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Original Article

Expression of two *Even-skipped* genes *eve1 and evx2* during zebrafish fin morphogenesis and their regulation by retinoic acid

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ABSTRACT Growth and patterning during fin regeneration depend, like for fin development, on the integrated expression of homeogenes. In the present work we have studied, by *in situ* hybridization, the expression and regulation of two vertebrate homologs *eve1* and *evx2* of the *Drosophila* pair-rule *even-skipped* gene family. Upon amputation of pectoral and caudal fins, both genes, expressed transiently in the mesenchyme during early stages of fin development of these fins, are turned on. During the formation of the blastema they are transcribed first in the mesenchyme located underneath the wound epidermis and then, their expression is restricted to the regenerating rays regions. These expression patterns are developmentally regulated since both genes are no longer transcribed when the bony rays are differentiating. Exposure of the regenerates to retinoic acid (RA) modifies the boundaries of *eve1* and *evx2* expression: the signal is down-regulated in the ray region and up-regulated in the interray region. Moreover, expression is induced in the wound epidermis. These results indicate that *eve1* and *evx2* products are part of the molecular signals involved in pattern formation of the fin and fin rays in connection with outgrowth. RA might alter growth and morphogenesis of the regenerating fins by a fine regulation of these genes among others.

KEY WORDS: *eve1, evx2, gene, development, regeneration, fin, zebrafish, retinoids*

Introduction

Homeobox containing genes are involved in limb pattern formation, axial patterning, and growth control, among other functions (Dollé *et al.*, 1989; McGinnis and Krumlauf, 1992). The *Drosophila* pair rule gene *even-skipped* itself is involved in the proper segmentation and neurogenesis of the fly (MacDonald *et al.*, 1986), but *even-skipped* expression differs in other species since it is not expressed in the segmentation of another insect, the grasshopper (Patel *et al.*, 1992). *Even-skipped* homologs have been found in the genome of vertebrates, including humans, where EVX-1 (Faiella *et al.*, 1992) and EVX-2 (D'Esposito *et al.*, 1991) have been isolated by virtue of their homology of sequence to *Drosophila*. In Amphibians, *Xhox3*, an *even-skipped* homolog, is first transcribed at the mid-blastula transition during *Xenopus* development and maximally expressed at the late gastrula- early neurula stage. It has been suggested that *Xhox3* plays a role in the pattern formation along the anteroposterior (A-P) axis (Ruiz i Altaba and Melton, 1989; Ruiz i Altaba *et al.*, 1991). In developing mouse, the expression pattern of *Evx-1* is compatible with a role in specifying posterior positional information along the embryonic axis (Bastian and Gruss, 1990). *Evx-1*, also expressed in the embryonic ectoderm before gastrulation, is suggested to play a role in the process of gastrulation itself, possibly in the determination of the dorsoventral (D-V) mesodermal cell fates (Dush and Martin, 1992). In the teleost zebrafish *Danio rerio*, *eve1* (Joly *et al.*, 1993) and *evx2* (Sordino *et al.*, 1996) have been recently cloned. During early development, Joly *et al.* (1993) showed that *eve 1* is involved in the formation of antero-posterior axis of the fish since it is expressed in the late blastula cells, then restricted to a crescent of ventral and lateral cells of the marginal zone of the gastrula and then to a few cells of the caudalmost (posterior) mesoderm of a 24 h post fertilization (hpf) embryo. On the other hand, *evx2* is transiently transcribed only in cells of the posterior mesenchyme of pectoral

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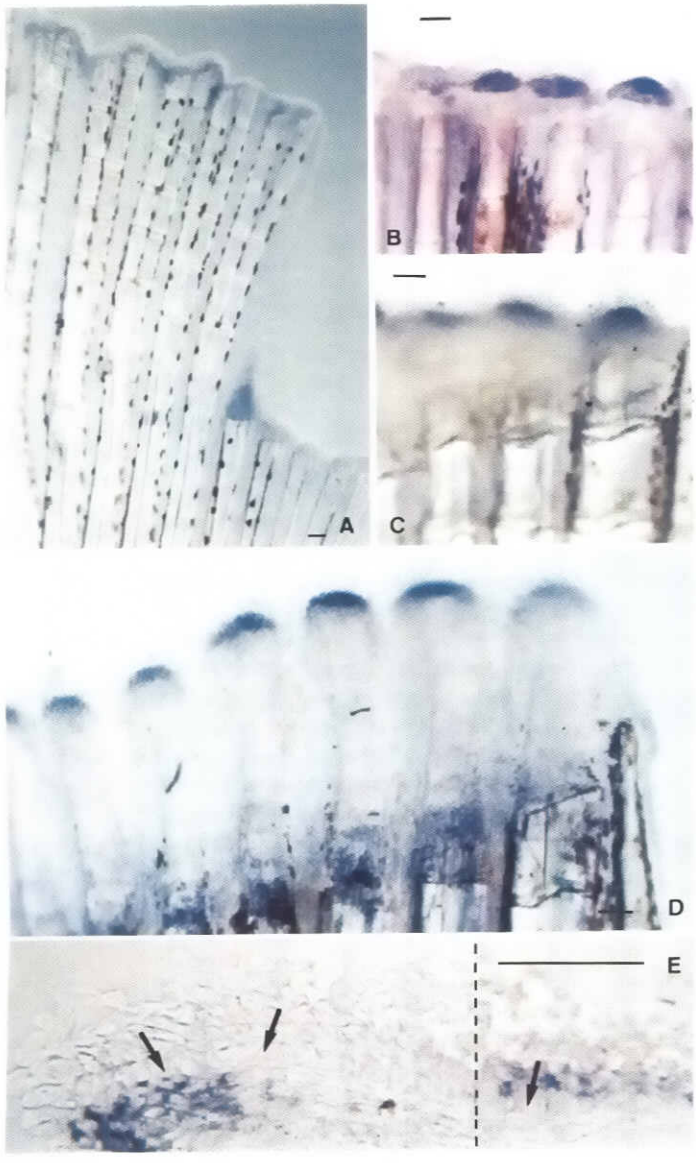

Fig. 1. *eve1* expression; *in situ* hybridization of whole-mount regenerating zebrafish fins. *Results are similar for caudal and pectoral fins.* **(A)** *Pectoral fin, 24 h post amputation (hpa). Amputation was done distal (left) and proximal to the first fork (right). eve1 is expressed in the mesenchymal cells of the blastema above and between the rays. No differential expression is observed along the proximo-distal axis of the regenerating fin.* **(B-C)** *Caudal fin 48 hpa and 72 hpa respectively. In (B) bony rays were stained with Alizarin Red S to enhance the contrast at the level of amputation. eve1 expression is restricted to the mesenchymal subepidermal cells located above the rays, in an arch-shaped fashion.* **(D)** *Caudal fin, 4 days after amputation. eve1 expression remains in the subepidermal mesenchymal cells of the apex of the ray while determined cells located between the level of amputation and the apex do not transcribe eve1.* **(E)** *Male pectoral fin. Longitudinal section showing eve1 transcripts (i) in the cytoplasm of subepidermal mesenchymal cells of the blastema (left) and (ii) in the upper layers of the stump epidermis at the level of the ornamentations (right). Note that eve1 is not expressed in the basal layer of the wound and stump epidermis (arrows). The dotted line shows the level of amputation. The apex of the regenerate is on the left, where the high signal is present in the mesenchymal cells of the blastema. Bar, 100μm.*

fin buds and in mesenchymal cells of the budding tail of embryos (Sordino *et al.*, 1996).

We are interested in the molecular and cellular aspects of fin regeneration compared to fin development, and especially in the expression of homeogenes involved in axial patterning. The present work focuses on *eve1* and *evx2* because of their involvement in axis determination during early development. Whether regeneration of a fin reiterates its genetic developmental program or whether a new combinatorial set of gene products is involved, is an interesting issue in developmental biology. More specifically, are genes involved in axial determination re-expressed during regeneration of the rays since the blastema develops on a stump that is already polarized?

Retinoic acid (RA) is an important signaling molecule which appears to play a significant role in vertebrate pattern formation. During zebrafish development, endogenous retinoids are involved in patterning of the retina (Marsh-Armstrong *et al.*, 1994), antero-posterior axis specification (Marsh-Armstrong *et al*, 1995; Costaridis *et al.*, 1996) and pectoral fin development. In the latter case, disulphiram exposure which inhibits aldehyde dehydrogenase/RA synthase activity, induces pectoral fin distortions of various kinds indicating that pectoral fin morphogenesis may depend on the regulated presence of endogenous RA (Marsh-Armstrong *et al.*, 1995). On the other hand, exogenous retinoids are known to modify positional information during development (rev., Means and Gudas, 1995) and limb regeneration (Maden, 1982; Thoms and Stocum, 1984; Pecorino *et al.*, 1996; rev., Géraudie and Ferretti, 1998). In the case of newt limb regeneration, there is evidence that the wound epidermis is a local source of synthesis and release of 9-*cis* retinoic acid possibly used in the system in cellular interactions between the two compartments of the blastema (Viviano *et al.*, 1995). In the developing zebrafish, exogenous retinoids affect brain organization (Holder and Hill, 1991; Hill *et al.*, 1995), the heart (Stanier and Fishman, 1992) and interestingly, induce ectopic expression of *Sonic hedgehog shh/vhh-1* gene in the anterior margin of pectoral fin buds (Akimenko and Ekker, 1995). As in other vertebrates, RA mediates its various effects in zebrafish through receptors (Joore *et al.*, 1994; White *et al.*, 1994) which act as ligand-dependent transcription factors on target genes which are not yet fully identified except for some *Hox* genes (Simeone *et al.*, 1991; Alexandre *et al.*, 1996), cytokeratin genes in the newt limb blastema (Ferretti *et al.*, 1991) and some retinoic acid receptors (RAR) themselves.

We have shown that retinoic acid (RA) induces dysmorphogenesis during fin regeneration. Teleost fins are made of segmented and branched bony rays (lepidotrichia) separated by soft tissue and attached at their proximal end to muscles connected to endoskeletal elements. Each lepidotrichium has its own identity along the antero-posterior and dorso-ventral axis as to the level of the branch (fork), the lepidotrichium located either on the dorsal or anterior edge of the fin being forkless. Fin regeneration, like limb regeneration proceeds through the formation of a blastema (epimorphic regeneration) made of a thickened wound epidermis covering a mound of mesenchymatous-like cells (blastema *sensu stricto*) capping the mature stump tissues. Amputation of a fin across its proximo-distal axis involves sectioning of all the ray and interray regions and, as a consequence, a fin blastema is a collection of adjacent ray and interray blastemas (Géraudie and Singer, 1992; Géraudie, in preparation). After RA treatment, fusions

of rays are observed along the dorsoventral axis and distalization of the forks is induced along the proximodistal axis (Géraudie *et al.*, 1994,1995). These effects are mediated through apoptosis (Ferretti and Géraudie, 1995; Géraudie and Ferrretti, 1997).

Here, we show that: (i) *eve1* is a marker of the mesenchyme of the pectoral fin bud territory which becomes restricted to the proximal region of the growing fin bud, while Sordino *et al.* (1996) showed that *evx2* is present only in the posterior region of the fin bud; (ii) *eve1* and *evx2* are both re-expressed during fin regeneration along the proximodistal axis in cells of the mesenchymal compartment of pectoral and caudal fin blastema in their growing distal regions; (iii) *eve1* and *evx2* expression is related to dermal bone differentiation; (iv) a single RA treatment during any stage of fin regeneration alters *eve1* and *evx2* expression in the mesenchyme of the blastema and induces gene transcription in the wound epidermis. These results suggest that *eve1* and *evx2* products are part of the molecular signals which are involved in pattern formation of the fin in connection with outgrowth.

Results

Expression of eve1 gene during fin regeneration

Control fins (unamputated) as well as stump tissues after amputation did not show *eve1* expression. Amputation of the pectoral and caudal fins was rapidly followed by healing of the wound and *eve1* expression was similar in both regenerating fins. There was no signal during the first 6 h post-amputation (hpa) but by 24 hpa, a subepidermal expression of *eve1* gene was observed in the mesenchymal cells filling the space between the wound epidermis and the level of amputation (Fig. 1A). During the growth of the blastema (48 and 72 hpa), only subepidermal cells located at the tip of each sectioned bony ray displayed a high signal (Fig. 1B-C). Then, during the subsequent stages of blastema growth and cell differentiation (4 days up), the signal remained in subepidermal cells of each regenerating ray. Cells located between the level of amputation and the subepidermal cells did not show any signal (Fig. 1D). Later in development (7 days) *eve 1* expression was down regulated in these blastema cells.

Histological sections confirmed that transcripts were restricted to apical subepidermal mesenchymal cells (Fig. 1E). However, it was noticeable that the epidermal ornamentations of the pectoral fins, a sexual secondary feature of the male zebrafish (Géraudie *et al.*, 1994), were expressing *eve1*, but only in the inner layers of the epidermis, with the exception of its most basal layer.

Expression of eve1 gene during fin development

eve1 transcripts were present in the circular and symmetrical pectoral fin presumptive territories of a 24 hpf embryo (Fig. 2A) and located in the mesenchymal cells, under the ectoderm (Fig. 2B). During subsequent stages, a high signal persisted in the proximal region of the mesenchyme (Fig. 2C) in cells of the presumptive cartilaginous pectoral girdle, while mesenchymal cells of the developing fin palette, distal to the girdle, expressed a faint signal (Fig. 2D). As for the caudal fin primordium, at 28 hpf *eve1* was highly expressed in the caudalmost mesenchymal cells underneath the unlabeled fin fold ectoderm (Fig. 2E). This expression was transient since no labeling was observed in the developing caudal fin of 48 hpf embryos and in later stages of development (up to 6 days). Cells of the vent region also expressed *eve1* gene (Fig. 2F).

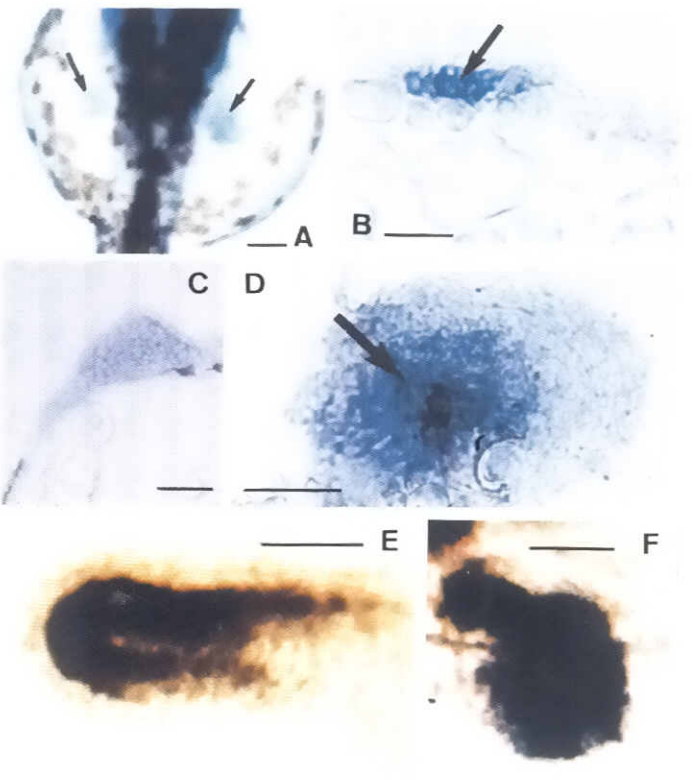

Fig. 2. *eve1* expression; *in situ* hybridization of whole-mount zebrafish embryos. *(A-D) Pectoral fin buds.* **(A-B)** *Twenty-four hpf embryo. In (A), the arrows point to the territory of the pectoral fin buds. Panel B shows a cross section of a pectoral fin bud: mesenchymal cells are labeled (arrow).* **(C)** *Section of a 48 hpf fin bud showing that the expression is restricted to the bulk of mesenchymal cells which is the presumptive territory of the fin endoskeleton.* **(D)** *Fifty-two hpf embryo pectoral fin bud showing beyond the strong signal in the area of endoskeleton (arrow), a weak signal in distal tissues except for the periderm which is devoid of transcripts.* **(E)** *Twenty-eight hpf embryo caudal fin. A high signal is observed in the mesenchyme of the growing tail. There is no expression in the ectoderm of the median fin fold.* **(F)** *eve1 is also expressed in the vent region as several Hox genes. Bar, 100 μm.*

Expression of evx2 gene during fin regeneration

Expression of *evx2* was similar to the spatio-temporal pattern of expression described for *eve1* in that it was also restricted to apical mesenchymal cells in an arch-shaped fashion (Fig. 3A-D) suggesting that the most apically located subepidermal cells co-expressed both genes. The medial part of the caudal fin regenerate, where the regenerated rays will be shorter than the more lateral ones to re-establish the original fin pattern, shows a weaker signal than the edges. There is a gradient of gene expression decreasing from the dorsal and ventral edges in direction of the medial part of the regenerate (Fig. 3 E). This has been observed with *eve1* also but the gradient was not as obvious as with *evx2*. Longitudinal sections confirmed that the epidermal cells do not transcribe *evx2* (Fig. 3F-G).

In the pectoral fin regenerates, this gradient of expression was observed as well (not shown). The signal was higher in the

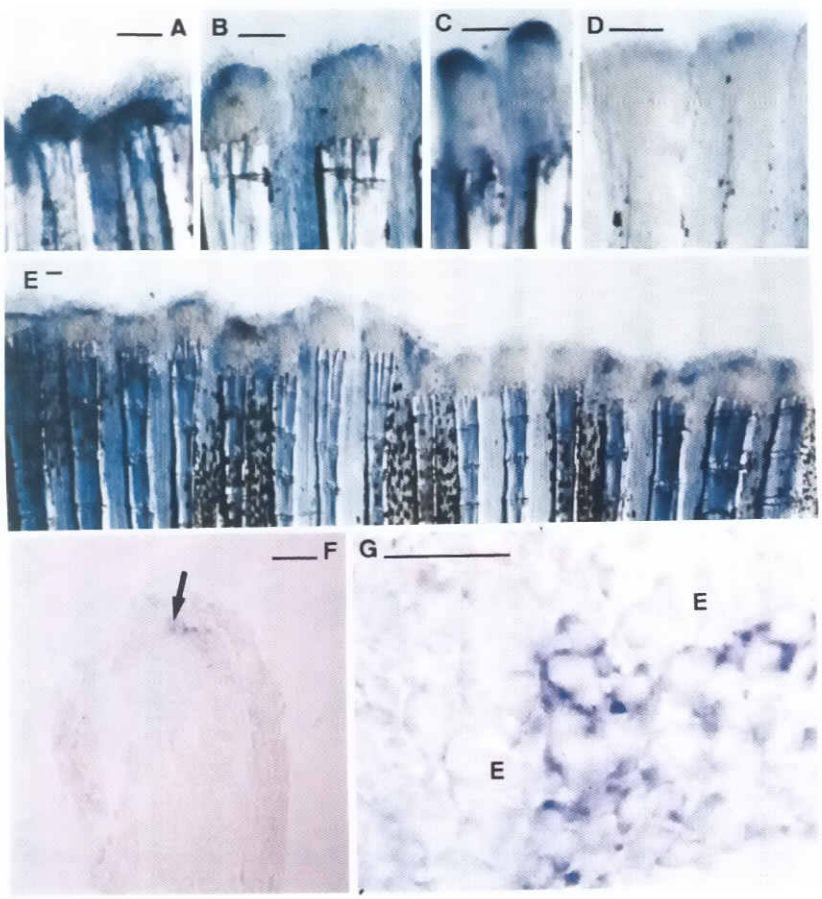

Fig. 3. *evx 2* expression; *in situ* hybridization of whole-mount regenerating fins. *Expression is similar in pectoral and caudal fins so that either caudal (A and C) or pectoral (B and D) fins are shown.* **(A-D)** *Time course expression of evx 2 during fin regeneration. Like for eve1, at 24 hpa, evx2 is expressed in all the mesenchymal cells (above and between the rays). Then, in subsequent stages of regeneration, 48, 72 and 96 hpa (B,C and D respectively), its expression is restricted to the mesenchymal subepidermal cells of the blastema located at the tip of the rays.* **(E)** *Caudal fin, 3 days after amputation. A decreasing gradient of evx2 signal is observed from the edges of the regenerate towards its medial part.* **(F-G)** *Longitudinal sections of a pectoral fin, 4 days after amputation. evx2 transcripts are observed only in subepidermal mesenchymal cells (arrow). (G) High magnification of the apical region showing that the cells of the wound epidermis (E) do not transcribe evx2. Bar, 100 μm.*

blastemas of the sturdy regenerating rays of the dorsal edge than in the opposite shorter and thinner rays of the ventral edge. Like *eve1*, *evx2* was developmentally regulated since it was no longer transcribed in blastemas when differentiation occurred in the regenerate.

Regulation by retinoic acid

Regenerating fins

A 24 h treatment with *all-trans* RA 10^{-6} M inhibits temporarily the growth of the fin regenerate whatever its developmental stage (Géraudie *et al.*, 1994). In the RA-treated fish, signals for *eve1* (Fig. 4A-C) and *evx2* (not shown) were faint but continuous in the distal edge of the regenerate while in DMSO controls, they were observed only in the blastema of the rays (not shown but similar to Fig. 1). Cross sections of the regenerate unexpectedly showed that *eve1* (Fig. 4C) and *evx2* (not shown) were also expressed in the wound in the basal layer. Thus, the immediate consequence of a single treatment seemed to be a decrease of *eve1* and *evx2* expression at the level of the rays, an induction of expression in the mesenchyme of the blastema located between the rays and in the wound epidermis (Fig. 4C).

Developing fins

One or two hours treatment of the embryos with RA 10^{-6} M (not shown) did not modify *eve1* expression in pectoral fin buds and did not induce obvious dysmorphogenesis in treated samples (Table 1). In contrast, treatment with a higher concentration of RA 10^{-5} M for the same length of time, down-regulated *eve1* expression and led to pectoral fin dysmorphogenesis (Table 1). The proximal region where endoskeleton will differentiate and which already forms a small pedicle at that stage of development, was reduced; altered pectoral fins were short and rounded (Fig. 5A-B). The caudal region of the embryo was shortened (Fig. 5C-D).

Discussion

Reinduction of eve1 and evx2 expression in regenerating fins

This work is the first to show that zebrafish genes of the *even-skipped* family, expressed during fin (Sordino *et al.*, 1996) and mammalian limb development (Dollé *et al.*, 1989,1994), are re-expressed during fin regeneration. This expression in adult zebrafish is regeneration-specific since it is restricted to regenerating tissue and since there are neither *eve1* nor *evx2* transcripts in the adult unamputated fins. In urodele amphibians, the only tetrapods capable, as adults, of complete limb regeneration, the expression of the *even-skipped* gene family has not yet been studied, to our knowledge, either during limb development and/or regeneration.

We show here that following fin amputation, at the time of wound healing (24 hpa), cells located under the wound epidermis express *eve1* and *evx2* in the ray and interray regions. Then transcripts persist only in subepidermal cells located at the level of each regenerating bony ray, no signal being observed in the interray regions. *eve1* and *evx2* expression is first observed during the

presumed phase of cell dedifferentiation occurring in the stump of the amputated fin which allows the emergence of the progenitor cells or blastema cells from mature stump tissues located in the vicinity of the plane of amputation, like after healing of the wound of amputated limbs (Hay, 1959; Wallace 1981; Brockes, 1998). This pattern of expression suggests a role of *eve1* and *evx2* in cell dedifferentiation in connection with cell migration and/or cell proliferation, two events necessary for the formation of the blastema. Then, during growth of the blastema of each regenerating ray, the signal is still observed in apical subepidermal cells. In that region, it has been shown that BrdU is incorporated (Santamaria *et al.*, 1996), which demonstrates that it is made up of proliferating cells. This apical region in the regenerate seems to be equivalent to the progress zone described during limb bud development (Summerbell *et al.*, 1973). No signal is observed proximally, where presumptive osteoblasts adjacent to the regenerating bone and interrays presumptive connective tissue cells fill up the space between the level of amputation and the apex. Since the signal is located only distally, this suggests that *eve 1* and *evx2* are markers of apical mesenchymal proliferating cells. In this latter territory of the regenerate the secretion of elastoidin takes place making up the actinotrichia. These actinotrichia are specifically found in the ectodermal fold of the developing teleost fin bud, before the development of the lepidotrichia (Géraudie, 1977; Géraudie *et al.*, 1998) and in the distal edge of adult fins (rev., Goss and Stagg, 1957). Since actinotrichia belong to the distal skeleton of the fin, their presence, early after onset of regeneration indicates that it is this distal part of the fin that is first specified during fin regeneration, which reiterates the developmental process. In this apical territory, whether mesenchymal *eve1* and *evx2* expression has an instructive or a permissive role in the establishment of the actinotrichia and further regeneration of the bony rays is unclear.

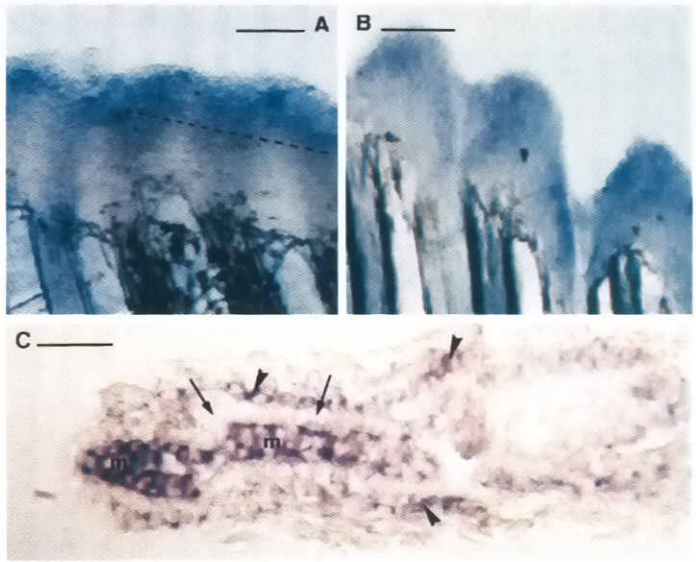

Fig. 4. *eve1* expression; *in situ* hybridization on whole-mount RA-treated pectoral and caudal fins. *The RA treatment was applied for 24 h starting at 24 hpa, so that the fins shown were fixed at 48 hpa.* **(A,C)** *Caudal fin.* **(B)** *Pectoral fin. A weak signal is observed all along the distal edge of the blastema, in the mesenchyme.* **(C)** *Cross section of a caudal fin regenerate done at the level of the dotted line shown in A. The signal is observed, as in the controls (see Fig. 1), in the mesenchymal cells (m) of the ray region and, in addition, in the interray mesenchymal cells. Note that eve1 is expressed in the cells of the wound epidermis (arrow heads) with the exception of its most basal layer (arrow). Bar, 100μm.*

TABLE 1

SINGLE RA 10^{-5}M TREATMENT OF ZEBRAFISH EMBRYOS: EFFECTS ON THE DEVELOPMENT OF PECTORAL FINS AND ON *EVE1* EXPRESSION OBSERVED BY *IN SITU* HYBRIDIZATION OF WHOLE-MOUNTS.

Hour postfertilization at the time of treatment with 10^{-5} MRA	Length of treatment (hours)	**a**. Phenotype 1 day later **b**. eve 1 expression	**a**. Phenotype 6 days later **b**. eve 1 expression
27	1	**a**. same as control **b**. same as control	**a**. slight dysmorphogenesis **b**. same as control
27	2	**a**. slight dysmorphogenesis **b**. weak signal	**a**. obvious dysmorphogenesis **b**. weak signal
42	1	**a**. growth arrest **b**. weak or no signal	**a**. strong dysmorphogenesis or growth arrest **b**. weak signal
42	2	**a**. growth arrest **b**. weak or no signal	**a**. growth arrest **b**. no signal

Data presented here demonstrate that re-expression of *eve1* and *evx2* is developmentally regulated since these genes are no longer transcribed during differentiation in the blastema. The weaker signal observed in the medial region of the early caudal fin regenerate, where rays will be shorter than on both edges in the fully regenerated caudal fin, indicates a down regulation of the gene expression in a specific region of the regenerate where growth is reduced, in connection with caudal fin morphogenesis. The gradient of expression of these genes along the DV axis reflects a correlation between *eve* expression, growth and patterning of the regenerating fins.

It is worthwhile to note that genes expressed during fin development and which are re-expressed during fin regeneration may be switched on and off with an unpredictable spatio-temporal pattern. For example, of all the genes of the *msx* family expressed in the mesenchymal cells of the developing fin bud, only *msx B* and *C* are transiently transcribed in the blastema cells of the regenerate. Similarly, transcription of *msxB* observed in fin ectodermal cells is not reinduced in the wound epidermis cells of the regenerate (Akimenko *et al.*, 1994b). These patterns of expression suggest the existence of new combinatorial sets of genes expressed during growth and patterning of fin regenerate compared to fin ontogenesis. On the other hand, re-expression of developmental genes may not be an obligatory step for fin regeneration to occur. Indeed,

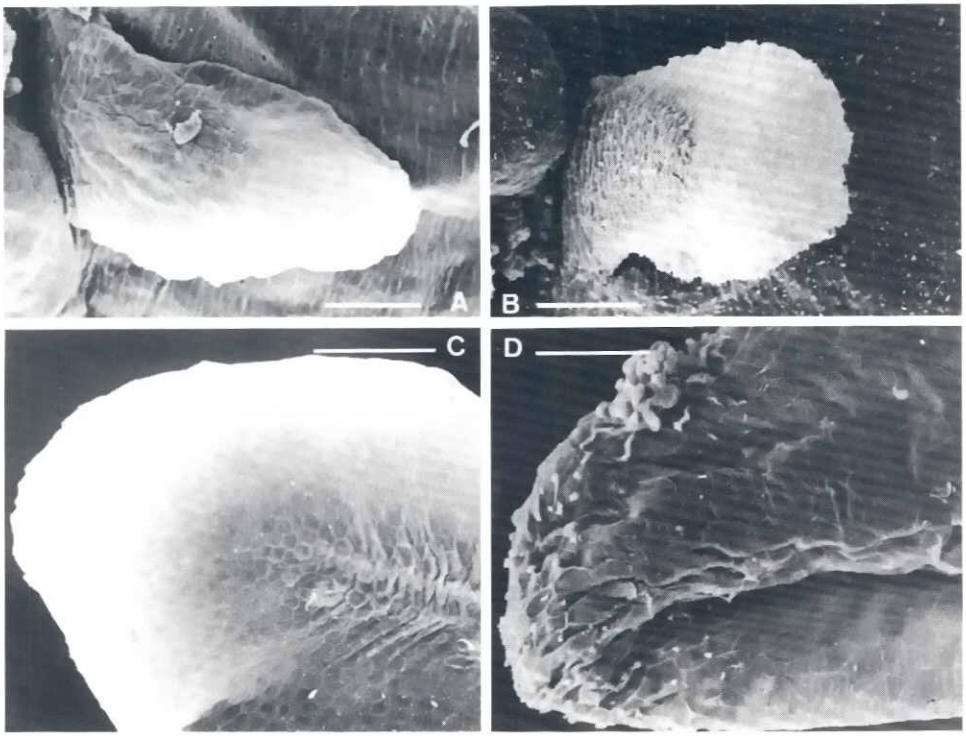

Fig. 5. SEM of control and RA-treated zebrafish pectoral and caudal fin buds. **(A-B)** *Compare the roundish and reduced size of the developing pectoral fin of a 10^{-5} M RA treated embryo for 1h (B) with a control embryo (A) fixed at the end of the treatment. A defect in growth and patterning is apparent in the treated embryo. Head is on the left and belly on top.* **(C-D)** *Note the truncated and wrinkled caudal region of the treated embryo (D) when compared to control (C). Head is on the right and belly on top. Bar, 100 μm.*

attempts to demonstrate a re-expression of any *engrailed* gene during pectoral and caudal fin regeneration failed (unpublished results). *Engrailed* is a segment polarity gene in *Drosophila* whose expression has been studied during early zebrafish development (Ekker *et al.*, 1992; Fjöse *et al.*, 1992). A signal is obtained for an engrailed-like homeoprotein at 24-32 h of development in the ectodermal cells located in the antero-ventral quarter of the pectoral fin bud which will contribute to the ventral surface of the adult fin (Hatta *et al.*, 1991).

Contrary to *evx2* gene expression restricted to the posterior mesenchymal cells of the pectoral fin buds (Sordino *et al.*, 1996), *eve1* gene is expressed in all the cells of early pectoral fin bud territory, and in cells of the tail bud mesoderm, before any phenotypic differentiation of the caudal fin proper. This suggests that *eve1* could be a marker of the territory of these fins, whatever their cellular origin, ectomesenchyme (neural crest) or mesoderm or both (Smith *et al.*, 1994).

Regulation by retinoic acid

We provide here evidence that *eve1* and *evx2* expression is altered in the blastema following RA exposure, whatever the developmental stage of the regenerate. This can be correlated with dysmorphogenesis of the regenerate (Géraudie *et al.*, 1994,1995). During fin development a decrease of *eve1* expression in buds after RA treatment can also be correlated with growth impairment. RA effects are mediated by a family of nuclear retinoid acid receptors (RAR) and retinoid X receptors (RXRs) which are ligand-dependant transcription factors (Means and Gudas, 1995). In zebrafish, RAR have been cloned (Joore *et al.*, 1994; White *et al.*, 1994) and RARγ and α transcripts are present in the apical mesenchymal cells of the regenerating rays (White *et al.*, 1994). In this specific region, we show that *eve1* and *evx2* are also expressed during regeneration. Following RA treatment , transcripts are no longer localized at the distal part of the rays but in the whole blastema, albeit at lower levels. This can be interpreted as a down regulation of expression in the distal mesenchymal cells of the rays region and an upregulation in the interrays region. However, it is unclear whether this regulation occurs in all the cells or in a subpopulation of cells.

We also show here that, following RA treatment, *eve1* and *evx2* are turned on in the epidermal cells above the basal layer, suggesting an upregulation of these genes. We have previously shown that RA induces apoptosis at random in the cells of the wound epidermis (Ferretti and Géraudie, 1995), but our data do not allow any correlation between *eve* expression and cell death. It has been shown that during newt limb regeneration, RARδ1 are present in half of the nuclei of epidermal and blastemal cells (Hill *et al.*, 1993). There is no data available on the expression of this receptor in the blastema of the zebrafish fin. However, it is possible that RARs are present in the wound epidermis and that the binding of exogenous RA on these receptors could activate *eve1* and *evx2* in this territory. Alternatively, RARs could be present only in the mesenchyme of the blastema and binding of exogenous RA could then be a signal transmitted to the wound epidermis, then activating *eve1* and *evx2*.

We have shown that RA temporarily inhibits growth of the fin regenerate (Géraudie *et al.*, 1994,1995). On the other hand, Schilthuis *et al.* (1993) have found that proliferation of newt limb blastema cells cultured *in vitro* is inhibited by RA, this effect being mediated through RARα1. In the fin blastema, RA could down regulate *eve* genes expression through an effect linked to a molecular cascade leading to cell proliferation.

Materials and Methods

Adult zebrafish *(Danio rerio)* were purchased from Sidoli (Noisy-Le-Grand, France) and maintained at 24°C ± 2°C. Prior to amputation, fish

were anesthetized in a 0.017% Tricaine (3-amino benzoic acidethylester or MS222, Sigma) solution according to the Zebrafish book (Westerfield, 1989).

Embryos, incubated at about 28°C, were obtained in the laboratory from natural crosses and staged according to the Zebrafish book (Westerfield, 1989). All samples (embryos, control fins and regenerates) were fixed overnight at 4°C in 4% paraformaldehyde in PBS, thoroughly rinsed in PBS and stored in methanol at -20°C from one night to several days.

Fin amputation

Fins were amputated at midlength, proximal to the first ray branching, either with fine scissors for the pectoral fins or a scalpel for the caudal one. To determine the existence of a putative gradient of expression along the proximo-distal axis, amputation of a single caudal fin was done at different levels in a staircase-like fashion, leading to distal amputation severing the fin above the first forks of the bony rays.

Retinoic acid treatment

A single 24 h 10^{-6} M *all trans*-retinoic acid (Sigma) treatment was applied at different time points after amputation as previously reported (Géraudie *et al.*, 1994). Harvest of the regenerates was done immediately after the end of the treatment.

A short treatment (1 or 2 h) with 10^{-6} M or 10^{-5} M *all trans*-retinoic acid was applied to 27 and 42 h old embryos. Fixation of the treated embryos in paraformaldehyde 4% was done either at the end of the treatment or 24 h later, when pectoral fins are well developed in a 48 hour-old control fish or 4 days later. An assessment of the effects of RA was done on embryos fixed for *in situ* hybridization using Scanning Electron Microscopy (SEM) according to routine procedure (Ferretti and Géraudie, 1995) except for post-fixation with osmium tetroxide which was omitted.

in situ hybridization

eve1 and *evx2* expression was studied by *in situ* hybridization on whole-mounts according to the method of Akimenko *et al.* (1994a). Antisense *eve1* RNA digoxigenin labeled UTP probe was transcribed by T7 RNA polymerase (Boehringer) from a 400bp fragment *(MscI-StuI)* of the *eve1* zebrafish gene provided by J.S. Joly.

evx2 RNA digoxigenin-labeled probe was transcribed by T3 RNA polymerase from a *SmaI-AflIII* bp genomic fragment kindly provided by P. Sordino (Sordino *et al.*, 1996). Signal detection was done for both genes with the Boehringer Dig-DNA labeling kit detection according to the manufacturer's instructions. Samples were mounted in glycerol for analysis or embedded in paraffin for 7 µm longitudinal and cross sectioning.

Data presented here have been obtained through six series of experiments. Staining of whole-mounts was carried out for comparable times in all samples for all experiments.

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