part of the fin, regenerates through continuous turnover [10]. The biochemical composition of the ECM varied between the blastema proper and the actinotrichial blastemic region. Previous studies proposed that cell-cell interactions in the proximal blastema were regulated by hyaluronate in the ECM [25]. Our studies also support the idea that hyaluronate is the main constituent of the blastemic ECM [22]. Once the scleroblasts initiate their differentiation, the lepidotrichia is formed by the local deposition of ECM. The composition of this dermal bone ECM was studied by transmission electron microscopy (TEM), which suggested the presence of type II collagen. These observations led

to the proposal that the ray of teleostean fins is similar to cartilage and that it could serve as a model system for cartilage regeneration [11].

An interesting study done in collaboration with Prof. G. S. Montes was the functional analysis of collagen during fin regeneration [4]. Fin regeneration was perturbed by using a number of drugs that affect collagen biosynthesis. Fin ray blastema formation and outgrowth were adversely affected by these drugs, suggesting that collagen has a morphogenetic, causative role during teleostean fin regeneration. These results provide an interesting perspective for future studies.

Prof. Montes inspired us in a very profound way. Many of our studies and ideas were directed by his insights and expertise which addressed practical aspects of biological research, including experimental design, the discussion of results, and the writing of papers. Our work for many years to come will be influenced by the knowledge conveyed by Prof. G. S. Montes. Our group and our university will miss him greatly.

ACKNOWLEDGMENTS

This work was supported by grants from DGESC (BOS2002-00347, SAF2002-02183) and Junta de Andalucía (grupo CV1217).

REFERENCES

1. Akimenko M-A, Marí-Beffa M, Becerra J, Géraudie J (2003) Old questions, new tools, and some answers to the mistery of fin regeneration. *Dev. Dyn*. **226**, 190-201.
2. Becerra J, Junqueira LCU, Bechara IJ, Montes GS (1996) Regeneration of fin rays in teleosts: a histochemical, radioautographic and ultrastructural study. *Arch. Histol. Cytol*. **59**, 15-35.
3. Becerra J, Montes GS, Bexiga SRR, Junqueira LCU (1983) Structure of the tail fin in teleosts. *Cell Tissue Res*. **230**, 127-137.
4. Bechara IJ, Joazeiro PP, Marí-Beffa M, Becerra J, Montes GS (2000) Collagen affecting drugs impair regeneration of teleost tail fins. *J. Submicrosc. Cytol. Pathol*. **32**, 273-280.
5. Géraudie J, Meunier F-J (1982) Comparative fine structure of the Osteichthyan dermotrichia. *Anat. Rec*. **202**, 325-328.
6. Géraudie J, Singer M (1977) Relation between nerve fiber number and pectoral fin regeneration in the teleost. *J. Exp. Zool.* **199**, 1-8.
7. Iovine MK, Johnson SL (2000) Genetic analysis of isometric growth control mechanisms in the zebrafish caudal fin. *Genetics* **155**, 1321-1329.
8. Johnson SL, Weston JA (1995) Temperature-sensitive mutations that cause stage-specific defects in zebrafish fin regeneration. *Genetics* **141**, 1583-1595.
9. Junqueira LCU, Cossermelli W, Brentani RR (1978) Different staining of collagens type I, II and III by Sirius red and polarization microscopy. *Arch. Histol. Jpn.* **41**, 267-274.
10. Marí-Beffa M, Carmona MC, Becerra J (1989) Elastoidin turnover during tail fin regeneration in teleosts. A morphometric and radioautographic study. *Anat. Embryol.* **180**, 465-470.
11. Montes GS, Becerra J, Toledo OHS, Gordilho MA, Junqueira LCU (1982) Fine structure and histochemistry of the tail fin ray in teleosts. *Histochemistry* **75**, 363-376.
12. Morgan TH (1900) Regeneration in teleosts. *Arch. Entw. Org.* **10**, 120-131.
13. Morgan TH (1901) *Regeneration*. Macmillan: New York.
14. Morgan TH (1902) Further experiments on the regeneration of the tail fins of fishes. *Arch. Entw. Mech*. **14**, 539-561.
15. Morgan TH (1906) The physiology of regeneration. *J. Exp. Zool*. **4**, 457-500.
16. Moss ML (1963) The biology of acellular teleost bone. *Ann. Nat. Acad. Sci.* **109**, 337-350.
17. Murciano C, Fernández TD, Durán I, Maseda D, Ruiz-Sánchez J, Becerra J, Akimenko M-A, Marí-Beffa M (2002) Ray-interray interactions during fin regeneration of *Danio rerio*. *Dev. Biol*. **252**, 214-224.
18. Murciano C, Ruiz J, Maseda D, Fernández TD, Durán I, Marín-Girón F, Becerra J, Marí-Beffa M (2001) Ray and inter-ray blastemas interact to control bifurcations of *Danio rerio* fin rays. *Int. J. Dev. Biol*. **45**, S129-S130.
19. Nelson JS (1994) *Fishes of the World*. 3rd edn. John Wiley: New York.
20. Poss KD, Keating MT, Nechiporuk A (2003) Tales of regeneration in zebrafish. *Dev. Dyn.* **226**, 202-210.
21. Quint E, Smith A, Avaron F, Laforest L, Miles J, Gaffield W, Akimenko, M-A (2002) Bone patterning is altered in the regenerating zebrafish caudal fin after ectopic expression of *sonic hedgehog* and *bmp2b* or exposure to cyclopamine. *Proc. Natl. Acad. Sci. USA* **99**, 8713-8718.
22. Santamaría JA, Becerra J (1991) Tail fin regeneration in teleosts: cell-extracellular matrix interaction in blastemal differentiation. *J. Anat*. **176**, 9-21.
23. Santos-Ruiz L, Santamaría JA, Ruiz-Sánchez J, Becerra J (2002) Cell proliferation during blastema formation in the regenerating teleost fin. *Dev. Dyn*. **223**, 262-272.
24. Tawk M, Tull D, Torrente Y, Vriz S, Paulin D (2002) High-efficiency gene transfer into adult fish: a new tool to study fin regeneration. *Genesis* **32**, 27-31.
25. Toole BP (1972) Hyaluronate turnover during chondrogenesis in the developing chick limb and axial skeleton. *Dev. Biol*. **29**, 321-329.

Received: March 3, 2003
Accepted: June 30, 2003