

# Regeneration of lower and upper jaws in urodeles is differentially affected by retinoic acid

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**ABSTRACT** The vitamin A derivative retinoic acid (RA) is a powerful teratogen which can induce severe craniofacial and limb malformations if administered at certain stages of gestation. In addition this compound has been shown to affect patterning in regenerating systems. A classical example is the induction of supernumerary structures along the proximodistal axis of the regenerating amphibian limb. We have investigated the effect of RA on other regenerating systems, the amphibian lower and upper jaws, both in developing and adult animals. We report here that RA does not induce formation of extra structures either in the lower or in the upper jaw of adult newts under experimental conditions where duplications of the regenerating limb occur. However, RA selectively induces severe malformations in the upper jaw regenerate that resemble those induced in avian and mammalian embryos. Analysis of the expression of the newt retinoic acid receptors  $RAR\alpha$  and  $\delta$  in upper and lower jaws showed that  $RAR\alpha$  was expressed at a significant level in the wound epidermis, but not in blastemal cells, whereas no  $RAR\delta$  could be detected in the regenerate either by *in situ* hybridization or by using an anti- $RAR\delta$  antibody. Therefore, unlike in the limb, in jaws  $RAR\delta$  is not up-regulated following amputation, and this difference in expression may be causally related to the different effects induced by RA on jaws and limbs. In order to establish whether retinoids affected regeneration of developing jaws in a similar fashion, their effects were studied in animals whose jaws had been amputated at different developmental stages. Under the experimental conditions used overall growth retardation and head defects were observed in the majority of embryos which had been amputated and treated with retinol palmitate (RP) between stages 26-28 and 38-39. In contrast, patterning of upper jaw regenerates in larvae amputated at stage 45 was not significantly affected by the treatment, although the early phase of regeneration was slower than in controls. The different responses to retinoids of regenerating facial structures in embryos, larvae and adults will be discussed.

KEY WORDS: *jaw, development, regeneration, retinoid, urodele amphibian, RAR*

## Introduction

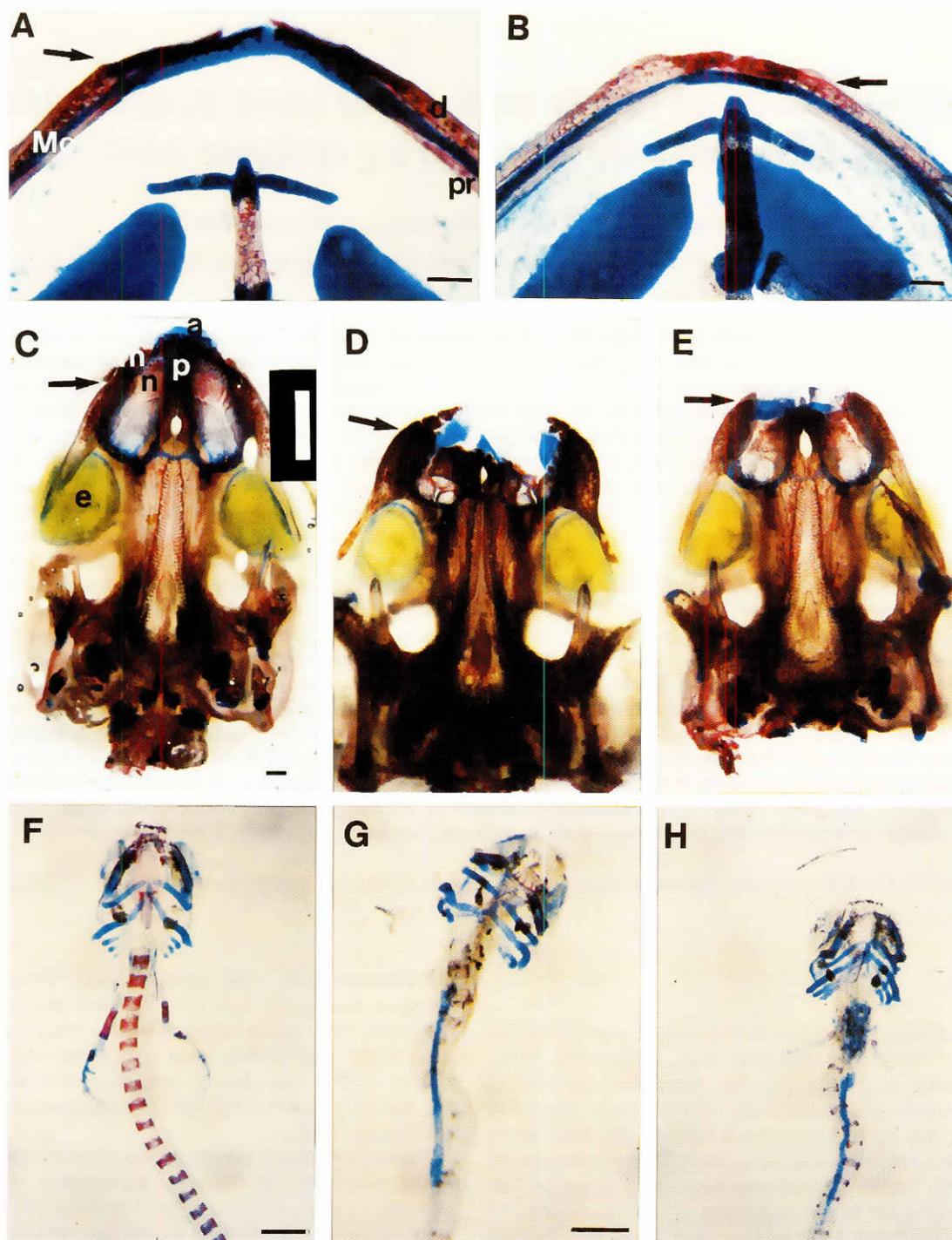
Formation of facial structures during development relies on complex tissue interactions (Wedden *et al.*, 1988; Milos, 1992; Thorogood, 1993) and errors in these processes that lead to serious craniofacial abnormalities are not infrequent. Vitamin A and its derivatives, such as retinol and retinoic acid (RA), affect morphogenesis in many developing systems. Their action appears to be specifically targeted to particular developing structures that are differently affected by RA depending on the developmental times at which the treatment is performed. It is well documented that RA induces a number of craniofacial abnormalities in vertebrates, including humans (Lammer *et al.*, 1985; Sulik *et al.*, 1988; Wedden, 1991; Morriss-Kay, 1993), and differentially affects upper and lower jaws in certain species. In fact, RA-treatment of chick embryos at stage 20, where the facial processes are already apparent, results in truncations of the upper jaw, but not of the lower

one (Tamarin *et al.*, 1984; Wedden, 1991). Differential effects of the same dose of RA have been reported also in the case of the amphibian limb, where retinoids induce morphogenetic duplications in the regenerating newt limb (Niazi and Saxena, 1978; Maden, 1982), while causing deletion of limb skeletal elements, and therefore hypomorphic limbs, during development (Scadding and Maden, 1986).

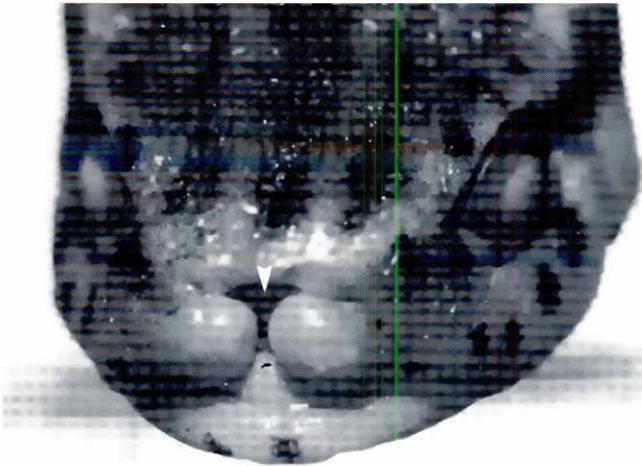
Retinoid effects are mediated by two families of nuclear receptors of the steroid/thyroid hormone superfamily, the retinoic acid receptors (RARs), and the retinoid X receptors (RXRs), that act as transcription factors and have distinct cellular functions (Leid *et al.*, 1992; Pecorino *et al.*, 1994, 1996). Three RARs and their spliced forms have been isolated in urodele amphibians,  $RAR\alpha1$  and 2,  $RAR\beta$ , and  $RAR\delta1$  and 2 (Giguere *et al.*, 1989; Ragsdale *et al.*,

*Abbreviations used in this paper:* RA, retinoic acid; RP, retinol palmitate; RAR, retinoic acid receptor.

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**Fig. 1.** Whole-mount preparations of either normal (A,C,F) or retinoid-treated (B,D,E,G,H) lower (A,B) and upper (C-E) adult jaw regenerates, and of embryos amputated at stage 26-28 and left to develop to stage 48-50 (F-H) stained for bone (red) and cartilage (blue). The level of amputation is indicated by arrows. (A) 12-week lower jaw regenerate from a DMSO-treated animal. (B) 12-week lower jaw regenerate from a RA-treated animal; no significant difference from the DMSO control is apparent. (C) 20-week upper jaw regenerate from a DMSO-treated animal; a, alary cartilage; p, premaxilla; m, maxilla; n, nasal; e, eye. (D-E) Examples of 20-week upper jaw regenerates from a RA-treated animal. Note the severe defects induced by the treatment; (F) control embryo amputated at stage 26-28 and left to develop to stage 48-50; control; (F-H) control embryos amputated at stage 26-28, treated for 2 days with  $10^{-7}$  M RP starting 1 day after amputation and left to develop to stage 48-50. RP treatment retards embryonic growth (note the difference in ossification between control (F) and treated (G-H) embryos) and affects regeneration of facial structures (note that the head is club-shaped). Scale bars, 0.5 mm. C-E, and F and H are at the same magnification; the black bar in C with the bright window (mask for data projection) is the reference scale of the 35 mm film cassette. For further details on jaw skeleton see Ghosh et al. (1994). Abbreviations: a, alary cartilage; d, dentary bone; e, eye; Mc, Meckel's cartilage; m, maxilla; n, nasal; p, premaxilla; pr, prearticular bone.



**Fig. 2. Gross morphology of a 20-week upper jaw regenerate from a RA-treated animal with a cleft lip and palate-like appearance. Note the midline clefting (arrowhead).**

1989, 1992). The newt alpha and the partial sequence of the beta receptor presently available display high homology with the higher vertebrate RAR $\alpha$  and RAR $\beta$ , respectively, and RAR $\delta$ 2 is likely to be the newt homolog of RAR $\gamma$ . In contrast, RAR $\delta$ 1, although related to RAR $\gamma$ , diverges significantly from it in its sequence at the N- and C-terminus.

We have recently investigated both at a morphological and molecular level the events underlying regeneration of both upper and lower jaws in urodele amphibians (Ghosh *et al.*, 1994; Ferretti, 1996). From this work it has emerged that the regenerating jaw represents an important model for studying the mechanisms underlying regeneration of facial structures and their patterning. Advances in our understanding of such mechanisms could be greatly accelerated by the possibility of perturbing this regenerating system, and it is therefore important to start to investigate the effects of molecules which are likely to affect regenerating jaws.

For the reasons outlined above it seems conceivable that RA treatment may affect regeneration of the jaw in amphibians, either by inducing formation of extra structures, as in the adult regenerating limb in urodeles (Stocum, 1991), or by causing deletion of skeletal elements, as in the developing amphibian limb and in the upper jaw of the chick embryo (Scadding and Maden, 1986; Wedden, 1991). Therefore, it will be important to assess the long-term effects of RA on regenerating upper and lower jaws, and to start with it will be logical to use the experimental conditions which induce duplications in regenerating limbs, since this will allow a direct comparison of the effect of RA on these different regenerating systems. Since RARs mediate the cell response to RA, it will also be important to know how the different RARs are distributed in jaw blastemas. In fact, although RAR expression has been widely studied in regenerating limbs, no information is yet available on the distribution of RARs in regenerating jaws. Information on the expression of RAR $\delta$  will be of particular interest because this is the most abundant RAR detected in regenerating limbs (Ragsdale *et al.*, 1992), and is related to the mammalian RAR $\gamma$ , which in the developing head is initially expressed in craniofacial mesenchyme and later becomes restricted to cartilaginous elements (Ruberte *et*

*al.*, 1990). Finally, no information on the response to retinoids during jaw development and regeneration is yet available, and this is essential if one is to use regenerating jaws as an additional model for the study of craniofacial abnormalities.

In order to begin to elucidate the effect of retinoids on regenerating jaws, how these may be mediated, and whether they affect developing and regenerating jaws in a similar fashion, we have studied the effects of RA on regenerating upper and lower adult jaws, the expression of RAR $\alpha$  and RAR $\delta$  in jaw blastemas by *in situ* hybridization and immunocytochemistry using an antibody against RAR $\delta$ , and compared the response to retinoids in developing and regenerating jaws.

The work presented here shows that 1) RA does not produce duplications of structures either in the lower or in the upper jaw, but selectively induces truncations in the upper jaw regenerate that resemble those induced in developing avian upper jaws; 2) RAR $\delta$  is not up-regulated in jaw blastemas, unlike in limb blastemas, although it is present in various tissues of the stump; 3) developing and regenerating jaws respond in a similar fashion to retinoid treatment, since deletions and hypomorphic structures, rather than duplications, are observed in all the experimental conditions tested. The relevance of these findings will be discussed within the context of regeneration and development of limbs and jaws.

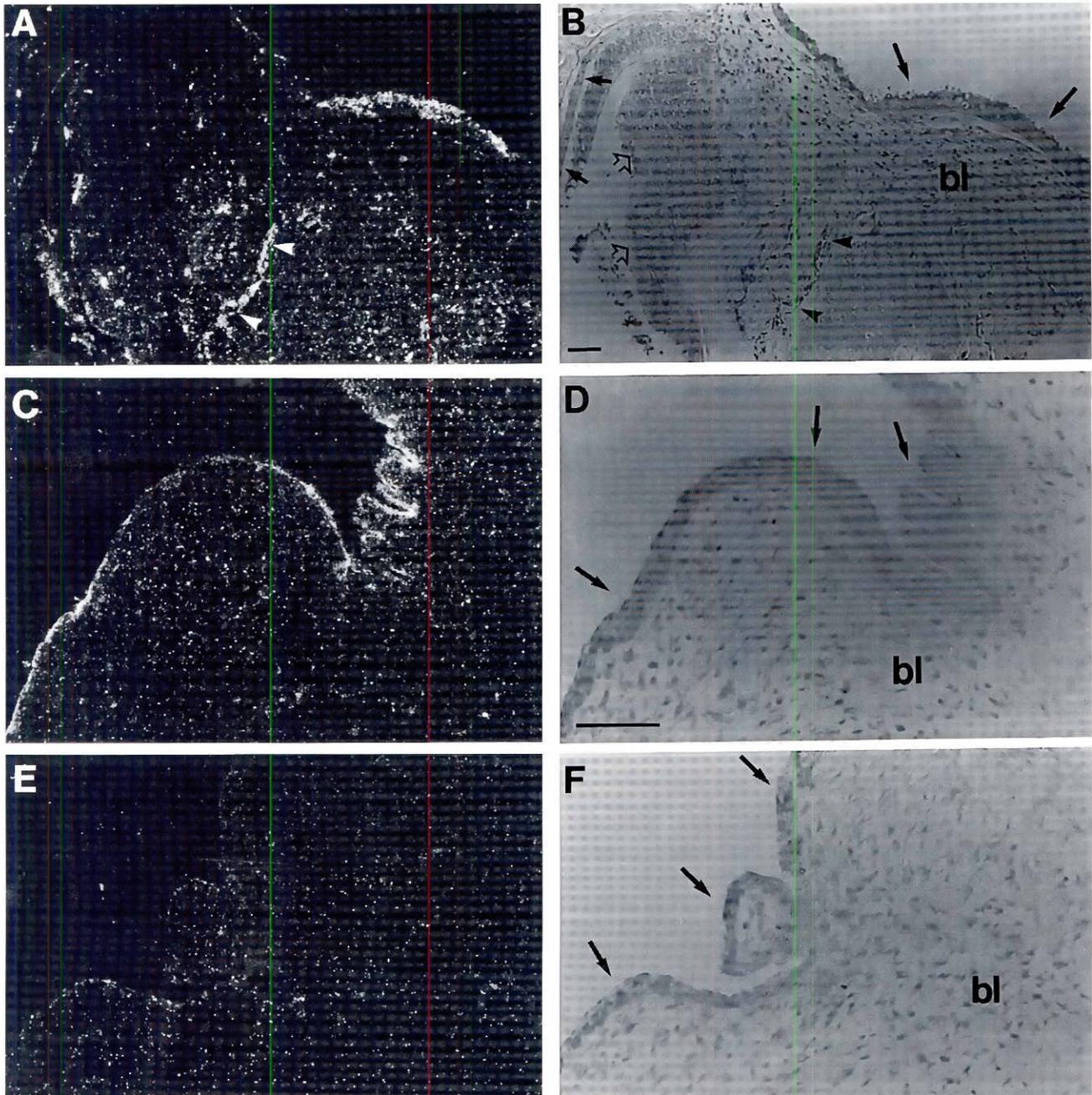
## Results

### **Effect of retinoic acid (RA) treatment on regeneration of lower and upper jaws**

In order to assess whether RA affected jaw regeneration, adult animals were injected with a dose of RA which is known to induce duplications in regenerating limbs, either one or two weeks after amputation of either lower or upper jaws. While mortality following injection of such a dose of RA in animals which have undergone limb amputation is almost non-existent (unpublished observation), animals regenerating their jaws appeared to be more sensitive to the treatment, and typically only between 30 to 50% of the injected animals survived the duration of the experiment. The data presented here do not include animals that died before the time points discussed, although the effects, or lack of them, described below were usually already apparent in animals which had survived for 5-6 weeks after surgery.

Independently from the time of injection, RA treatment appears to slow down regeneration of both upper and lower jaws for a few weeks, as detected by regular observation of regenerating animals and by analysis of whole-mount preparations. However, by 12 weeks after amputation, when jaw regeneration is virtually complete, no significant difference in lower jaw regenerates is observed between animals treated with either DMSO or RA 1 week after amputation (Fig. 1A-B).

In contrast, RA treatment appeared to affect significantly the regenerating upper jaw and major abnormalities were clearly apparent 12 weeks after amputation in all of the surviving RA-treated animals (5). Our previous work had shown that, although there is a higher variability in the regeneration time of upper jaws in comparison to lower jaws, upper jaw regeneration is very advanced 12 weeks after amputation. Nonetheless, in order to rule out completely the possibility that the differences observed could be due to growth delay induced by the treatment, animals injected with RA 1 week after amputation were left to regenerate for up to 20 weeks (Fig. 1C-E). The animals injected only with DMSO, which



**Fig. 3.** Localization of  $RAR\alpha$  (A-D) and  $RAR\delta$  (E-F) by *in situ* hybridization in upper (A-B) and lower (C-F) regenerating jaws. (A)  $RAR\alpha$  expression in a 2-week upper jaw regenerate and (B) corresponding Nomarski image. The transcript is detected in the wound epidermis (arrows), but not in the blastema mesenchyme (bl); note that also the nasal cartilage (short arrows) and the nasal epithelium (empty arrows) are negative; a highly pigmented area, which is detected also in dark field, is indicated by arrowheads; (C)  $RAR\alpha$  expression in a 3-week lower jaw regenerate and (D) corresponding Nomarski image. Note the presence of the transcript in the wound epidermis (arrowheads), but not in the blastema mesenchyme (bl); (E)  $RAR\delta$  expression in 3-week lower jaw regenerate (same as in C-D) and (F) corresponding Nomarski image. Note that both the wound epidermis (arrows) and the blastema mesenchyme (bl) are negative. Scale bars, 100  $\mu$ m. C-F are at the same magnification.

is the RA vehicle, appeared normal at gross morphological level (Fig. 1C). However, minor defects such as slight asymmetry in the size of the naris, slightly incomplete regeneration of the nasal fenestration, delayed ossification of maxilla and premaxilla were observed in half of them. In the RA-treated group 2 of the 7 surviving animals analyzed at 20 weeks presented minor defects

similar to those observed in some of the DMSO-treated ones, whereas the remaining jaws were severely misshapen (Fig. 1D-E). Altogether, 10 of the 12 RA-treated animals analyzed presented major abnormalities which were never observed in DMSO-treated animals. In some cases the abnormalities observed were reminiscent of a cleft lip and palate syndrome (Figs. 1D and 2), whereas

TABLE 1

SUMMARY OF RAR $\alpha$  AND RAR $\delta$  DISTRIBUTION IN JAWS

	jaw blastema	jaw WE*	normal jaw
RAR $\alpha$	-	+	muscle, teeth, glands
RAR $\delta$	-	-	cartilage <sup>§</sup> , muscle, teeth, glands, normal epidermis

\*wound epidermis; <sup>§</sup>expression in cartilages was consistently detected by RP6 immunoreactivity, but rarely by *in situ* hybridization.

in other animals little regeneration of the premaxilla, maxilla and cartilages had occurred (Fig. 1E), and a misshapen cartilage had formed at the amputated surface. In particular, the premaxilla and the regenerated cartilage, which as previously reported (Ghosh *et al.*, 1994) replaces nasal bone and the vomer in controls, were either not present, or present only in "patches". Furthermore, the nasal septum, if present at all, was incomplete.

#### Expression of retinoic acid receptors (RARs) in adult jaw blastemas

In order to establish whether there was any correlation between the effect of RA on patterning of upper jaw regenerates in adult animals and expression of the retinoic acid receptors (RARs), RAR $\alpha$  and RAR $\delta$ , we studied their distribution by *in situ* hybridization and, in the case of RAR $\delta$ , also by immunocytochemistry (Figs. 3 and 4) in jaw blastemas. Two- and three-week blastemas were used because at earlier stages of jaw regeneration not many blastemal cells are present and it can be difficult to clearly identify them in sections which have undergone the harsh processing involved in the *in situ* hybridization technique.

Neither RAR $\alpha$  nor RAR $\delta$  transcripts appear to be expressed in the blastemal cells of either lower or upper regenerating jaws (Fig. 3). In contrast, clear expression of RAR $\alpha$  (Fig. 3A-D), but not of RAR $\delta$  (Fig. 3E-F), was detected in the wound epidermis of both jaws. No RAR $\alpha$  was detected in nasal cartilage of the upper jaw stump (Fig. 3A), but in a few instances some RAR $\delta$  hybridization product was observed in discrete areas of nasal cartilage (not shown). Therefore, unlike the limb blastema (Giguere *et al.*, 1989; Ragsdale *et al.*, 1989, 1992), jaw blastemas do not appear to express significant amounts of either RAR $\alpha$  or RAR $\delta$  in mesenchymal progenitor cells, and expression of RAR $\alpha$  appears to be restricted to the wound epidermis, although various tissues of the stump express these receptors as summarized in Table 1 (see also Fig. 4).

In order to confirm the results obtained by *in situ* hybridization and establish whether the pattern of expression of the RAR $\delta$  protein reflected that of the transcript, we used an antibody to RAR $\delta$ 1, RP6, previously developed and characterized by Hill *et al.* (1993). Analysis of regenerating lower (Fig. 4A-B) and upper jaw blastemas (not shown) confirmed that RAR $\delta$ 1 is not up-regulated in these blastemal cells, since no RP6-positive nuclei could be detected in 3-week lower jaw regenerates. The wound epidermis was also devoid of RAR $\delta$ 1 reactivity (Fig. 4A-B), but normal epidermis, glands and muscle were strongly positive (Fig. 4A-E). However, as exemplified in Figure 4A-D, the number of positive epidermal and gland cells varied, and, whereas in some areas virtually all the nuclei of these tissues were positive, in others

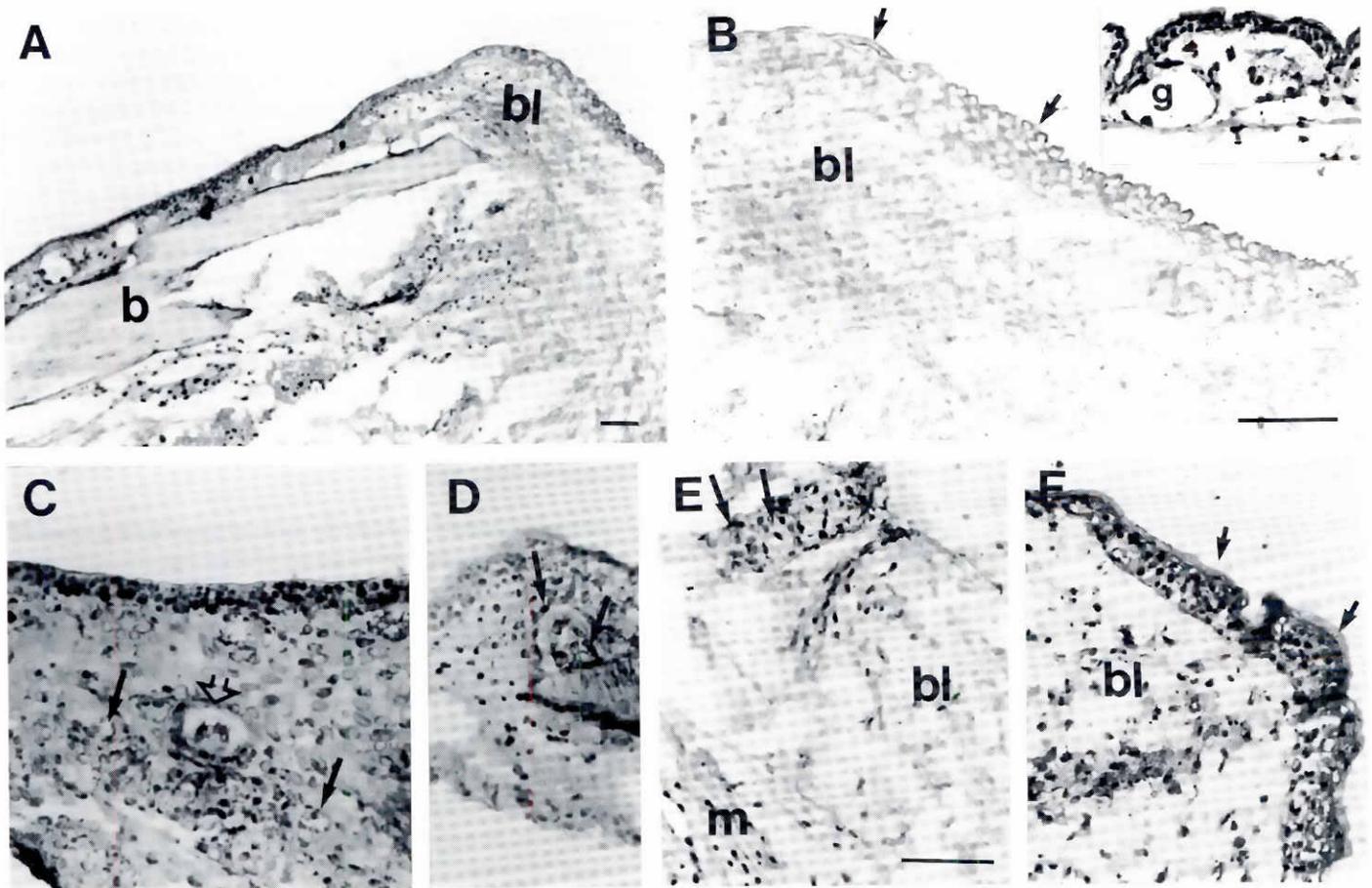
numerous negative cells were present. In addition, RAR $\delta$  is expressed in the dental lamina, in the dental pulp and in the enamel epithelium of the tooth (Fig. 4C-D) as also observed by *in situ* hybridization (not shown). However, whereas the RAR $\delta$  transcript was seldom detected in jaw cartilages by *in situ* hybridization, strong RP6 reactivity was observed both in Meckel's cartilage (Fig. 4A,D) and nasal cartilage (not shown). It is not clear at present whether this discrepancy may be due to differences in the turnover of the RAR $\delta$  transcript and protein. Nevertheless, the reactivity of RP6 antibody in lower and upper jaw blastemas and stump tissues paralleled the distribution of the transcript, and confirmed that RAR $\delta$  is neither expressed in jaw blastemal cells nor in the wound epidermis (Fig. 4). RAR $\delta$  expression in jaw blastemas is therefore significantly different from that in limb blastemas, since, as previously reported (Hill *et al.*, 1993) and confirmed here (Fig. 4E), RP6 strongly reacts with both mesenchymal and epidermal cells of the newt regenerating limb.

#### Retinol palmitate (RP) effect on jaws of developing animals

Analysis of developmental events in *Notophthalmus viridescens* presents some difficulties since they cannot be easily bred in captivity. In order to study the effect of retinoids during their development we selected pregnant females from stocks of animals collected in September and January, since these are the reproductive periods in this species, and we injected them with HCG (human chorionic gonadotropin) to induce spawning, on the assumption that the females might have been fertilized in the wild before collection (Ghosh *et al.*, 1994). These method allowed us to generate a reasonable number of embryos, but we were still limited in the number of experimental conditions we could investigate. Therefore we used retinol palmitate (RP) instead of retinoic acid since this retinoid is soluble in water, and a single control group, rather than two as in the case of animals treated with RA solubilized in DMSO, is required. RP has been shown to affect limb development and regeneration in a similar fashion to RA (Scadding and Maden, 1986).

The concentration of RP we chose,  $10^{-7}$  M, induces patterning abnormalities during limb development and regeneration in axolotl larvae. Because of the high mortality rate observed in pilot experiments, treatment was carried out for 2 days rather than 4 days as reported in axolotl. Even under these experimental conditions a high degree of mortality was observed especially at the earliest developmental stages (26-28) studied (Table 2). The highest rate of mortality occurred within the first week from the beginning of the experiment. In the absence of RP treatment, mortality was higher in amputated than in non amputated animals, especially in the younger embryos, probably due to the higher degree of difficulty and variability in the surgery at these earlier developmental stages (Table 2, Fig. 5). In all experiments the effects of RP treatment was monitored daily. Its effect on skeletal development was studied in whole-mount preparations stained for bone and cartilage in animals left to develop to stage 48-50. A summary of the different experimental groups, of the number of animals analyzed in whole-mount preparations and of the results obtained is given in Table 2, and examples of the type of defects observed are shown in Figure 1G-H.

In stage 26-28 embryos, the effect of RP treatment was evaluated both in unamputated embryos and in embryos whose distal portion of the jaw primordia had been removed (Figs. 1, 5). In these embryos RP treatment induced a high mortality rate both in



**Fig. 4.** Examples of the pattern of expression of RAR $\delta$ 1 protein either in normal or regenerating lower jaw (A-B, E), upper jaw (C-D) and limb (F). (A) Three-week lower jaw regenerate. Note that whereas in the stump positive nuclei are observed in numerous tissues (glands, epidermis, muscle and cartilage; see also B and E), no significant immunoreactivity is detectable either in blastemal cells or in the wound epidermis (arrows); b, bone; bl, blastema. (B) Higher magnification of the blastema (bl) shown in A and of a patch of normal skin and glands (g). Note the difference in staining between the normal epidermis (insert) and the wound epidermis (arrows) and in the dental lamina (arrows) and in the tooth bud (empty arrow). (C) Stump of a 2-week lower jaw regenerate. Note staining in the dental lamina (arrows) and in the tooth bud (empty arrow). (D) Stump of a 2-week upper jaw regenerate. Note the strong staining in the enamel cells and in the dental pulp of the mature tooth (arrows). (E) Stump of a 3-week lower jaw regenerate. Note staining in the Meckel's cartilage (arrows) and in muscle (m), but not in the blastema (bl). (F) Mid bud limb blastema. Note that both blastemal cells (bl) and cells of the wound epidermis are positive. Scale bars: 100  $\mu$ m in A and B, and 50  $\mu$ m in E, C, D and F are at the same magnification.

unamputated and amputated controls, and not more than 50% of the RP-treated embryos survived up to stage 48-50. As in the case of RA in adult animals, RP always appeared to delay regeneration. In addition, it significantly decreased body growth in embryos treated at stage 26-28 (Fig. 1F-H). All of the RP-treated animals which reached this developmental stage, and were therefore processed for whole-mount staining of bone and cartilage, displayed abnormal jaws, limbs and gills both in amputated and unamputated embryos (Table 2, Fig. 1G-H). The most striking feature in the head of RP-treated embryos was its club-like rather than elongated shape in comparison to untreated regenerates; no significant difference between untreated regenerates and unamputated animals was observed (Table 2). Head abnormalities were clearly evident also in all the RP-treated embryos which did not survive to stage 48-50, although exact details of the defects could not be examined in whole-mount preparations.

The effects of RP were less dramatic and more variable when the treatment was performed at later developmental stages (Table

2), and only half of the embryos treated at stage 38-39, when the transition from embryo to larva occurs, show clear abnormalities. However, RP delayed regeneration in all of the experimental groups studied.

Although we had previously studied regeneration of larval lower jaws, the regenerative ability of the larval upper jaw had not been examined. Upper jaws of stage 45 larvae (3 digits in the forelimb) were amputated, and skeletal preparations of regenerated jaws examined 4 weeks after amputation (Table 2). The regenerated jaw appeared to be a rather faithful replacement of the missing part, and small differences observed in some of the specimens were consistent with a slight developmental delay probably as a consequence of surgery. In order to establish whether retinoids have the same teratogenic effects on regenerating jaws of developing animals as on those of adult animals, we treated regenerating jaws of stage 45 larvae with RP as described above. Although an initial delay in growth occurred as in all the other retinoid-treated groups, 4 weeks after amputation we could not observe any significant

TABLE 2

EFFECT OF AMPUTATION AND RETINOL PALMITATE<sup>#</sup> (RP) TREATMENT ON THE DEVELOPMENT OF UPPER AND LOWER JAWS IN NEWT EMBRYOS AND LARVAE

Stage of operation	Unamputated	Amputated	Treatment	Survival after 7 d	Survival at stage 48-50*	Defects observed**
26-28	4	5	Control	4	4	none
			Control	2	2	none
	10	8	RP	3	2	smaller body size than untreated
			RP	4	4	club head, short gills, microphthalmia (1), smallerbody size, limb abnormalities
32-33	4	11	Control	4	4	none
			Control	6	6	none
			RP	7	5	club head (3)
34-36	2	3	Control	2	2	none
			Control	2	2	none
			RP	4	3	club head
38/39	2	5	Control	2	2	none
			Control	5	5	none
			RP	5	5	club head (3), short gills (3), microphthalmia (1), limb abnormalities (3)
45 <sup>§</sup>	5	4	control	5	5 <sup>§</sup>	none
			control	4	4 <sup>§</sup>	none
			RP	9	9 <sup>§</sup>	none

<sup>#</sup>Amputated embryos were left to recover for 1 day before being treated with  $10^{-7}$  M RP for 2 days. \*This stage was reached between 3 and 4 weeks from the beginning of the experiment. \*\*Unless specified in brackets all the specimens analyzed presented the abnormality described. <sup>§</sup>In this group only the upper jaw was amputated (see text), and the larvae left to regenerate for 4 weeks.

difference between treated and untreated regenerates in whole-mount skeletal preparations, indicating that, at least under the experimental conditions used, RP does not affect regenerating larval upper jaws (Table 2).

## Discussion

### RA affects differently regeneration of upper jaws, lower jaws and limbs in adult newts

The work presented here shows that retinoids initially affect regeneration of both upper and lower jaws in adult newts in a similar fashion, since they induce a delay in the growth of RA-treated regenerates in comparison with untreated and DMSO controls. Growth retardation, however, is only temporary and is presumably directly related to the concentration of RA, or one of its metabolites, present in the jaw at different times following injection. This effect of RA on growth retardation is common to other regenerating systems, such as the newt limb and the zebrafish fin (Maden, 1983; Ferretti and Géraudie, 1995). In contrast, the effect of retinoids on patterning of regenerating jaws is significantly different from that induced in regenerating limbs and fins (Fig. 6), where proximodistal duplications of the limb (Niazi and Saxena, 1978; Maden, 1982) and narrowing of the fin occur (Géraudie *et al.*, 1994).

Once normal growth is eventually resumed, lower jaw regeneration in RA-treated animals appears to proceed normally and no significant patterning defect is observed in the full regenerate. On the contrary, patterning of the upper jaw is severely affected under the same experimental conditions, demonstrating a different sensitivity to the drug by tissues of upper and lower jaws, and indicating that RA effects on growth and patterning can be dissociated. The range of defects observed in upper jaw regenerates which have been treated with RA varies from partial to almost complete

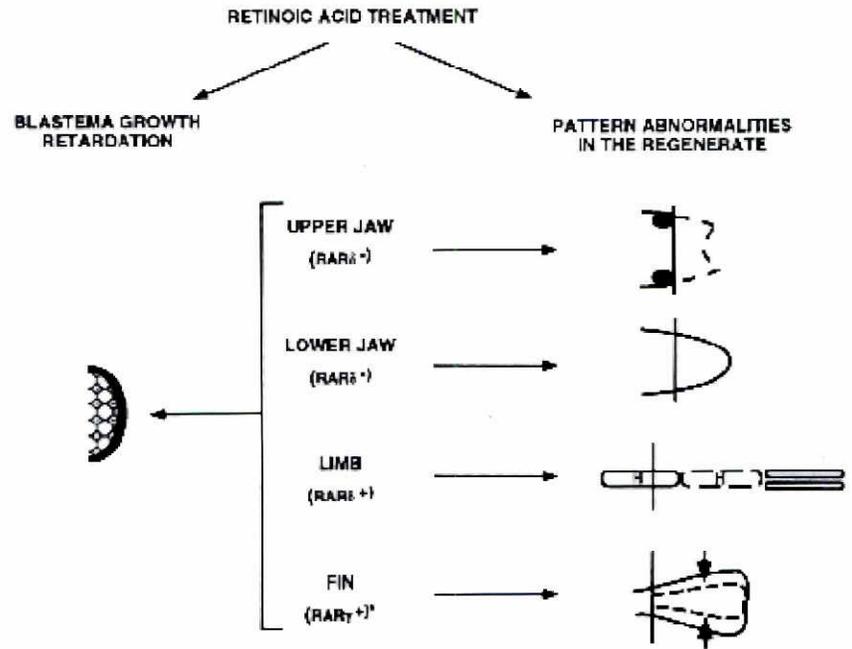
inhibition of regeneration. Such variability is probably linked to a certain individual variability in the population studied (*i.e.* age), as the animals are collected in the wild, and possibly to slight differences in the dose of RA (for example due to some leakage at the injection point) to which each animal was exposed.

It seems most unlikely that RA-treated animals that have partially regenerated their upper jaws 20 weeks after amputation could fully regenerate if left for a much longer period of time, because of the type of malformations observed and the fact that in controls the regenerative process is very advanced by 12 weeks post-amputation (Ghosh *et al.*, 1994). It appears therefore that injection of a dose of RA which induces duplications in regenerating limbs either impairs upper jaw regeneration or results in formation of upper jaw regenerates significantly less developed than controls. Furthermore, upper and lower jaws appear to respond differently to the same concentration of RA. This resembles the teratogenic response induced by RA in developing avian jaws (Tamarin *et al.*, 1984; Wedden, 1991), suggesting that the ability to respond differently to this compound is maintained in an adult animal, and that such response is likely to be mediated through the same molecular mechanisms.

A significant difference in the pattern of expression of RAR $\alpha$  and RAR $\delta$  in regenerating upper and lower jaws was observed in the



Fig. 5. Schematic drawing of a stage 28 embryo where the level of amputation is indicated by a straight line.



**Fig. 6. Summary cartoon** showing that whereas RA treatment temporarily delays growth in jaw, limb and fin blastemas, its effect on the patterning of these regenerating structures is significantly different (the dashed lines indicate the pattern abnormalities in the different regenerates; the vertical lines indicate the level of amputation). Note that the regenerated upper jaw is hypomorphic and misshapen, the regenerated lower jaw is normal, the regenerated limb contains supernumerary structures (H, humerus), and the regenerated fin is narrower (indicated by dotted arrows) than the original one, but the length of its skeletal elements has not been affected. \*RAR $\gamma$  is more closely related to RAR $\delta$  than to other newt RARs (White *et al.*, 1994), but its function in fin regeneration is unknown.

wound epidermis, where only RAR $\alpha$  could be detected. The fact that the RAR $\alpha$  transcript was found both in upper and lower jaw wound epidermis suggests that the teratogenic effects selectively induced by RA on regenerating upper jaws are not mediated by this receptor.

In apparent contrast to regenerating limbs, neither RAR $\alpha$  or RAR $\delta$  transcripts were expressed at a significant level in jaw blastemal cells, as assessed by *in situ* hybridization and, in the case of RAR $\delta$ 1, by immunocytochemistry. The fact that the RAR $\delta$ 1 protein appears to be expressed in the same tissue types in normal jaws and limbs, rules out the possibility that the different expression observed in blastemal cells of limbs and jaws is due to an artefact, and may be causally related to the different effects of RA on these two regenerating systems. Whereas the RP6 antibody recognizes only the RAR $\delta$ 1 protein (Hill *et al.*, 1993), the probe used in the *in situ* hybridization experiments detects both RAR $\delta$ 1 and RAR $\delta$ 2 (Ragsdale *et al.*, 1992), suggesting that neither of these receptors is expressed in jaw blastemas. RAR $\delta$ 1 is the most highly represented form in limb blastemas, and it has been shown to mediate the induction of a marker of the limb wound epidermis, WE3, but is unable to change the proximodistal identity of blastemal cells in the limb (Pecorino *et al.*, 1994). In contrast, RAR $\delta$ 2, although expressed at lower levels in limb blastemas, can mediate the proximalizing effect of RA (Pecorino *et al.*, 1996). Therefore, lack of these receptors in blastemal cells of regenerating jaws seems to correlate with the different patterning defects induced by RA in jaws and limbs. It is likely that either RAR $\beta$ , of which a partial newt sequence has been identified (Giguere *et al.*, 1989; Ragsdale *et al.*, 1989), or one of the RXRs yet to be isolated in the newt, may be important in mediating the effects of retinoids on regenerating jaws, and their distribution in this system will have to be investigated.

#### **Sensitivity to RP is different at different developmental stages**

We have found that RP treatment overall retards embryonic growth and affects patterning of head and face in newt embryos amputated between stage 26 and 39, indicating that regenerating

facial structures are sensitive to retinoids both in embryos and adults. However, direct comparison between regeneration in embryos and adults is not as straightforward as in the case of the limb (Fekete and Brockes, 1988), because the surgery performed up to stage 38-39 involves removal of distal structures which will contribute both to upper and lower jaws. It is only from stage 40 onwards that lower (Ghosh *et al.*, 1994) and upper jaws (this paper) can be independently amputated. Nonetheless, it clearly appears that both in adults and embryos retinoids produce hypomorphic facial structures, and not opposite effects as in the case of the limb where in the adult duplications, rather than hypomorphic limbs, are observed following retinoid treatment (Scadding and Maden, 1986). The fact that in adults only the regenerating upper jaw seems to be sensitive to retinoids, whereas in embryos the pattern of lower jaws is also affected, is likely due to the high regulative capability of the embryo, which can therefore coordinate the growth of upper and lower jaws in response to amputation and teratogenic insult.

The decrease with increasing embryonic age of the severity of RP-induced malformations in regenerating facial structures may be partly due to a change in concentration/body weight ratio. Although embryos at stage 38-39 can still respond to  $10^{-7}$  M RP, this treatment does not appear to affect regeneration of upper jaws amputated at larval stage 45. This is rather surprising, since retinoids clearly affect upper jaw regeneration in adult newts. Various explanations, not mutually exclusive, can be invoked to account for this difference between embryos, larvae and adults. As mentioned above it is possible that the concentration of RP used has either become insufficient to induce a teratogenic effect given the increased body size, or that at stage 45 treatment by immersion is no more effective. It is known that duplications of structures in regenerating adult limbs cannot be induced by using this administration route. Alternatively, blastema formation may be slower in larvae than in embryos, and a longer treatment with RP might be necessary to evoke the same effect. It has indeed been shown that

the time and length of the treatment is important both in limb and fin regeneration (Thoms and Stocum, 1984; Johnson and Scadding, 1992; Géraudie *et al.*, 1994, 1995). Finally, it is also possible that a change in the sensitivity to retinoid teratogenic effects may be related to the transition from embryo to larva which occurs around stage 40. This would imply that teratogenic and growth retardation effects are separable, since RP still has a retarding effect on jaw regeneration in stage 45 amputees, and is consistent with differences in the reversibility of retinoid effects in regenerating fins and limbs (Johnson and Scadding, 1992; Ferretti and Géraudie, 1995).

The work presented here demonstrates clear differences in response to retinoid-treatment of regenerating upper and lower jaws. These observations raise many questions on the origin of different skeletal structures following amputation, their regenerative ability, and their sensitivity to RA. Thus, regeneration of the amphibian jaw is not only an interesting system for addressing issues concerning regeneration of complex body structures in vertebrates, but also another potentially important model for studying differential effects of RA on facial development.

## Materials and Methods

### Animals and surgery

All experiments were carried out on either adults or larvae of the red-spotted newt *Notophthalmus viridescens* (supplied either by Nasco Ltd, USA or Sullivan & Co, USA). Adult newts were maintained at 25°C for all the duration of the experiment and fed shredded bovine heart on alternate days. Collection and staging of embryos was carried out as previously described (Gallien and Durocher, 1957; Ghosh *et al.*, 1994). Briefly, spawning was induced by daily injection in pregnant females of 100 units of HCG (human chorionic gonadotropin, Sigma, Poole, UK) for 3 to 5 days, and embryos were grown at 22–24°C in sterile tap water. Larvae were fed daily with brine shrimps and reared in individual containers.

Adult newts were anesthetized in 0.1% tricaine (3-aminobenzoic acid ethylester methanesulphonate salt, Sigma, Poole, UK) and larvae in a 1/3000 dilution in sterile tap water of a 10% tricaine solution. Amputation of lower jaws was performed by transversally cutting the distal third of the jaw, but leaving the tongue and hyoid apparatus intact, and adult upper jaws were amputated by a transverse section just proximal to the external naris (Ghosh *et al.*, 1994; Ferretti, 1996). Mid bud blastemas were obtained by amputating the limb proximal to the elbow. After surgery animals were allowed to recover from anaesthesia in a shallow aqueous solution of 0.5% sulfamerazine (Sigma, Poole, UK) for 18–24 h before being returned to the tanks. The animals were sacrificed at different times after surgery, and jaws collected in order to analyze the regenerated skeletal structures. The type of amputations performed in developing jaws depended on the developmental stage. At the earlier stages of development, before opening of the mouth, (from stage 26–28 to stage 38–39) the whole distal portion of the jaw primordia was removed using tungsten needles (Fig. 5), whereas at later stages (stage 45) only the distal third upper jaw was amputated as in adult animals.

### Retinoic acid (RA) and retinol palmitate (RP) treatment

Treatment with retinoic acid (RA; all trans retinoic acid, Sigma, Poole, UK) in adults was performed by injecting intraperitoneally (i.p.) 10 µl of RA solution (30 mg/ml) in DMSO (dimethyl sulfoxide, Sigma, Poole, UK) either one or two weeks after jaw amputation. Control animals were injected i.p. with 10 µl of DMSO. Regenerating jaws from both control and RA treated animals were collected either 5 weeks after surgery, in the case of animals treated with RA 2 weeks after amputation, or 4, 12 and 20 weeks after surgery, in the case of animals treated with RA one week after amputation. The degree of regeneration in the different experimental groups was regularly monitored for the entire duration of the experiments.

Developing newts were treated with retinol palmitate (RP), rather than with RA because of its solubility in water. Treatment by immersion in 10<sup>-7</sup> M RP was carried out at different stages of development on newts which had been mechanically dechorionated. Both intact animals and animals whose jaws had been amputated immediately before treatment were maintained in RP for 2 days. Controls of equivalent stages were maintained in water. Both control and treated animals were monitored daily. Animals from experimental groups between stage 26 and 39 were left to develop up to stage 48–50 before being sacrificed with an overdose of tricaine and their skeletal development analyzed in whole-mount preparations. The skeletal structure of larvae whose upper jaw was amputated at stage 45 was examined in whole-mount preparations 4 weeks after amputation.

### Analysis of regenerating jaws

Regeneration of cartilages and bones was studied in whole-mount preparations stained for these tissues with alcian blue and alizarin S red by minor modifications of Simon and Van Horn's method (1971).

### In situ hybridization

The RAR probes used in this study, E3Kd (RARα) and E1d (RARδ), have been described by Ragsdale *et al.* (1992), and *in situ* hybridization was basically performed according to Wilkinson and Green (1990). After harvesting normal jaws and jaw blastemas were rinsed in cold A-PBS (0.1 M phosphate buffer, 0.12 M NaCl, pH 7.4), and fixed overnight at 4°C in 4% paraformaldehyde in A-PBS. The jaws were decalcified by treatment with 0.5 M EDTA, pH 7.5, for 3 to 5 days. After rinsing in the same buffer the tissues were dehydrated in graded ethanol, embedded in paraffin wax, and 6 µm sections cut. After dewaxing, sections were fixed in 4% paraformaldehyde for 20 min, treated with 20 µg/ml of proteinase K for 5 min, post-fixed in 4% paraformaldehyde for 5 min, acetylated with acetic anhydride in triethanolamine buffer, and dehydrated. The slides, covered with a coverslip, were hybridized overnight at 55°C with 10<sup>6</sup> cpm/µl of the transcribed riboprobes, which had been purified on a Sephadex G-50 drip column, in a hybridization mixture consisting of 50% formamide, 20 mM Tris-HCl pH 8.0, 0.3 M NaCl, 5 mM EDTA, 0.5 mg/ml yeast tRNA, 1xDenhardt's solution, 10% dextran sulphate. The slides were washed twice for 30 min in 5xSSC, 10 mM DDT at 50°C, the coverslips removed, and then washed at high stringency for 30 min at 65°C with 50% formamide, 2xSSC, 10 mM DDT. After three 10-min washings with NTE buffer (0.5 M NaCl, 10 mM Tris-HCl pH 8.0, 5 mM EDTA), the slides were treated with 20 µg/ml of RNAase A for 30 min at 37°C and washed for 15 min with NTE. The high stringency washing was then repeated, followed by a washing with 2xSSC and one with 0.1xSSC of 15 min each. Following dehydration, the slides were processed for autoradiography and exposed for either 5 or 6 days at 4°C before being developed and counterstained with toluidine blue.

### Immunocytochemistry

The RARδ protein was localized in 8 µm cryostat sections of jaw and limb regeneration blastemas fixed as previously described by using the RP6 polyclonal antibody (Hill *et al.*, 1993). The bound antibody was detected by a horseradish peroxidase-conjugated-swine-anti-rabbit-immunoglobulin antibody (Dako, Denmark).

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