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Original Article

Caudal fin regeneration in wild type and *long-fin* mutant zebrafish is affected by retinoic acid

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ABSTRACT Zebrafish *(Danio rerio)* represents an ideal experimental model to tackle fundamental issues concerned with organogenesis during development and regeneration of complex body structures. We discuss here the development of the skeleton in zebrafish caudal fins, their regenerative ability in wild type and *long-fin* mutant adult fish, and how retinoic acid (RA), which induces duplications along the proximodistal axis in regenerating limbs, affects regeneration of the caudal fin. The dorsal and ventral lobes of zebrafish caudal fins are apparently symmetrical along the dorsoventral axis, but all of the skeletal elements and most of the soft tissues of both lobes originate from the ventral part of the embryo, as demonstrated by whole-mount staining of developing fish. Analysis of caudal fin regenerates in wild type adults does not reveal any difference in the regenerative ability of the two lobes, and in the length of the regenerate in comparison with the amputated part. In contrast, in the *long-fin* mutant the regenerated caudal fin is always somehow defective in that the original asymmetry in the length of the two lobes observed in this mutant is not reproduced in the regenerate. Furthermore, in the majority of the batches studied the regenerate is much smaller in size than the amputated part. This suggests that this mutant may be valuable to further our understanding of the mechanisms underlying growth control and patterning during regeneration. Finally, we show that the regenerating caudal fin is sensitive to RA-treatment, and clear teratogenic effects on the dorso-ventral axis are observed under many of the experimental conditions investigated both in wild type and *long-fin* mutants. However, RA can neither induce formation of extra-long fins in the wild type, nor restore mutant fins to their original length.

KEY WORDS: *development, regeneration, caudal fin, retinoic acid, long-fin mutant*

Introduction

Understanding the molecular events underlying growth, differentiation and patterning of vertebrate appendages during development and regeneration is of fundamental importance in biology. The zebrafish fin represents an ideal experimental model to study both development and regeneration, since it develops over a short time and regrows rapidly in the adult fish following amputation. Furthermore, zebrafish is amenable to genetic studies and while some fin mutants such as *long-fin* which was selected in the seventies in Russia, have been known and bred for a long time, many more have been recently isolated in laboratories in the USA and in Germany. In the *long-fin* mutant all fins grow much longer than in wild type zebrafish, but no other developmental defect is apparent. Although this mutant is easily available, analysis of the regenerative ability of its fins is still in its infancy (Géraudie *et al.*, 1993), and nothing is known about genetic heterogeneity within the population.

Fin regeneration represents an example of epimorphic regeneration since it occurs through formation of a blastema, a growth zone of rapidly proliferating progenitor cells (blastemal cells). It is not known whether the blastemal cells originate through a process of dedifferentiation similar to that believed to occur in the regenerating amphibian limb, but by 3 or 4 days after amputation they have accumulated at the end of the stump beneath the wound epidermis (Goss, 1969). Most studies on fin regeneration have focused on pectoral fins of *Fundulus heteroclitus* and other teleosts (Goss and Stagg, 1957; Géraudie and Singer, 1979; Wagner and Misof, 1992), and characterization of the regenerative process in zebrafish fins has only recently begun. We started this line of investigation by studying the regenerative ability of pectoral fins (Géraudie *et al.*, 1993, 1994), and have now extended our analysis to the caudal fin.

While the zebrafish pectoral fin is a paired bilateral structure, the caudal fin is a medial structure consisting of two lobes which are nearly perfect mirror images of each other along the dorso-ventral axis. The skeleton of each lobe is made up of 9 major rays (lepidotrichia) and the more dorsal and ventral of the rays, unlike the others, do not branch. Striated muscles connect the proximal

Abbreviations used in this paper: RA, retinoic acid; DMSO, dimethyl-sulphoxide.

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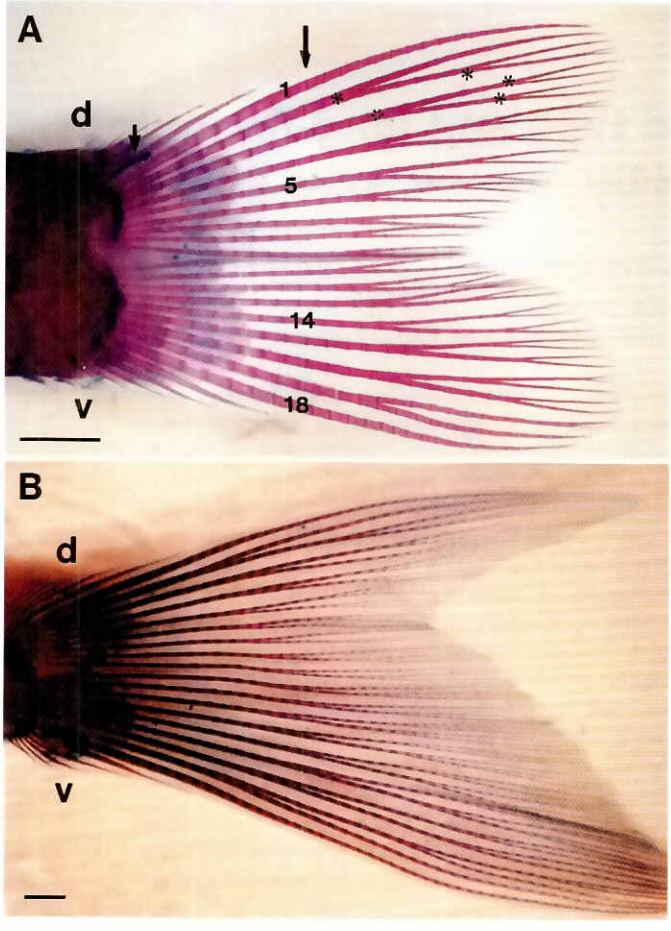

Fig. 1. Photograph of whole-mount preparations of unamputated caudal fins stained for cartilage (blue) and bone (red). (A) *Wild type zebrafish. The level at which fins are normally amputated is indicated by an arrow and the major lepidotrichia are indicated by sequential numbering starting from the most dorsal one (from 1 to 18). The short arrow points at a skeletal element present in the dorsal, but not in the ventral lobe. The dichotomies of rays 2 and 3 are indicated by stars.* **(B)** *Long-fin mutant zebrafish; in this example the ventral lobe is longer than the dorsal one. d, dorsal; v, ventral. Scale bar, 1 mm.*

end of the rays to the hypural bones and are present only at the base of the caudal fin which is covered by three large, adjacent scales. The rays are composed of segments of dermal bone which forms by mineralization of a collagenous basal lamella (Landis and Géraudie, 1990), and each segment is composed of 2 hemisegments which, in cross section, appear like a parenthesis (Becerra *et al.*, 1983). The rays grow in a proximal to distal direction by sequential addition of segments which, once formed, can become increasingly thicker but cannot elongate. Branching of the ray is originated by repeated splitting along the proximodistal axis, but the mechanisms controlling such patterning are not yet understood.

Recently, we have investigated the effects of exogenously administered retinoic acid on the regenerating zebrafish pectoral fin to establish whether patterning of the fin could be affected by this putative morphogen as observed in other regenerating systems (Niazi and Saxena, 1978; Maden, 1982; Stocum, 1991; Kelley *et al.*, 1993). Our work has shown that depending on the concentration and the experimental schedule, RA can affect both the dorsoventral and the proximodistal axis of the regenerating pectoral fin (Géraudie *et al.*, 1994). In fact, narrowing of the space between the rays, fusion of rays (teratogenic effect), and formation of more segments between the amputation level and the first fork (morphogenetic effect) were observed. Because of these results we have become interested in establishing whether patterning of the regenerating caudal fin can also be altered by RA, and whether the defects induced by RA in regenerating caudal fins parallel those observed in pectoral fin regenerates.

In a preliminary report on regeneration of the caudal fin in zebrafish, we have shown that amputation of the caudal fin distal to the point of insertion of the rays onto the basal bones results in the regeneration of the missing part, and that regeneration can be affected by RA (Géraudie *et al.*, 1993). The work presented here further describes the effects of RA treatment on caudal fin regeneration under different experimental conditions, and suggests that there is a different sensitivity to the same RA treatment in regenerating caudal and pectoral fins.

Results

Morphology and development of the caudal fin

The skeletal structure of the adult caudal fin is clearly visible in whole-mount preparations reacted with alcian blue which stains cartilage, and alizarin red which stains bone (Fig. 1A). We have numbered the major rays of the caudal fin from 1 to 18, 1 being the most dorsal and 18 the most ventral ray; these are the only major rays which never branch. A variation in the number of dichotomies (usually 2 to 7) in equivalent rays from different zebrafish is observed in all of the other rays. Furthermore, a different number of dichotomies is occasionally apparent within the same caudal fin between rays in the dorsal lobe and their counterparts in the ventral one (e.g. rays 2 and 17).

In long-fin mutants the fins are much longer than in wild type (Fig. 1B; see also Fig. 4D), and display much higher variation in the number of dichotomies. Analysis of whole-mount preparations shows that the abnormal length of these fins is not due to an augmented growth of individual segments, which do not appear to be significantly longer than in wild type, but to an increase in the number of segments formed in each ray. While in wild type the dorsal and ventral lobes of the caudal fin are equal in length, in the mutant a difference between the length of the lobes is usually observed, and animals in which either the dorsal lobe is shorter than the ventral, or vice versa, are observed. Furthermore, in some of the mutants the number of rays in the dorsal and ventral lobes is not the same, and the lobes are therefore asymmetrical along the dorso-ventral axis, suggesting that not all of the specimens studied might carry the same mutation (Fig. 1B). The total number of rays present in each caudal and pectoral fin examined, however, is always the same as in wild type.

Although in wild type zebrafish the 9 dorsal and 9 ventral rays of the caudal fin mirror each other, analysis of whole-mount preparations stained to detect the fin skeleton reveals a slight asymmetry between the dorsal and the ventral lobes due to the presence of an extra axial skeletal element at the base of the dorsal lobe (Fig. 1). Since internal asymmetry has been observed in other teleosts (Goodrich, 1930) and it is the consequence of a dorsal bending of the notochord which occurs during development, we have monitored the formation of the caudal skeleton in whole-mount preparations of developing zebrafish stained for bone and cartilage. As

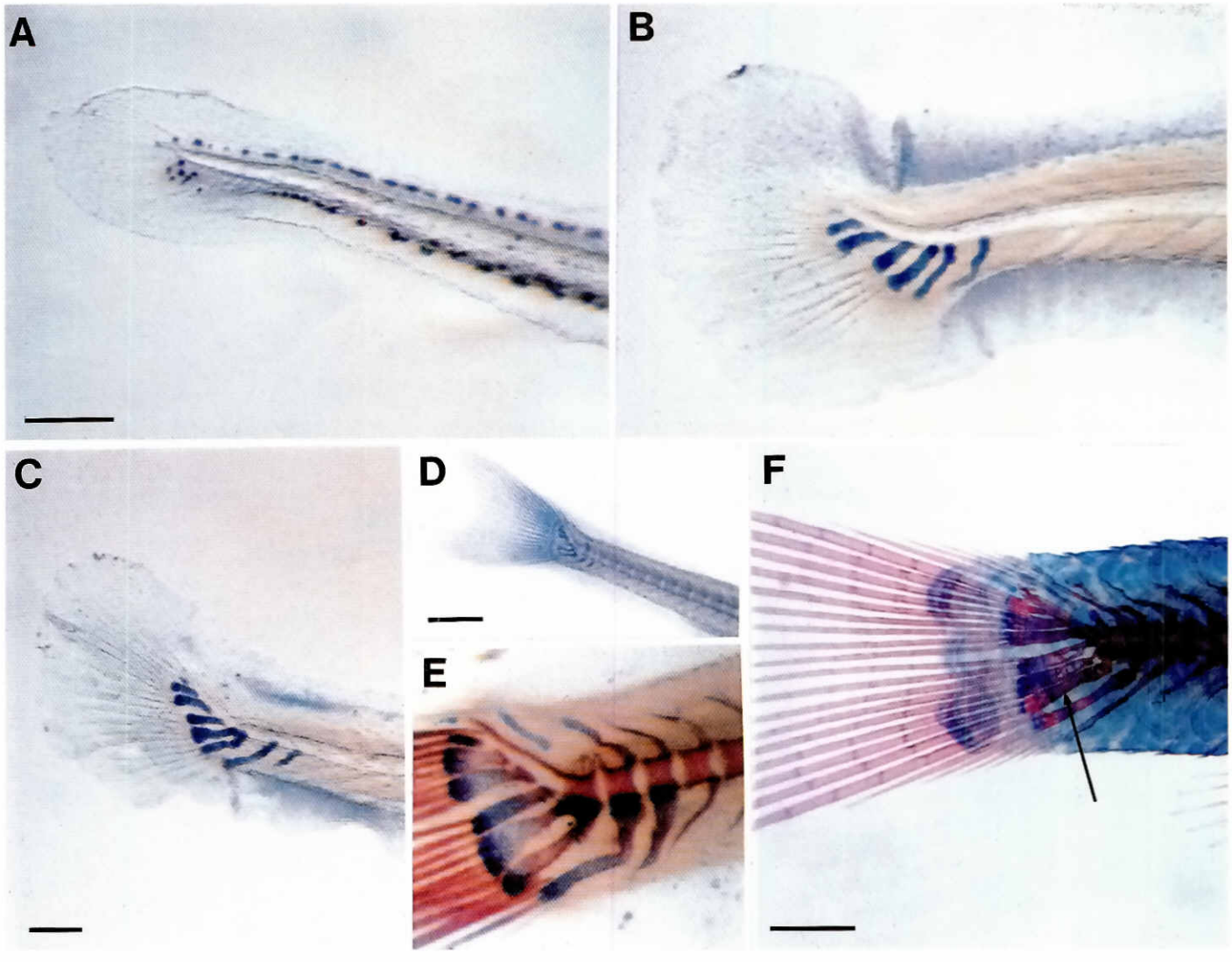

Fig. 2. Analysis of caudal fin development in whole-mount preparations of developing zebrafish stained for cartilage (blue) and bone (red). *Note the progressive bending of the notochord* **(A-D)**, *and ossification of the endoskeletal elements, hypurals, onto which the dermal rays articulate. The arrow in* **F** *points at one of the hypurals* **(A-F)**. *Scale bars: A,C, 0.2 mm; D,F, 0.5 mm. A,B and E are at the same magnification.*

shown in Fig. 2, the endoskeletal elements of the caudal fin originate ventrally from condensation of cartilage at the caudal end of the notochord, and skeletal elements are sequentially added dorsally, while the dorsal curvature of the notochord progressively increases (Fig. 2A-D). A curvature in the vertebral column is still clearly visible during endochondral ossification of the caudal fin

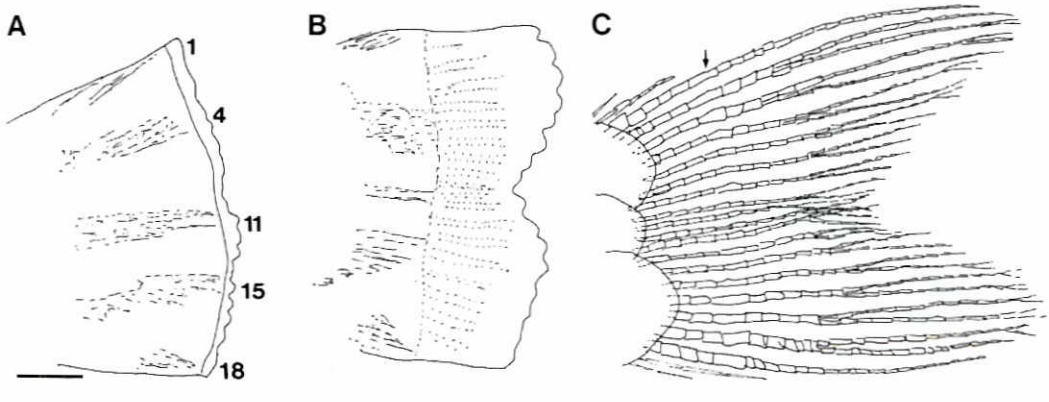

Fig. 3. Camera lucida drawings of regenerated fins at different times after amputation. *In* **(A)** *and* **(B)** *the pigments running along some of the rays in the stump are represented.* **(A)** *2-day regenerate (pigments running along rays 1, 4, 11, 15, 18).* **(B)** *7-day regenerate. All of the pigments of the regenerate are represented (black dots); note that their density is lower than in the stump.* **(C)** *The rays of a full regenerate 35 days after amputation are represented; the level of amputation is indicated by an arrow. Scale bar, 1 mm.*

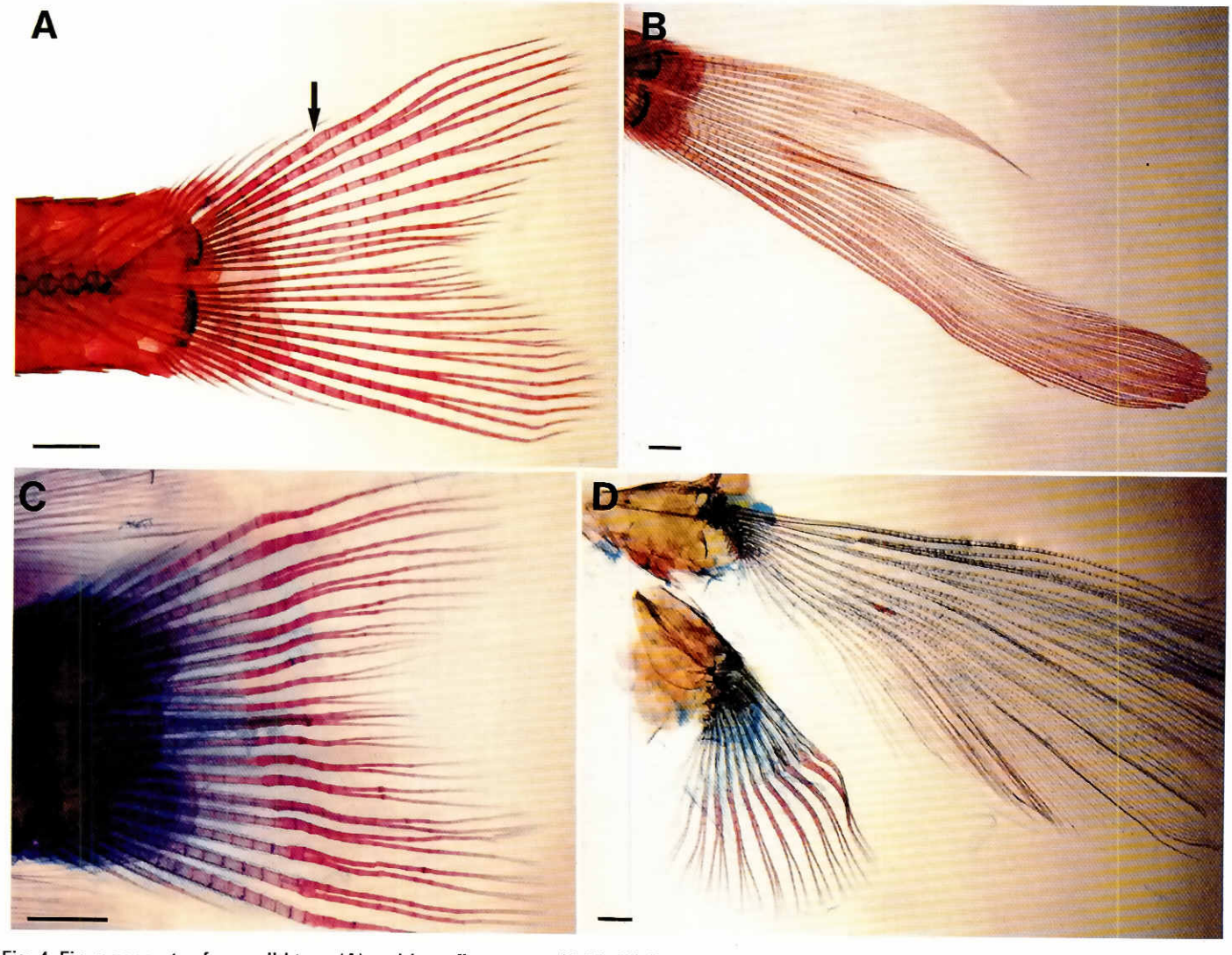

Fig. 4. Fin regenerates from wild type (A) and *long-fin* mutants (B-D). (A) *Dorsal lobe regenerate in the caudal fin of a DMSO-treated fish: DMSO does not appear to have any effect on the regenerative process. Note that the level of amputation is apparent (arrow).* **(B)** *long-fin mutant regenerate after amputation of the dorsal lobe of the caudal fin.* **(C)** *long-fin mutant regenerate 2 months after amputation of both lobes of the caudal fin.* **(D)** *Untreated long-fin pectoral regenerate and controlateral unamputated fin. Scale bar, 1 mm.*

cartilaginous elements (Fig. 2E), but it is hardly apparent at later stages of development (Fig. 2F). The rays, which are part of the dermal skeleton, originate from the ventral fin fold and are connected with skeletal structures of ventral origin (Fig. 2E), with the exception of the minor and more dorsal rays which seem to originate from the dorsal fin fold and articulate with the dorsal endoskeleton (Fig. 2F).

Caudal fin regeneration in wild type and long-fin mutants

When amputation is performed at the level of the articulation of the ray with the fin endoskeleton, or across the endoskeleton, regeneration does not occur (not shown). After amputation at the level shown in Fig. 1, the cut surface of the fin is rapidly covered by a wound epithelium, but no growth is apparent at macroscopic level 2 days after amputation in fish maintained at 25°C, as recorded by camera lucida drawings (Fig. 3A). A growth zone of undifferentiated mesenchymal cells, the blastema, is clearly visible in 3-4-day regenerates (not shown), and, a week after

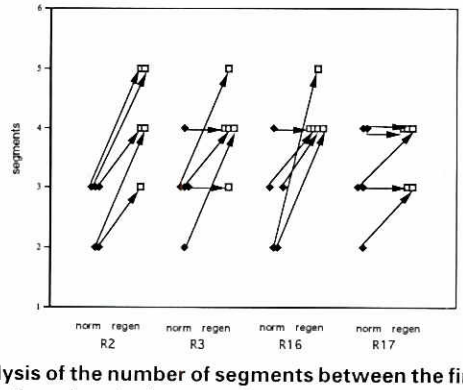

Fig. 5. Analysis of the number of segments between the first fork and the amputation plane in the amputated part of the fin (■) and in the corresponding regenerate (❑) of 5 fish. *Each arrow joins the number of segments in the normal (■) and regenerated (❑) fin from an individual animal. A significant difference between the number of segments is revealed by paired Student's t test in rays 2 and 16 ($p<0.05$ and 0.02 respectively), but not in rays 3 and 17.*

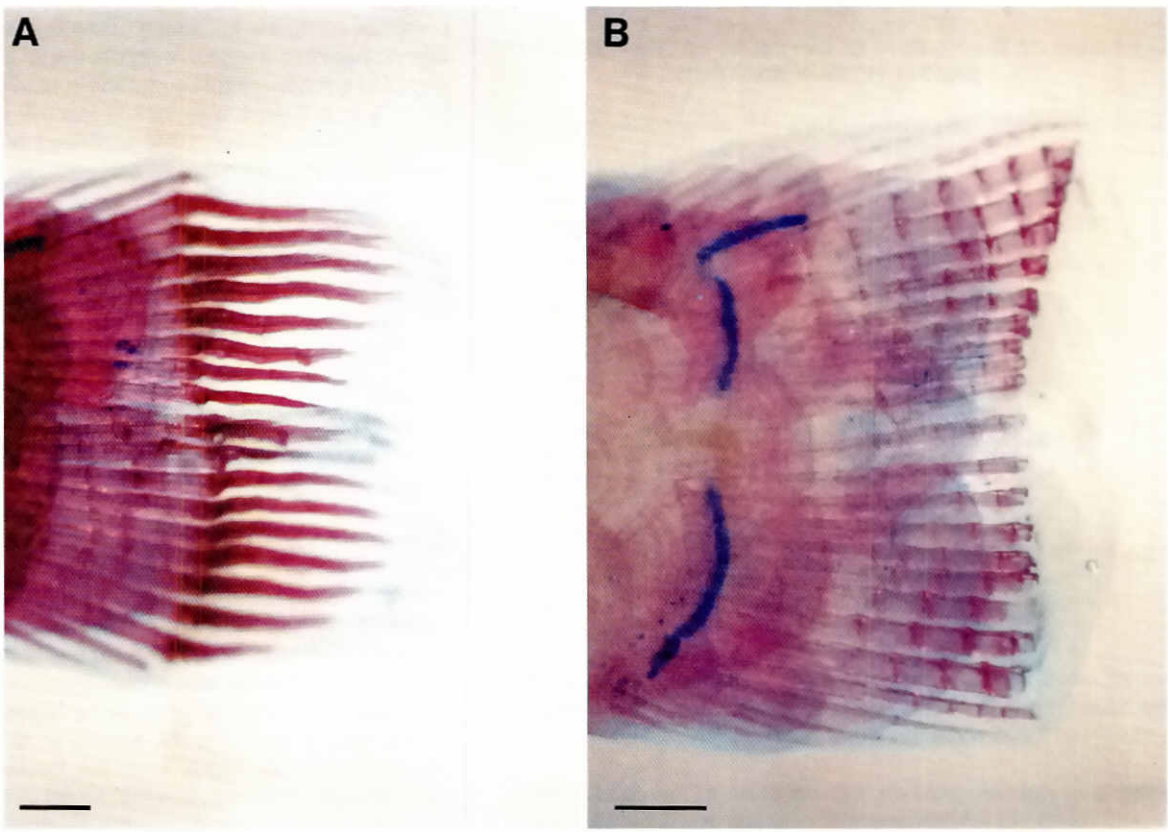

Fig. 6. Whole-mount preparations of 12-day fin regenerates treated for 7 days from the time of amputation with either DMSO (A) or 10^{-6} M RA (B). *Note that regeneration is more advanced in DMSO than in control treated fins. Scale bars, 0.5 mm.*

amputation, differentiation of the bony rays has begun (not shown) and is well advanced in a 12-day regenerate (Fig. 6A). By this stage pigmentation is present, but the density of the melanophores is lower and more scattered than in the unamputated part (Fig. 3B) and will maintain a similar appearance in the full regenerate (not shown). Differentiation proceeds in a proximal to distal direction, and the regenerated fin has regrown to its original length by 30-35 days after amputation in animals kept at 25°C (Fig. 3C). However, regeneration can be complete as early as 3 weeks after amputation if the fish are maintained at 28°C. The level of amputation is often apparent in full regenerates (Figs. 3C, 4A), as the ray segments at the level of amputation tend to be longer and slightly thicker than the others.

Regenerated fins from either untreated wild type zebrafish (Fig. 3C), or wild type zebrafish treated with DMSO (Fig. 4A), which is the solvent used to solubilize RA (see below), always grew to be equal in length to the amputated part (Fig. 5A). On the contrary, in 3 of the 4 batches of long-fin mutants studied, the regenerated caudal fins were not as long as the control ones, and amputation of either the dorsal (Fig. 4B) or ventral half (not shown) of the caudal fin proximal to the first dichotomy resulted in a lobe much smaller than the amputated one. In addition, while in the unamputated fin one of the lobes is usually shorter than the other, no difference in size between dorsal and ventral lobes was observed in regenerated mutant fins following amputation of all the fin proximal to the first dichotomy (Fig. 4C). Defective growth following amputation was not confined to the caudal fin but was also observed in pectoral fin regenerates (Fig. 4D). In order to establish that this was not simply due to a slower rate of regeneration, we followed these regenerating mutant fins for as long as 5 months, but in 3 batches the regenerated fins never grew bigger than that of a wild type zebrafish. However, we found that fish from one of the batches studied could regrow their fins to their original length but, as in the other *long-fin* mutants, the 2 regenerated lobes were equal in length.

To examine in more detail how faithfully the branching pattern of the caudal fin skeleton was re-established in the wild type regenerate, we carried out morphometrical analysis of fully regenerated wild type fins. We counted the number of segments and measured the distance between the amputation plane and the first branching point of the rays in the amputated part and in the corresponding regenerate either after total caudal fin amputation or amputation of only the dorsal or ventral half. Since analysis of the number of segments and of the distance in mm gave comparable results, only the data expressing the number of segments are presented here. From this analysis it appears that the first fork is often more distally located in the regenerated than in the control fin, but variability, not only among rays from different fish, but also among different rays in the same fish, was observed (Fig. 5). For example, statistical analysis of the data presented in Fig. 5 shows that the number of segments between the amputation plane and the first fork is significantly higher in regenerated rays 2 and 16 ($p<0.05$ and 0.02, respectively), but not in rays 3 and 17, as compared to the corresponding amputated rays. Despite this variability in the number of regenerated segments, the newly formed rays never started to branch more proximally than the

TABLE 1

SUMMARY OF THE EFFECTS OF DIFFERENT RA TREATMENTS ON CAUDAL FIN REGENERATION

RA concentration amputation	day after (days)	length of treatment regenerate	effect on full
$5x10^{-6}$ M	3	1	none
10^{-6} M	0	1, 2	none
	0	3, 4, 7	teratogenic
	1	1, 2	none
	1	3	teratogenic
	2	1	none
	2	2	none
	2	3	teratogenic
	3	1	none
	3	2	teratogenic
	3	3	teratogenic
	4	1	none
	4	2	teratogenic
	4	3	teratogenic
10^{-7} M	0	2	none
10^{-7} M	1	3	none
10^{-8} M	0	4	none

control ones. Similar results were observed when only one half of the caudal fin was amputated (not shown), indicating that dorsal and ventral lobes have the same regenerative ability.

Effects of retinoic acid on regeneration of the caudal fin

The effects of RA-treatment at concentrations between $5x10^{-6}$ and 10^{-8} M, and at different times after amputation of wild type fins (Table 1), were evaluated by external observation of the regenerate *in vivo* and analysis of the skeletal structure in whole-mount preparations stained for bone and cartilage (Figs. 6 and 7). The skeletal abnormalities observed (teratogenic effects) consisted of a small to significant dorsoventral reduction of the width of the fin and partial or complete fusion of some of the rays, but the overall length of the regenerate was comparable to that of control fins.

Under all of the experimental conditions studied RA slowed down the regenerative process (Table 1; Fig. 6A-B). However, neither fish treated with 10^{-7} and 10^{-8} M RA, nor fish treated with 10^{-6} M and $5x10^{-6}$ M RA for one day at different times after amputation showed any skeletal abnormality in their regenerated fins (Table 1). Treatment with 10^{-6} M RA for 2 days also failed to affect regeneration when started at the time of amputation, but it induced abnormalities if started at least 3 days after amputation (Fig. 7A), the time at which accumulation of blastemal cells occurs. These results indicate that the teratogenic response to RA depends not only on the concentration used, but also on the time at which the treatment is performed. When fish were treated for more than 2 days with 10^{-6} M RA teratogenic effects were always apparent, independently from the time at which the drug was added to the water after amputation (Table 1, Fig. 7B-F). However, the effect of RA increased with longer treatment, and the regenerated fins became progressively narrower and displayed a higher number of fused rays (Fig. 7B-F). From this analysis it is apparent that, to induce a teratogenic effect, RA must be present in the regenerating fin for at least 2 days at stages at which a large number of blastemal cells are present.

None of the rays seemed to be preferentially affected by RA, and the number of fusions usually did not exceed 2 per fin, although up to 5 fused rays could be present. The fusion was not always complete along the full length of the ray, and often a bony bridge formed between 2 adjacent rays at the level of amputation. Examples of fully regenerated caudal fins from wild type zebrafish treated for 2, 3 and 4 days with 10^{-6} M RA are shown in Fig. 7A-C. A response to RA similar to that described in wild type zebrafish was also observed in both caudal and pectoral regenerates in *long-fin* mutants (Fig. 7D-F).

Morphometrical analysis was performed in wild type caudal fins to establish whether, as previously observed in the pectoral fin, RA treatment could induce distalization of the first fork by increasing the number of segments proximal to it. No significant difference between the number of segments counted in DMSO and RA-treated fins was observed in any of the experimental conditions studied (Table 1); examples are given in Fig. 8, which shows the analysis of individual rays 2, 5 and 15 in full regenerates which had been treated either with DMSO or 10^{-6} M RA for 7 days from the time of amputation.

Discussion

We have studied the development of the caudal fin skeleton in zebrafish, its regenerative capability in wild type and *long-fin* mutants, and how exogenous administration of retinoic acid, a vitamin A metabolite which can alter patterning of many body structures in vertebrates, affects the regenerative process.

We have shown that the caudal fin of zebrafish, as in other teleosts(Goodrich, 1930), is of the homocercal type, since the external symmetry between the dorsal and ventral lobe does not reflect an internal symmetry, but is obtained through a progressive ventral to dorsal bending of the notochord. Therefore, the dorsal and ventral lobe do not have a dorsal and ventral origin respectively, but they both originate mainly from the ventral fin fold. The major endoskeletal elements of the caudal fin, the hypurals, form by broadening and elongation of the most caudal of the ventral haemal spines, and only a minor contribution to the caudal fin endoskeleton comes from the caudal neural spines which support the shortest lepidotrichia in the dorsal part of the caudal fin.

In wild type zebrafish the caudal fin regenerates fairly accurately and, apart from a decrease in the level of pigmentation, no significant defect either in the shape or length of the fin is detectable by external observation. However, a certain variation in the structure of the skeleton of regenerated fins, such as thickening of the lepidotrichia at the amputation plane, length of the most proximal regenerated segment, number of segments proximal to the first dichotomy, and the number of dichotomies, has been noticed in stained specimens. We have previously reported that in pectoral fins the number of segments proximal to the first dichotomy is rather constant, and that their number is significantly increased following amputation (Géraudie *et al.*, 1994). In contrast, analysis of the number of segments proximal to the first dichotomy carried out in normal and regenerated caudal fins has revealed that in both groups the variability between animals and between rays is much higher than in the pectoral fin(Géraudie *et al.*, 1994). Examination of individual rays in caudal fins, however, shows that overall the number of segments proximal to the first dichotomy tends to be higher in a regenerated ray than in the same ray before amputation.

We have compared the regenerative capability of fins in wild type and *long-fin* mutant zebrafish, a long established mutation which is still poorly characterized. Analysis of regeneration in this mutant has indicated a significant genetic diversity within the

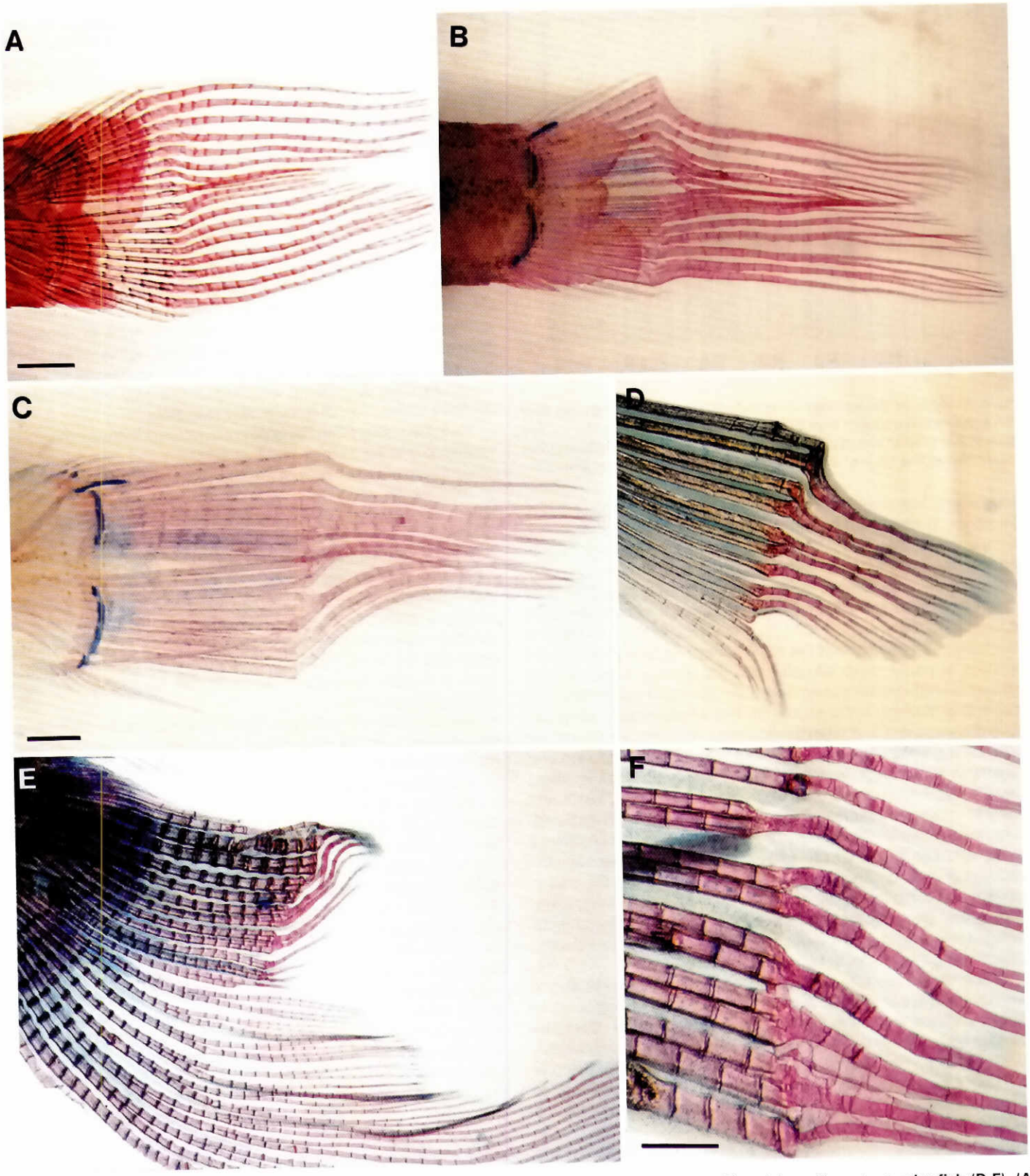

Fig. 7. Whole-mount preparations of fin regenerates treated with 10^{-6} M RA from wild type (A-C) and *long-fin* mutant zebrafish (D-F). (A) *Regenerate from a fish treated 4 days after amputation with RA for 2 days;* **(B)** *regenerate from a fish treated 1 day after amputation with 10^{-6} M RA for 3 days;* **(C)** *regenerate from a fish treated for 4 days from the time of amputation;* **(D)** *pectoral fin regenerate from a fish treated for 7 days with RA from the time of amputation;* **(E)** *caudal fin dorsal regenerate from a fish treated as in* **D. (F)** *Caudal fin ventral regenerate from a fish treated as in* **D**. *Scale bars: A, 1 mm; C,F, 0.5 mm. A,B,D and E are at the same magnification.*

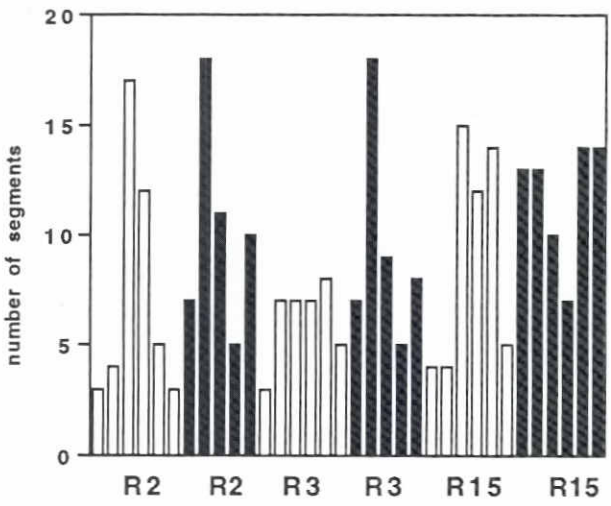

Fig. 8. Analysis of the number of segments between the first fork and the amputation plane in rays (R) 2, 3 and 15 of fish treated for 7 days with either DMSO (empty bars) or 10^{-6} M RA (solid bars) starting the treatment at the time of amputation. *Each bar represents a single ray.*

population. Nonetheless, in all of the mutant fish studied, the difference in size between the dorsal and ventral lobes observed in the unamputated caudal fin is not reproduced in the regenerate, independently from its final length. Furthermore, all of the fish from 3 of the 4 batches used in our study were unable to regrow their caudal and pectoral fins to their original size, as if amputation could at least partially revert the long-fin phenotype to the wild type. In conclusion, it appears that the regenerative capability of long-fin mutants is partly impaired, and that the defects observed are concerned with growth control both in pectoral and caudal fins, and also with patterning in the caudal fin. The differential growth of the fin in development and regeneration may be a useful indicator for establishing when and how the transition between regulation and regeneration occurs, in that the embryo loses its ability to "readjust the fate map of an undifferentiated primordium" (Slack, 1980) and starts to undergo epimorphic regeneration as the adult in response to removal of tissue. Altogether, it appears that *long-fin* mutants will prove valuable to further our understanding of the mechanisms underlying growth control during regeneration of the fin, and to provide additional information on the relationship between regeneration and development and the events underlying these processes. However, *long-fin* mutant lines will have to be selected before using this model to tackle these issues.

The endogenous role and mechanisms of action of RA during development are still controversial, but this compound appears to play a fundamental role in the patterning of many body structures (Holder and Hill, 1991; Osmond *et al.*, 1991; Bryant and Gardiner, 1992; Mohanty-Hejmadi *et al.*, 1992; Morriss-Kay, 1993; Chen *et al.*, 1994). Exogenous RA has been shown to induce teratogenic effects on developing systems and to affect regenerating systems such as the newt limb by inducing duplications of limb segments along the proximo-distal axis (Tamarin *et al.*, 1984; Scadding and Maden, 1986; Brockes, 1989; Stocum, 1991). In addition, we have reported that RA can induce both teratogenic and morphogenetic effects in the regenerating pectoral fin of zebrafish (Géraudie *et al.*, 1994). The work presented here shows that the regenerating caudal fin is also affected by RA treatment, but its effects are in some respects different not only from those observed in the newt limb, but also in pectoral fins under the same experimental conditions. Nonetheless, unlike the regenerating limb, neither pectoral nor caudal fins ever grow longer than controls when treated with RA.

In the pectoral fin certain concentrations of RA, which are not teratogenic or very mildly so, induce formation of extra bone segments between the amputation plane and the first dichotomy, although the overall length of the fin is similar to that of controls. However, we have not convincingly observed a similar morphogenetic effect in caudal fin regenerates. On the contrary, clear teratogenic effects are observed under many of the experimental conditions investigated both in wild type and *long-fin* mutants also at doses which do not have teratogenic effects on the pectoral fin, indicating a different sensitivity to RA of these 2 structures. Expression of retinoic acid receptors in caudal fin blastemas has been recently demonstrated (White *et al.*, 1994), but it is not known yet whether the different sensitivity to RA we have observed in pectoral and caudal fins may be related to a different distribution of retinoic acid receptors.

As in the pectoral fin, RA appears to affect selectively the dorsoventral axis of the caudal fin both in wild type and *long-fin* mutants, since it induces narrowing of the fin which usually results in partial or total fusion of several rays. On the contrary, no significant difference between DMSO and RA-treated caudal fins along the proximodistal axis has been observed. We cannot exclude the possibility that a small effect on the number of segments forming between the amputation plane and the first dichotomy, similar to that reported in the pectoral fin might occur since the high variability among animals might mask it. Nevertheless, the random internal variation observed between rays in untreated regenerates suggests that this is not the case, and that the caudal fin skeleton is quite a flexible structure in terms of the number of segments constituting each ray.

The extent of the defects induced by RA in caudal fin regenerates depends on the drug concentration and the length of treatment. In addition, the time at which the treatment is started seems to be important, since 10^{-6} M RA for 2 days is teratogenic if started 3 days after amputation, but not at earlier stages of regeneration. Therefore, it appears that RA is most effective in inducing teratogenesis after the phase of accumulation of blastemal cells has been completed. Cellular analysis of the changes induced by RA during regeneration supports this view, and suggests that the teratogenic effects observed may be the result of significant RA-induced apoptosis in the wound epidermis, which would decrease its width and consequently affect patterning of the underlying blastemal mesenchyme (Ferretti and Géraudie, 1994). Interestingly, although both regenerating limbs and fins are most sensitive to RA treatment when blastemal cells have accumulated and started to proliferate, the range of effects observed on caudal fins are different from those on limbs and pectoral fins, in that only teratogenic, but not morphogenetic effects can be elicited. Understanding the molecular basis underlying these differences between limbs, paired fins and caudal fins will have to wait for a thorough comparative analysis of these systems both at the cellular and molecular level.

Our results show that although the caudal fin is more sensitive to RA than the pectoral fin, the number of segments proximal to the first fork is apparently not affected by RA treatment, and that RA can neither induce formation of extra-long fins in the wild type, nor restore to their original length fins of mutant zebrafish. Teratogenic

doses of RA induce the same type of defects in caudal and pectoral fins, suggesting that equivalent cell populations are the targets of RA action in these 2 systems, and that regenerating fins can provide a relatively simple and accessible in *vivo* model to study the cellular and molecular mechanisms of RA-induced teratogenesis.

Materials and Methods

Animals

Wild type and mutant adult zebrafish, *Danio rerio* were obtained from Sidoli SARL (Noisy-le-Grand, France) and Wundpet (Birmingham, UK). Both stock and experimental animals were kept at 25°C and fed daily. Animals were anesthetized in a 0.1% solution of MS222 (Sigma) and either the whole caudal fin, or only the dorsal or ventral half was amputated proximally to the first dichotomy (Fig. 1). Each experimental group consisted of 6 to 8 animals. The regenerative process was followed by regular observation of the animals before sacrifice. Animals were sacrificed by treatment with an overdose of MS222 between 1 day and 2 months after amputation and whole-mount preparations of regenerating fins were compared either with fins from unamputated animals or with the unamputated half from the same animal in the case of partial amputation.

Morphological and morphometrical analysis

In order to analyse the fin skeleton, whole-mount preparations were stained with alcian blue and alizarin red as previously described (Simons and Van Horn, 1971; Géraudie *et al.*, 1994). Morphometrical analysis was always performed in fully regenerated animals which had been amputated at least 45 days before collection, by counting the number of segments or by measuring the length between the level of amputation and the first dichotomy of the ray in whole-mount preparations of fins stained with alcian blue and alizarin red (Géraudie *et al.*, 1994). At least 6 rays from each fish were analyzed in each experiment. Statistical evaluation of the results was by paired Student's *t* test.

Retinoic acid treatment

The effect of different concentrations of retinoic acid (RA, all-trans retinoic acid; Sigma) administered under different experimental schedules was assessed by adding the appropriate amount of a 5 mg/ml stock solution of RA solubilized in DMSO (dimethyl-sulphoxide, Sigma) to the water as previously described (Géraudie *et al.*, 1994). The RA concentrations studied ranged between 10^{-6} and 10^{-8} M, and treatments were performed at different times after amputation and for different lengths of time (Table 1). Untreated animals and animals treated with the RA in DMSO were included in each experiment as controls. Statistical evaluation of the results was by unpaired Student's t test.

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