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 (Dolle 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 eve1 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
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 (II) in the cytoplasm of subepidermal mesenchymal cells of the blastema (left) and (III) 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, 100 μm.

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 homegenes 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 fork is induced along the proximodistal axis (Géraudie et al., 1994, 1995). These effects are mediated through apoptosis (Ferretti and Géraudie, 1995; Géraudie and Ferretti, 1997).

Here, we show that: (i) even-1 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 evx-2 is present only in the posterior region of the fin bud; (ii) even-1 and evx-2 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) even-1 and evx-2 expression is related to dermal bone differentiation; (iv) a single RA treatment during any stage of fin regeneration alters even-1 and evx-2 expression in the mesenchyme of the blastema and induces gene transcription in the wound epidermis. These results suggest that even-1 and evx-2 products are part of the molecular signals which are involved in pattern formation of the fin in connection with outgrowth.

Results

Expression of even-1 gene during fin regeneration

Control fins (unamputated) as well as stump tissues after amputation did not show even-1 expression. Amputation of the pectoral and caudal fins was rapidly followed by healing of the wound and even-1 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 even-1 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) even-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 even-1, but only in the inner layers of the epidermis, with the exception of its most basal layer.

Expression of even-1 gene during fin development

even-1 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 even-1 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 even-1 gene (Fig. 2F).

Expression of evx-2 gene during fin regeneration

Expression of evx-2 was similar to the spatio-temporal pattern of expression described for even-1 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. 3E). This has been observed with even-1 also but the gradient was not as obvious as with evx-2. Longitudinal sections confirmed that the epidermal cells do not transcribe evx-2 (Fig. 3F-G).

In the pectoral fin regenerates, this gradient of expression was observed as well (not shown). The signal was higher in the
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 (Sardino et al., 1996) and mammalian limb development (Dolle 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 eve1 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 telost 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

**TABLE 1**

**SINGLE RA 10^(-6)M TREATMENT OF ZEBRAFISH EMBRYOS: EFFECTS ON THE DEVELOPMENT OFPECTORAL FINS AND ON EVE1 EXPRESSION OBSERVED BY IN SITU HYBRIDIZATION OF WHOLE-MOUNTS.**

<table>
<thead>
<tr>
<th>Hour postfertilization at the time of treatment with 10^(-6) MRA</th>
<th>Length of treatment (hours)</th>
<th>a Phenotype 1 day later</th>
<th>b eve1 expression</th>
<th>a Phenotype 6 days later</th>
<th>b eve1 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>1</td>
<td>a same as control</td>
<td>a slight dyssmorphogenesis</td>
<td>b same as control</td>
<td>a obvious dyssmorphogenesis</td>
</tr>
<tr>
<td>27</td>
<td>2</td>
<td>a slight dyssmorphogenesis</td>
<td>b weak signal</td>
<td>a strong dyssmorphogenesis</td>
<td>b weak signal</td>
</tr>
<tr>
<td>42</td>
<td>1</td>
<td>a growth arrest</td>
<td>a growth arrest</td>
<td>b no signal</td>
<td>a growth arrest</td>
</tr>
<tr>
<td>42</td>
<td>2</td>
<td>a growth arrest</td>
<td>a growth arrest</td>
<td>b no signal</td>
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</tbody>
</table>
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; Fjose 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-dependent transcription factors (Means and Gudas, 1995). In zebrafish, RAR have been cloned (Joore et al., 1994; White et al., 1994) and RAR 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 internrays 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, RARα1 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 acid ethyl ester or MS222, Sigma) solution according to the Zebrafish book (Westferder, 1989).

Embryos, incubated at about 28° C, were obtained in the laboratory from natural crosses and staged according to the Zebrafish book (Westferder, 1985). 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.

Retinoid acid treatment
A single 24 h 10-5 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 were 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 fixation with osmium tetroxide which was omitted.

in situ hybridization
ev1 and ev2 expression was studied by in situ hybridization on whole mounts according to the method of Akimenko et al. (1994a). Antisense ev1 RNA digoxigenin labeled UTP probe was transcribed by T7 RNA polymerase (Boehringer) from a 400bp fragment (MscI-Stul) of the eve1 zebrafish gene provided by J.S. Joly.
ev2 RNA digoxigenin-labeled probe was transcribed by T3 RNA polymerase from a SmaI-AflII 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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