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Effects of Localized Application of Retinoic Acid on *Xenopus laevis* Development

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In order to more accurately determine the mechanism by which retinoic acid causes embryonic defects, we have developed a simple method of locally applying retinoic acid rather than immersing the whole embryo in retinoic acid solutions. Retinoic acid was suspended in corn oil and then injected between the surface and the deep ectodermal layers of an early gastrula *Xenopus* embryo. When droplets containing retinoic acid were injected into the presumptive head region, the embryos exhibited inhibited development of anterior structures near the injection site. Development of the eye, cement gland, hatching gland, olfactory pits, and expression of engrailed protein were all disrupted near the injection site. Inhibited development of anterior structures was far greater on the injected side of the embryo than on the uninjected side. The retinoic acid droplet did not cause an anterior shift of structures on the injected side relative to the uninjected side. These experiments suggest that retinoic acid does not cause global respecification of axial level in the head, but rather suppresses development of anterior structures. Retinoic acid injected into presumptive trunk regions had no discernible effect. © 1994 Academic Press, Inc.

INTRODUCTION

The nervous system of *Xenopus laevis* is induced by a combination of signals which emanate from the dorsal mesoderm (Spemann, 1938; Dixon and Kintner, 1989; Sharpe and Gurdon, 1990; Doniach *et al.*, 1992). In normal embryos, the characteristic anterior-posterior polarity of the neural tube is thought to result from a two-step process. The nervous system is first specified to an anterior fate and then a gradient of transforming factor, highest at the posterior end, transforms the nervous system to its normal fate (Nieuwkoop, 1952).

When *Xenopus* embryos are immersed in retinoic acid, the anterior-posterior polarity of the embryo is maintained, but there is a loss of anterior ectodermal structures (Durston *et al.*, 1989; Sive *et al.*, 1990; Sive and Cheng, 1991). One hypothesis to explain these teratogenic effects is that retinoic acid acts as the transforming factor of Nieuwkoop's activation-transformation model, respecifying anterior structures to a more posterior fate (Durston *et al.*, 1989). Addition of exogenous retinoic acid causes normally anterior structures to take on a posterior fate.

Several lines of evidence support this possibility. Retinoic acid is present in *Xenopus*, during gastrulation, at concentrations that can have biological effects (Durston *et al.*, 1989). Increased expression of posterior neural markers after retinoic acid treatment has been shown in *Xenopus* (Sharpe, 1991; Sive and Cheng, 1991), mouse (Morriss-Kay *et al.*, 1991), and chick (Sundin and Eichele, 1992). Respecification of hindbrain structures by retinoic acid has been demonstrated in mouse (Marshall *et al.*, 1992). In addition, retinoic acid has been shown to alter the expression of homeotic genes (Simeone *et al.*, 1990) that may mediate axial determination.

Another hypothesis to explain the teratogenic effects of retinoic acid in *Xenopus* could be that retinoic acid acts by suppressing the differentiation of anterior structures (Drysdale and Elinson, 1991; Papalopulu *et al.*, 1991) but does not respecify axial level. We sought to distinguish between these two possibilities by locally perturbing retinoic acid concentrations. Asymmetric application of retinoic acid might be expected to displace structures anteriorly on the treated side if retinoic acid plays a role in axial specification (Fig. 1). Alternatively, if retinoic acid suppresses anterior differentiation, then the embryo's treated side should display inhibited development compared to contralateral structures (Fig. 1).

We have devised a method of locally perturbing patterns of differentiation by microinjecting small quantities of retinoic acid suspended in oil. This enabled us to give a higher dose of retinoic acid to one side of the embryo. In doing so, we found that retinoic acid acted to

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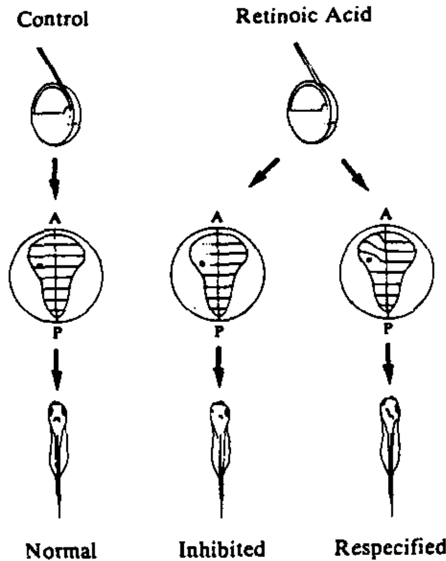

FIG. 1. Possible patterns of development following retinoic acid microinjection. Injection of retinoic acid between the deep and the surface ectodermal layers of a stage 10.5 gastrula embryo (top drawings) might have one of two effects upon subsequent head development. Control injections of carrier medium alone (open circle) should have no effect upon endogenous concentrations of retinoic acid. The resulting neural plate would have anterior-posterior specification and a normal tadpole would develop. Injection of retinoic acid (closed circles) might alter pattern formation by locally inhibiting differentiation of anterior structures so that they fail to develop on the injected side (represented by faded lines). Alternatively, if the axial level is specified by a gradient of retinoic acid, on the injected side the injected retinoic acid would supplement the endogenous retinoic acid gradient, thereby respecifying (represented by the upturned lines) the axial coordinates in the neural plate. In subsequent embryos, structures would then appear more rostral than contralateral ones.

suppress anterior differentiation but did not appear to respecify the axial level of anterior structures.

MATERIALS AND METHODS

Embryo Manipulations

Embryos were fertilized, dejellied, and raised according to Drysdale and Elinson (1991) and staged according to Nieuwkoop and Faber (1967). In preparation for microinjection, retinoic acid was suspended by sonication in corn oil (Mazola) and aliquots of this suspension were added to corn oil saturated with the lipophilic, fluorescent lineage marker DiI(C_{16}) (a gift from M. Terasaki, NIH). Final mixtures were apportioned at 2.5 (8.3 μM), 100 (335 μM), 200 (670 μM), 250 (833 μM), 500 (1.6 mM), and 1000 (3.2 mM) μg retinoic acid/ml. As retinoic acid does not solubilize well, the concentrations are only estimates.

Embryos to be injected had their fertilization envelope manually removed and were injected in Danilchik's medium (Keller *et al.*, 1985). Approximately 0.5–1 nl was injected between the surface and the deep ectodermal layers of stage 10–11 embryos by means of an air pressure injection system. The droplet was injected into presumptive head or trunk based on the fate maps of Keller (1975). Once the injection wound had healed, embryos were transferred to 20% Steinberg's solution and incubated at 18°C. Control embryos were injected with oil saturated with the lineage marker. This injection method was used because the beads traditionally used for this type of experiment (Eichele *et al.*, 1985) were quickly expelled from the *Xenopus* embryo. The expulsion of beads prevented us from obtaining consistent phenotypes or determining the exact position of the bead relative to the later phenotype.

The injected droplet could be traced by observing the whole embryo under a Leitz Orthoplan fluorescent microscope. The oil droplet remained in place throughout gastrulation, the period during which retinoic acid is thought to act (Durston *et al.*, 1989). The possibility of droplet movement later in development was not directly examined, but the bead was often in positions which would indicate that no obvious movement had occurred. In addition, cells in close proximity to the droplet would take up lineage tracer and these were usually adjacent to the droplet throughout development. As injections based on a fate map are only approximations, the fluorescence tracing was helpful in interpreting the final phenotypes.

Phenotype Observations

To observe morphology, embryos were fixed in 2% trichloroacetic acid in phosphate-buffered saline. The bead position was determined using the epifluorescent microscope and gross morphology was observed using a dissecting microscope. The morphology of the hatching gland was determined using an antibody against tyrosine hydroxylase as described in Drysdale and Elinson (1991). The distribution of engrailed protein was determined using the polyclonal antibody αEnhb-1 (kindly donated by A. Joyner) as outlined in Davis *et al.* (1991). The embryos were photographed after clearing in benzyl alcohol/benzyl benzoate (Dent *et al.*, 1989).

Electron Microscopy

Following injection with retinoic acid or with carrier medium alone, some embryos were fixed after 1.5, 3.0, or 14 hrs postinjection. Embryos were fixed for 2 hrs in 2.5% gluteraldehyde, 2.0% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) at room temperature. Embryos were then washed for 2 hrs with several changes in phosphate buffer, postfixed for 2 hrs in 1% osmium tetroxide, rinsed in distilled water, dehydrated through

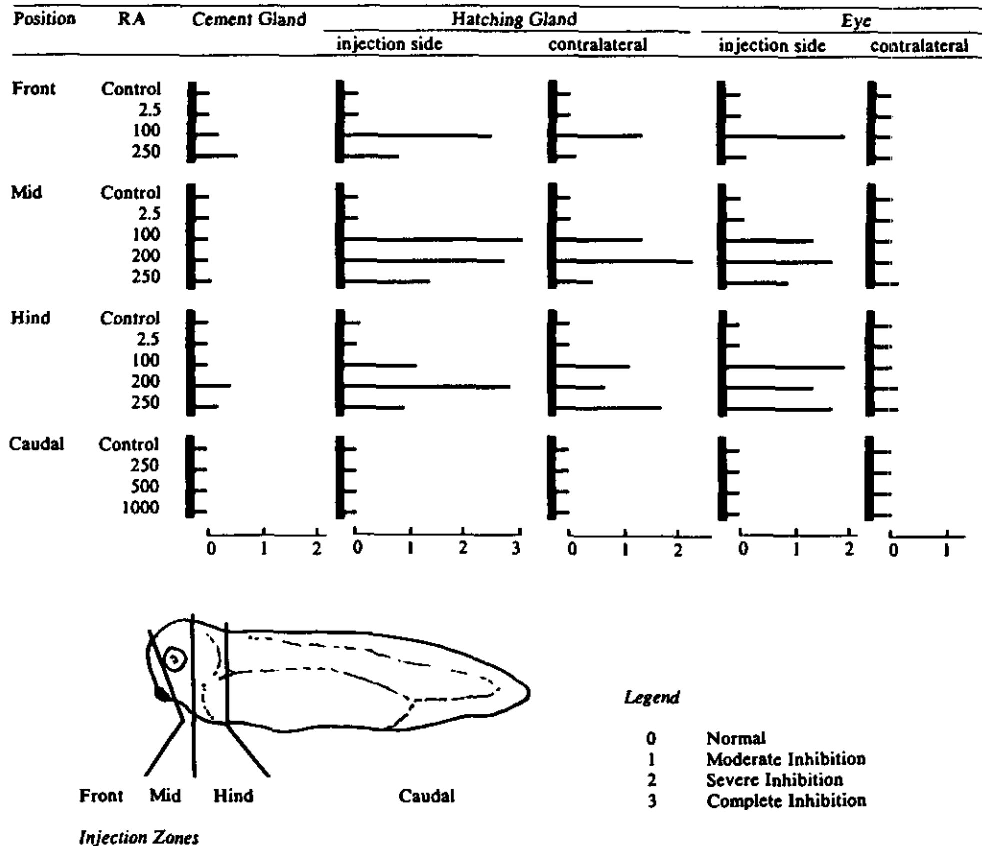

FIG. 2. Effects of droplet position and retinoic acid concentration on subsequent development. Embryos were categorized according to retinoic acid concentration (μg/ml) and droplet placement. The effects were scored according to the degree of inhibition from normal (0) to complete inhibition (3). The means of the scores are graphed. Cement gland was inhibited only when the injected droplet was near the front of the embryo. The injected side showed higher levels of hatching gland and eye inhibition than the contralateral side, indicating that the effects of the injection were graded and localized to the droplet region. Hatching gland was most sensitive to the retinoic acid, and both sides of the hatching gland pattern were often affected. Eyes were less sensitive. The eye on the injected side was usually only reduced in size with no apparent effect on the contralateral one. Occasionally, the eye on the injected side was absent, with the contralateral eye appearing normal.

an acetone series (25, 50, 70, 90, 95, 3× 100%), and then critical point dried in CO_2. Specimens were mounted on aluminum stubs and sputter coated with 20-nm gold for viewing under a Hitachi S 2500 scanning electron microscope.

RESULTS

Effects Resulting from Point Sources of Retinoic Acid

Perturbations of head pattern were seen around the retinoic acid injection sites. These sites were easily observed by monitoring the DiI fluorescence, which could be seen well past the tailbud stage. The perturbations were restricted to head structures and retinoic acid, injected into presumptive tail or trunk regions, had no discernible effect on the embryo regardless of concentration (Fig. 2). This differs from retinoic acid immersion, which caused defects in the tail (Durston *et al.*, 1989). This difference may be a result of the difficulty in delivering the droplet to the small region of the embryo fated to be tail or the possibility of prolonged mesoderm inducing activity in the tail (Woodland and Jones, 1988).

The perturbations of head structures caused by retinoic acid droplets were asymmetric; structures on the injected side of the embryo were affected more severely than those on the uninjected side (Fig. 2). We therefore assume that a gradient of retinoic acid was created by the injection. The distribution and steepness of the gradient was not determined. However, as the effects of the retinoic acid droplets on the observed anterior structures was similar to the phenotypes observed in retinoic acid immersion experiments (Durston *et al.*, 1989; Sive *et al.*, 1990), we can estimate the concentrations that are being seen by the embryo. Most embryos showed partial inhibition on the injected side (Fig. 2) and this phenotype corresponds to that seen with a 5–20 min immer-

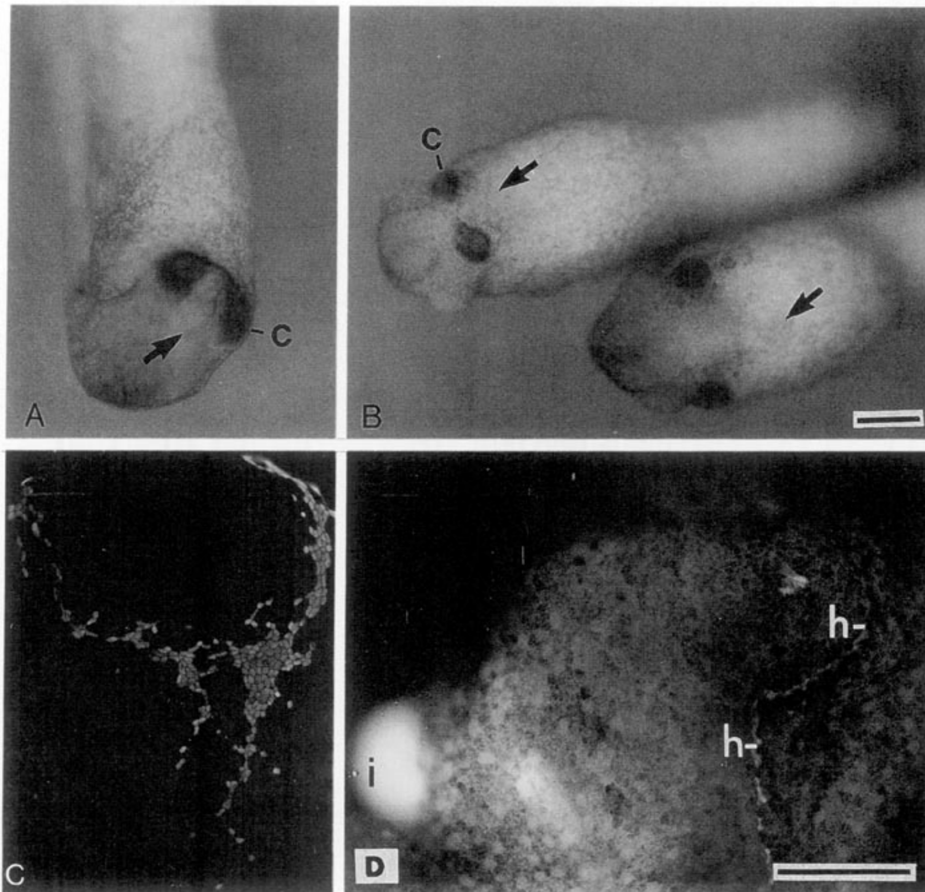

FIG. 3. Local inhibition of cement and hatching gland by retinoic acid. (A) A retinoic acid droplet (arrow) lying posterior to the cement gland causing local inhibition of the cement gland. (B) Two examples of embryos that had the retinoic acid droplet (arrow) injected anterior to the cement gland. In both cases the entire middle of the cement gland was inhibited, resulting in two patches of cement gland on either side of the droplet. Scale bar, 200 μm. (C) Hatching gland, visualized under epifluorescence, was strongly inhibited when in close proximity to the retinoic acid droplet. The droplet, not visible in this view, was on the left side, where there are fewer, more dispersed cells in the Y pattern. Note that the overall pattern is still symmetric. Anterior is up. (D) The injected droplet (i) can be visualized on the left side of the embryo because of the DiI fluorescence. The hatching gland (h) is completely absent on the left side of the embryo. Anterior is up. Scale bar, 100 μm.

sion treatment with 1 μM retinoic acid (Sive *et al.*, 1990). This would indicate that the majority of retinoic acid stays in the bead. This would be expected as retinoic acid is lipid soluble.

Because one side of the embryo was more severely affected than the other, we were able to test whether retinoic acid eliminated head structures by respecifying them or by inhibiting their differentiation (Fig. 1). At no time did the affected structures on the injected side appear further anterior than the contralateral structures. Figures 3A and 3B illustrate cases in which proximity to injection sites had locally inhibited formation of cement gland. There were no other structures in the inhibited region, suggesting that the cement gland was directly inhibited and that ectoderm was not respecified to a posterior fate. Cement gland and olfactory pits appeared less sensitive to perturbation than did eyes or hatching gland cells (Fig. 2).

Hatching gland near the injection site had fewer cells that were more dispersed than on the contralateral side (Figs. 3C and 3D). The dispersed appearance is similar to the hatching gland phenotype of embryos immersed in retinoic acid (Drysdale and Elinson, 1991). The Y pattern of hatching gland cells was maintained on both sides of the embryo without any anterior shifting of the hatching gland pattern on the injected side (Figs. 3C and 3D). Eyes on the injected side of the embryo were smaller or absent when compared to those on the uninjected side (Fig. 4) but did not appear in a more anterior position.

The pattern of *En-2* expression, at the midbrain/hindbrain junction, was reduced and more dissipated than the expression on the uninjected side (Fig. 5). In these examples, the *En-2* band was contiguous with the *En-2* staining on the uninjected side and did not appear to be shifted more anterior by the presence of retinoic

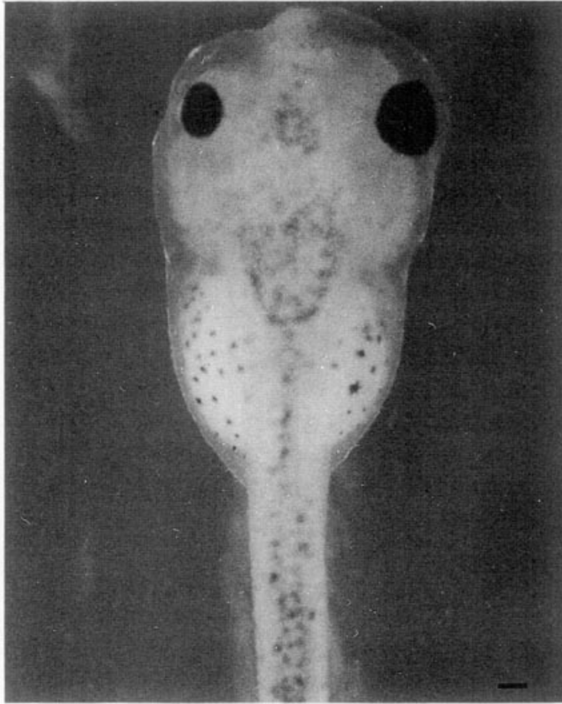

FIG. 4. Inhibition of eye by a retinoic acid droplet. The eye on the injected side (left) is inhibited, showing a smaller size. However, it is not more anterior than the contralateral eye. Scale bar, 100 μm.

acid. The pattern of *En-1* expression in the spinal cord, also detected by the antibody (Davis *et al.*, 1991), was unaffected by the presence of retinoic acid droplets.

It remains possible that the retinoic acid acts by specifically killing cells in the anterior region of the embryo rather than inhibiting their differentiation. Although the phenotypes we see are generally mild and have not been associated with extensive cell death in previous studies (Durston *et al.*, 1989; Sive *et al.*, 1990), we examined the injection sites, using scanning electron microscopy, 3 hrs after droplet injection to see if the cells still appear healthy. Both cells of the surface ectoderm (Fig. 6A) and the deeper cells (Fig. 6B) appeared normal and no cellular debris was obvious. This suggests that close proximity to the injected droplet is not lethal.

DISCUSSION

Treatment of vertebrate embryos with retinoic acid causes marked alterations of the central nervous system and is present in the embryo, lending it a putative role in specifying the anterior–posterior axis (Green, 1990; Balling, 1991). Determining the potential role for retinoic acid in normal embryogenesis has proven exceedingly complex as there are many proteins that can transduce or modulate its signal (Maden and Holder, 1992; Zhang *et al.*, 1992; Kliewer *et al.*, 1992). In most biological systems where a role for retinoic acid is hypothesized, it is thought to act in a gradient fashion but establishing the presence of endogenous retinoic acid concentration gradients is very difficult. Comparison between normal embryos and those treated with retinoic acid has demonstrated specific defects in the embryo, but the global application of retinoic acid makes it difficult to determine the root cause of these defects because the application of exogenous retinoic acid to *Xenopus* embryos has only been done by immersion. Immersion does not explicitly test whether retinoic acid acts in a gradient because it merely supplements any endogenous gradients. We have tried to more accurately determine the potential role for retinoic acid by applying the retinoic acid locally. Local application of retinoic acid has helped determine its possible role in limb (Tickle *et al.*, 1985; Izpisúa-Belmonte *et al.*, 1992) and feather (Chuong *et al.*, 1992) patterning.

Retinoic Acid Ablates Anterior Structures but Does Not Globally Respecify Axial Levels

If endogenous retinoic acid were supplying a graded positional signal (Green, 1990; Balling, 1991), then localized sources of the compound might be expected to cause nearby tissues to be respecified to a more posterior fate. Structures near a retinoic acid injection site should then appear more rostral than the contralateral structures (Fig. 1). This was not the case. We found no evidence for respecification of pattern to yield posterior structures, particularly as elements were observed to diminish in size without changing position relative to contralateral structures (Fig. 4). In addition, no posterior structures were observed to differentiate in place of suppressed head elements. This implies that retinoic acid suppresses the differentiation of anterior ectodermal structures but does not determine the axial level of those structures.

Alterations to the fore and midbrain can be achieved in *Xenopus* and mouse, but these changes have been likened to compressions (Papalopulu *et al.*, 1991; Conlon and Rossant, 1992; Marshall *et al.*, 1992). It is likely that the compression of anterior structures in *Xenopus* (Drysdale and Elinson, 1991; Papalopulu *et al.*, 1991) is the same phenomenon that we observe when smaller anterior structures are found on the injected side of the embryo. Whether this compression is due to inhibition of differentiated neural tissue, or a reduction in the amount of neural tissue originally induced, must await further studies.

Retinoic acid is known to cause respecification in mouse (Marshall *et al.*, 1992) and an increase in the level of posterior markers in *Xenopus* (Sive and Cheng, 1991;

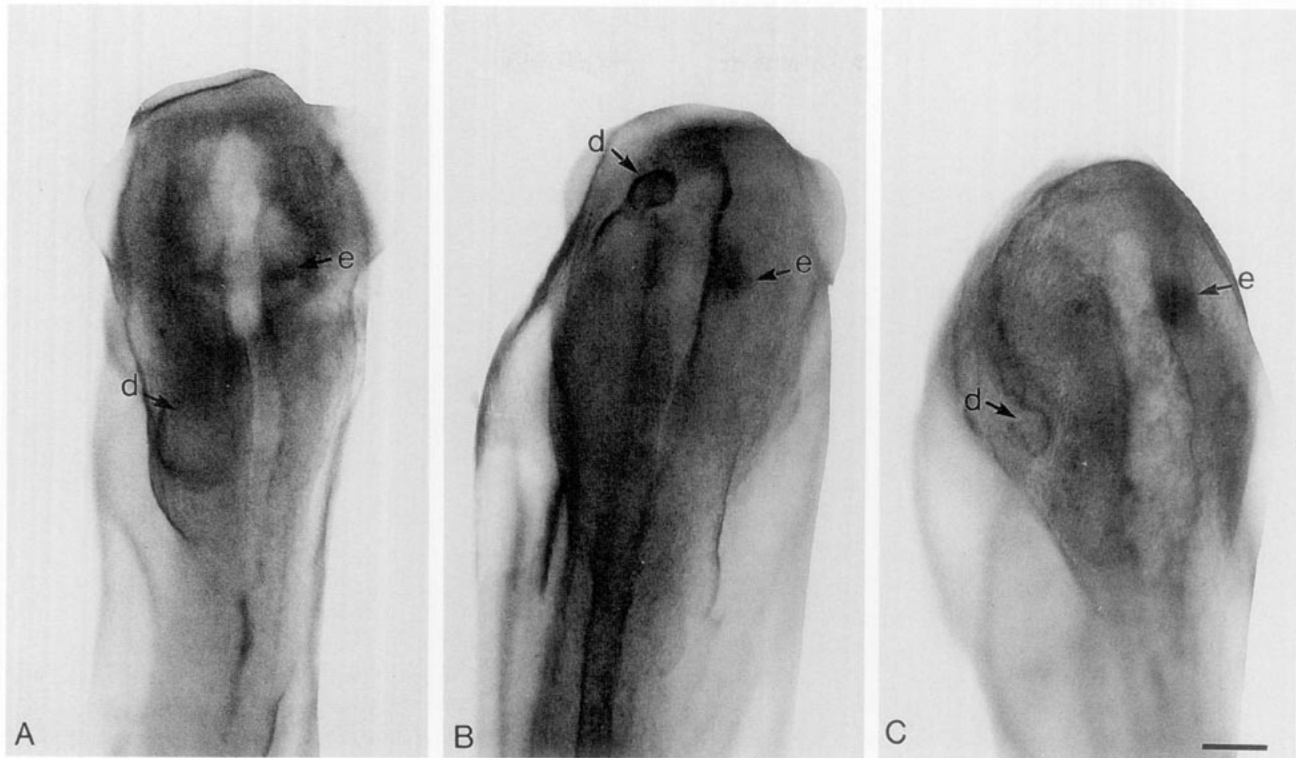

FIG. 5. Engrailed expression is inhibited by retinoic acid injection. (A) The band of engrailed staining (e) is normal and symmetric when a control droplet (d) is injected. When a retinoic acid droplet (d) is injected either anterior (B) or posterior (C) to the normal engrailed position (e), the engrailed band is inhibited on the injected side. No anterior shifting of the engrailed band was seen. Scale bar, 100 μm.

Sharpe, 1991), chick (Sundin and Eichele, 1992), zebrafish (Holder and Hill, 1991), and mouse (Morriss-Kay *et al.*, 1991; Conlon and Rossant, 1992; Marshall *et al.*, 1992). The identified respecification and ectopic expression events have been primarily within the hindbrain region. Ectopic expression of posterior markers in the fore and midbrain is not found in mice (Morriss-Kay *et al.*, 1991; Conlon and Rossant, 1992; Marshall *et al.*, 1992). The markers we have examined are not specific to the hindbrain. The use of hindbrain molecular markers in conjunction with the retinoic acid injections should be able to demonstrate the degree of respecification in the *Xenopus* hindbrain in response to retinoic acid.

We suggest that retinoic acid may still act as a transforming factor, suppressing differentiation of anterior structures, and possibly play a role in patterning the hindbrain, but retinoic acid does not normally play a role in determining the axial level of anterior ectodermal structures. The lack of a role for retinoic acid in determining head structures is supported by the findings that in the early mouse embryo there is no detectable transcription of a reporter gene that contains the retinoic acid response element, anterior to the brain–spinal cord boundary (Rossant *et al.*, 1991; Mendelsohn *et al.*, 1991). This implies that retinoic acid is not making a ligand–receptor–response element complex in the vertebrate head at this early stage.

Retinoic Acid Can Directly Suppress Ectodermal Differentiation

Evidence suggests that retinoic acid acts by directly altering the response of ectoderm to inductive signals (Durston *et al.*, 1989; Sive *et al.*, 1990; Sive and Cheng, 1991). Alternatively, retinoic acid could act on potential mesodermal inducers (Ruiz i Altaba and Jessell, 1991; Sive and Cheng, 1991). In our injection experiments, the droplets were in direct contact with the ectoderm. The regions of ectoderm that were suppressed corresponded to the position of the bead rather than the position of potential inducers. This was most obvious when the cement gland was suppressed (Fig. 3) and provides direct evidence that retinoic acid can act directly on the ectoderm.

Retinoic acid is a substance that acts through a complex array of cellular components, making it difficult to determine if it is actively working in a particular region. We have developed a novel method of locally applying retinoic acid that makes questions concerning the role of retinoic acid in axis formation more testable. Use of

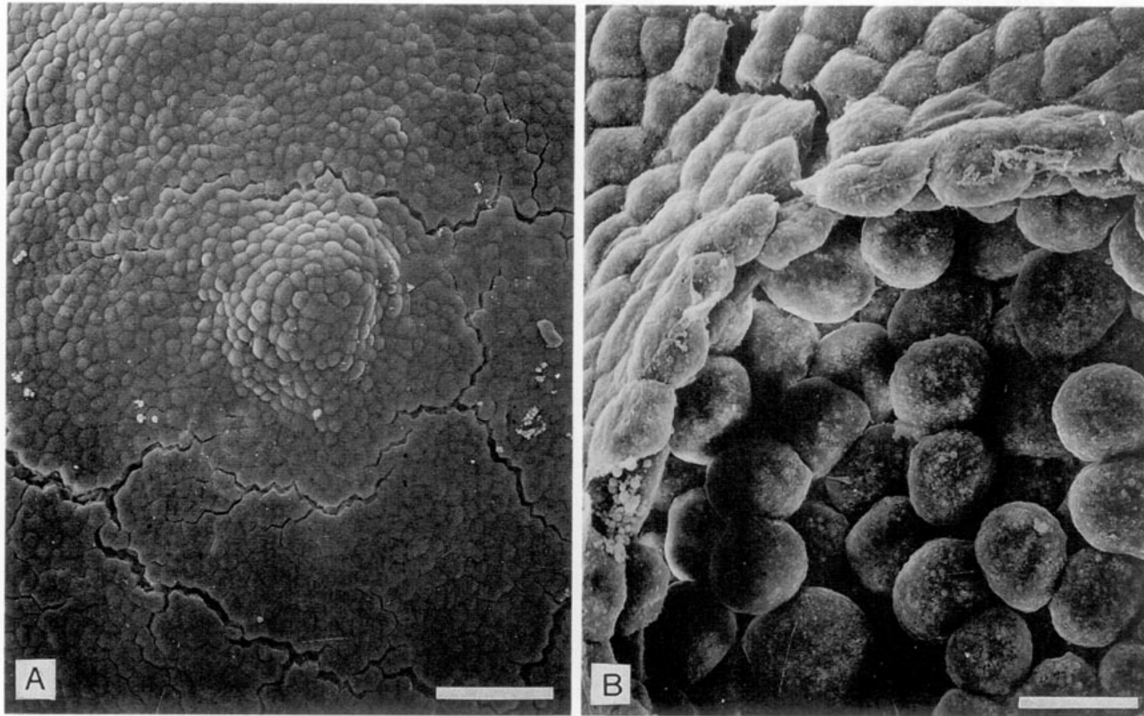

FIG. 6. (A) Microinjection of control and retinoic acid-containing droplets appeared to have no effect upon ectoderm integrity. This specimen, fixed 3 hrs following retinoic acid injection, appears normal, with the exception that the surface ectoderm has bulged slightly. No cytotoxicity was visible either 1.5, 3.0, or 14 hr postinjection. Scale bar = 100 μm. (B) This photomicrograph is of a 3-h postinjection specimen, the injection site of which was broken open just prior to sputter coating. Such specimens demonstrated that injections were being successfully placed between deep and surface ectoderm layers, and furthermore, that cells in both layers appear to suffer no obvious ill effects from the administration of retinoic acid. The compound seems unlikely to inhibit anterior structures by cytotoxic effects. Scale bar, 30 μm.

this technique has demonstrated that, although retinoic acid inhibits differentiation of anterior structures, it does not globally respecify them to a more posterior fate. Local application of retinoic acid by droplet injection will be useful in examining how retinoic acid exerts its teratogenic effects on the vertebrate embryo.

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