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v-erbA and citral reduce the teratogenic effects of all-*trans* retinoic acid and retinol, respectively, in *Xenopus* embryogenesis

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SUMMARY

Treatment of late blastula/early gastrula stage *Xenopus* embryos with all-*trans* retinoic acid results in disruption of the primary body axis through effects on both mesoderm and neuroectoderm. This effect of retinoic acid, coupled with the known presence of retinoic acid in *Xenopus* embryos has led to the proposal that retinoic acid may be an endogenous morphogen providing positional information in early development. To further elucidate the role of retinoic acid in early *Xenopus* development, we have attempted to interfere with the retinoic acid signalling pathway both at the level of retinoic acid formation, by treatment with citral (3,7-dimethy-2,6-octadienal), and at the level of nuclear retinoic acid receptor function, by microinjection of *v-erbA* mRNA. The feasibility of this approach was demonstrated by the ability of citral treatment and *v-erbA* mRNA injection to reduce the teratogenic effects of exogenous retinol and retinoic acid, respectively, in early *Xenopus* development. Interestingly, *v-erbA* mRNA injection and citral treatment of gastrula stage embryos resulted in tadpoles with a similar set of developmental defects. The defects were chiefly found in tissues that received a contribution of cells from the neural crest, suggesting that at least a subset of neural crest cells may be sensitive to the endogenous level of retinoic acid. In accord with this proposal, it was found that the expression patterns of two early markers of cranial neural crest cells, *Xtwi* and *XAP-2*, were altered in embryos injected with *v-erbA* mRNA. These results indicate that structures in addition to the primary axis are regulated by retinoic acid signalling during early *Xenopus* development.

Key words: *Xenopus*, all-*trans* retinoic acid, retinol, *v-erbA*, citral

INTRODUCTION

All-*trans* retinoic acid (RA) is a vitamin A metabolite that exhibits diverse effects on cellular differentiation and organismal development (De Luca, 1991). RA is a potent teratogen in vertebrates affecting pattern formation at many developmental stages. For example, exogenous RA can induce digit duplication along the anterior-posterior axis in chick limb buds (Tickle et al., 1982; Summerbell, 1983) and can respecify vertebral identity in the developing mouse embryo (Kessel and Gruss, 1991; Kessel, 1992). In *Xenopus*, exogenous RA alters primary axis formation through effects on the determination of both mesoderm and neuroectoderm (Durston et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991a, 1991b; Sive and Cheng, 1991). Similar effects have also been observed in zebrafish (Holder and Hill, 1991). The ability of exogenous RA to alter pattern formation coupled with the detection of endogenous RA in chick (Thaller and Eichele, 1987) and *Xenopus* (Durston et al., 1989) embryos has led to the suggestion that RA may be an endogenous morphogen providing positional information during development (Green, 1990).

Biological responses to RA are believed to be mediated by the nuclear retinoic acid receptors (RARs), members of the steroid/thyroid hormone receptor super-family (Leid et al., 1992). These receptors act as ligand-modulated transcription factors and thus affect expression of specific genes. More recently, a second set of retinoid modulated nuclear receptors has been defined, the retinoid 'X' receptors (RXRs; Mangelsdorf et al., 1990), which appear to mediate the effects of 9-*cis* retinoic acid (Heyman et al., 1992; Levin et al., 1992). These receptors may also alter the transcriptional responses to a number of compounds, including all-*trans* RA, through the formation of heterodimers with the nuclear receptors for these compounds (Leid et al., 1992). Two members each of the RAR family and the RXR family have been cloned from *Xenopus* and the temporal expression of transcripts from these genes are consistent with a role for retinoids in the early development of this organism (Ellinger-Ziegelbauer and Dreyer, 1991; Blumberg et al., 1992).

Proof that RA plays a role in early development requires the elimination of RA or components of its signalling pathway from early embryos. However, the lack of detailed

knowledge concerning the enzymes involved in retinoid metabolism and the inability to perform gene knock-out experiments in *Xenopus* has forced us to take the two approaches outlined below in an attempt to eliminate or reduce RAR function or RA production in *Xenopus* embryos. In the first approach microinjection of *v-erbA* mRNA was utilized in an effort to interfere with RAR function. v-erbA is the protein product of the virus-transduced form of the chicken *c-erbA* gene, which encodes thyroid hormone receptor α. v-erbA differs from c-erbA in that retroviral gag sequences replace the N-terminal twelve amino acids of the c-erbA protein, and nine amino acids found at the C terminus of c-erbA have been deleted in v-erbA. In addition, thirteen internal amino acid substitutions are present in the virus-transduced form of erbA (Sap et al., 1986). Functionally, both c-erbA and v-erbA are able to bind similar specific DNA sequences, although v-erbA appears to bind some of these sequences with an apparent lower affinity. In contrast, v-erbA is severely impaired in its ability to bind thyroid hormone and activate transcription when compared to its c-erbA counterpart (Privalsky, 1992). In fact a number of reports have demonstrated that v-erbA is unable to mediate thyroid hormone responses and instead acts as a constitutive repressor of thyroid hormone induced gene expression in vertebrate cells (Privalsky, 1992). In addition, the v-erbA protein can repress ligand-induced gene expression mediated by RARs and estrogen receptors; notably the oncogenic effects of v-erbA in erythroblasts is highly correlated with its ability to suppress RAR function (Sharif and Privalsky, 1991). Similarly, v-erbA has been shown to abrogate the growth-inhibitory effects of RA on chicken embryo fibroblasts, presumably by overcoming repression of AP-1 activity mediated by RAR (Desbois et al., 1991). These results raised the possiblity that v-erbA could be used in a dominant negative fashion to block RAR function in *Xenopus* embryos. Our second approach makes use of the food additive citral (3,7-dimethyl-2,6-octadienal), which has been reported to inhibit the oxidation of retinol to retinoic acid in mouse epidermis, thereby interfering with the biological activity of retinol (vitamin A) in this epithelial tissue (Connor and Smit, 1987; Connor, 1988), suggesting that citral might be potentially useful in reducing the formation of RA in *Xenopus* embryos.

In this report we demonstrate that microinjected *v-erbA* mRNA is able to partially rescue *Xenopus* embryos from the teratogenic effects of exogenous RA. Similarly, citral treatment rescues embryos from the teratogenic effects of high levels of exogenous retinol. Furthermore, microinjected *v-erbA* RNA and citral treatment of embryos results in specific developmental defects, suggesting that endogenous RA plays a role in early *Xenopus* development. It is suggested that these tools will be useful in further elucidating the role of retinoids in early vertebrate development.

MATERIALS AND METHODS

Materials

All-*trans* retinoic acid, all-*trans* retinol, citral (3,7-dimethyl-2,6-octadienal), and 4-methylpyrazole were purchased from Sigma. Nerol (3,7-dimethyl-2,6-octadien-1-ol) and 3,7-dimethyl-2,6-octadienenitrile were purchased from Aldrich.

Embryo culture

Fertilized *Xenopus* embryos were prepared as previously described (Newport and Kirschner, 1982). The jelly coat was removed by treatment with 2% cysteine, pH 7.8 and the embryos were rinsed with 0.1× MMR (1× MMR is 0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM Hepes, pH 7.8.) Embryos were maintained in 0.1× MMR at 16°-23°C and developmental stages determined according to Nieuwkoop and Faber (1956).

Retinoid and citral treatment of embryos

Stage 9.5-10.5 embryos were treated in the dark with retinoids diluted 1:1000 into 0.1× MMR from stocks dissolved in dimethyl sulfoxide or ethanol. Retinoic acid treatment (2 μM) was typically 25-40 minutes (duration of treatment was varied chiefly due to individual batch sensitivity of embryos to RA), while retinol (25 μM) treatment was overnight (approx. 16 hours). Citral and related compounds were prepared as stocks in acetone or ethanol and were diluted 1:1000 (final concentration 20-60 μM) into 0.1× MMR for treatment of embryos, 2.5-16 hours as stated in the figure legends.

RNA microinjection and western immunoblots

cDNAs encoding wild-type *v-erbA* or the *v-erbA* mutants S61G or 82t (Privalsky et al., 1988; Sharif and Privalsky, 1991) were inserted into the unique *Bgl*II site of plasmid pSP64T (Krieg and Melton, 1984) utilizing *Bgl*II linkers. Capped, synthetic mRNA was transcribed from *Xba*I linearized plasmid using SP6 RNA polymerase (Krieg and Melton, 1984). 5-20 nl of solution containing 0.15-0.4 mg/ml of RNA was microinjected into one- to four-cell embryos as described (Moon and Christian, 1989). Dorsal or ventral blastomere RNA injections were performed on four-cell embryos that had been previously 'tipped' and marked on the dorsal side (Peng, 1991).

Embryo extracts from control and *v-erbA* mRNA-injected embryos at various stages of development were prepared as described (Moon and Christian, 1989) and the extract from 2 embryo equivalents per sample was fractionated by electrophoresis in 10% SDS-PAGE gels, electroblotted to nitrocellulose and *v-erbA* immunoreactive polypeptides detected utilizing a rabbit polyclonal antiserum (1:200 dilution) directed against the v-erbA portion of a *lacZ/v-erbA* fusion protein (Bonde and Privalsky, 1990) and goat anti-rabbit secondary antibody (1:2500; Boehringer-Mannheim) coupled to alkaline phosphatase. The immunoblot was developed as previously described (Schuh et al., 1992).

RNAse protection assays

Animal pole cell explants were isolated from uninjected stage 8-9 embryos or embryos previously injected at the 2-cell stage with 82t or *v-erbA* RNA (2-4 ng) as previously described (Kimelman and Maas, 1992). *Xlim-1* expression was induced by treatment of animal caps with recombinant porcine activin A (4 ng/ml) or all-*trans* retinoic acid (5 μM). Animal caps were harvested after 6-7 hours of treatment and RNA was prepared as described (Krieg and Melton, 1984). RNAse protection assays were carried out on RNA from 8-10 animal caps with a 254 bp antisense transcript from the *Xlim-1* gene (Taira et al., 1992) (*Pst*I-*Ssp*I fragment encoding amino acids 285-369) synthesized using T7 RNA polymerase in the presence of [^{32}P]UTP (Krieg and Melton, 1984). An EF-1α (Krieg et al., 1989) probe prepared at reduced specific activity was added to the RNAse protection assays as a control for sample loading. RNAse treatment of hybridized transcripts was for 1 hour at 30°C with 10 μg/ml and 0.5 μg/ml RNAse A and T1, respectively. Protected RNA species were detected by autoradiography

of dried gels exposed to Hyperfilm-MP (Amersham) for 2-4 days with an intensifying screen.

Histology

Whole embryos were fixed in Bouin's reagent and paraffin embedded samples were sectioned and stained (Kimelman and Maas, 1992). Alternatively, embryos were fixed in Romeis' fixative (Hausen and Riebesell, 1991) and paraffin embedded samples sectioned as above. Mercury was removed from rehydrated tissue sections by incubating in Lugol's iodine (1 g iodine and 2 g potassium iodide/300 ml water) for 15 minutes, rinsing with water for 1 minute, clearing in 5% sodium thiosulfate and washing in running water for 10 minutes. After mercury removal, sections were stained using the fuelgen/light green/orange G method as described above. Sections were photographed under bright-field illumination with a Zeiss Axioplan microscope fitted with a Zeiss MC 100 camera containing Kodak Ektachrome film.

In situ hybridization

Whole-mount in situ hybridization was performed using digoxigenin-labelled RNA probes (Harland, 1991). An anti-sense probe corresponding to the 1050 bp *Eco*RI fragment of *XAP2-6a* (Winning et al., 1991) cloned into the *Eco*RI site of pBS+ (Stratagene) was synthesized using *Hin*dIII linearized plasmid and T7 RNA polymerase. An *Xtwi* anti-sense probe was prepared from a 550 bp cDNA fragment containing the entire protein coding sequences (Hopwood et al., 1989) cloned into pBluescript II SK+ (Stratagene) that was linearized with *Xho*I and transcribed using T3 RNA polymerase.

Retinoid extraction and analysis

All procedures were carried out using amber light. 100 *Xenopus* embryos (approx. 100-120 mg wet weight) from the designated stages of development were collected into a vial and rapidly frozen. To eliminate variability between different frogs in our outbred colony, all embryos for a single experiment were collected from a single mother. Immediately prior to HPLC analysis, 100 μl of isopropanol was added to the embryos and they were then sonicated on ice. The homogenate was vortexed for 1 minute and centrifuged at 4°C for 20 minutes at 4000 ***g***. The supernatant was injected directly into the HPLC system.

HPLC

A binary gradient system allowed separation of 10 retinoids within 20 minutes (Creech Kraft et al., 1988). A precolumn (20×4 mm cartridge prepacked with Lichrosorb RB 18 10 μm) and an analytical column (125×4.6 mm slurry packed with Spherisorb ODS II 3 μm) were used. The mobile phase was a mixture of 40 mM ammonium acetate (pH 7.3) and methanol. The gradient was formed by mixing these solvents such that the initial methanol concentration was 55%, reaching 100% at the termination of the run. The flow rate was 1.6 ml/minute and the eluate was monitored at 354 nm. Calibration was performed by a previously described external standard method (Creech Kraft et al., 1988). Each HPLC analysis was run in duplicate. Run to run variation was approximately 2% (Creech Kraft and Juchau, 1992).

RESULTS

v-erbA reduces the teratogenic effects of retinoic acid in *Xenopus*

In light of recent results describing the effects of v-erbA on retinoic acid induced gene expression in cell culture (Privalsky, 1992), we reasoned that v-erbA could potentially be used to selectively inhibit retinoic acid receptor function in early *Xenopus* development, and therefore be useful in elucidating the role, if any, of RA in early *Xenopus* development. As an initial test, we asked whether *v-erbA* mRNA could rescue *Xenopus* embryos from the teratogenic effects of exogenous RA. RA treatment of blastula and early gastrula stage embryos has been shown to cause graded anterior truncations of *Xenopus* embryos, resulting in loss of eyes, cement gland, and hatching gland cells as well as specific disruptions of central nervous system (CNS) structure (Durston et al., 1989; Drysdale and Elinson, 1991; Papalopulu et al., 1991). At high levels (micromolar concentrations) of RA, treatment of embryos for 30 minutes or longer results in severe axial deficiencies in both the anterior and posterior regions (Durston et al., 1989); an example of such RA-treated embryos is shown in Fig. 1C. Notably, microinjection of *v-erbA* mRNA into 1- or 2-cell embryos (at two random sites approx. 180° from one another) prior to RA treatment resulted in a distinct reduction in the teratogenic effects of RA (Fig. 1A,C). RA-treated embryos injected with *v-erbA* transcripts all hatched compared with only one-third of uninjected RA-treated embryos. In addition, *v-erbA* mRNA-injected embryos showed marked increases in the number of embryos displaying cement glands and eye tissue, particularly embryos with two eyes, when compared with uninjected embryos (Fig. 1A). The ability to inhibit the teratogenic effects of RA appeared to be a specific effect of the erbA portion of *v-erbA* since the 82t mutant of *v-erbA* (Privalsky et al., 1988), which contains a translational termination codon near the beginning of the erbA portion of *v-erbA*, was unable to rescue embryos from the teratogenic effects of RA.

RA treatment of *Xenopus* embryos has been reported to primarily affect dorsal structures, thus it might be anticipated that dorsal injection of *v-erbA* mRNA would be more effective than ventral injection of *v-erbA* mRNA in protecting embryos from RA teratogenicity. In accord with expectations, injections of *v-erbA* mRNA on the dorsal side produced significant rescue of RA-treated embryos, whereas ventral *v-erbA* mRNA injections were unable to do so (Fig. 1B). For unexplained reasons, ventral *v-erbA* mRNA injections actually resulted in a significant reduction in the number of embryos with cement glands (Fig. 1B). Thus v-erbA rescue of *Xenopus* embryos from the teratogenic effects of RA in early development was dependent upon the presence of v-erbA in the dorsal region of the embryo, implying that, in agreement with previous reports, the teratogenic effects of RA are primarily due to effects on dorsal structures.

v-erbA represses RA-induced expression of *Xlim-1* in animal pole cell explants

The biological effects of RA appear to be mediated by nuclear RARs that act as ligand-modulated transcription factors. Interference with RA/RAR-modulated gene expression was therefore a likely mechanism by which v-erbA might be functioning to reduce the teratogenic effects of RA in early *Xenopus* development. The homeobox-containing gene *Xlim-1* has been shown to be inducible in animal pole cell explants from blastula stage *Xenopus* embryos by both RA and activin, a peptide growth factor from the transforming growth factor-β family (Taira et al.,

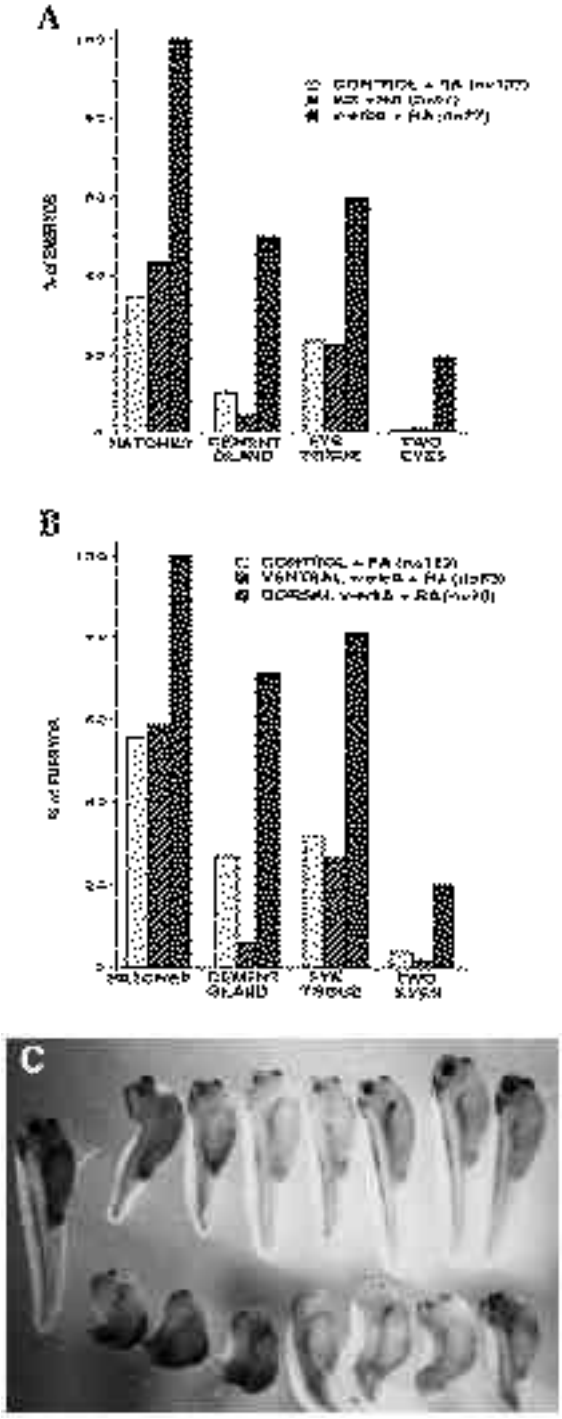

Fig. 1. v-erbA rescue of RA-treated embryos. (A) Uninjected embryos and embryos microinjected with *v-erbA* mRNA or the *v-erbA* mutant 82t mRNA were treated with 2 μM RA for 25-40 minutes at stage 9.5-10.5. The number of hatched embryos and the presence of cement glands, eye tissue or two complete eyes was scored on day 3 or 4 (*n* = number of embryos scored). (B) 4-cell embryos were injected in two dorsal or two ventral blastomeres prior to RA treatment as described for A above. (C) Example of v-erbA rescue of RA-treated embryos; left, 1 untreated embryo; bottom row, range of phenotypes of RA-treated embryos; top row, range of phenotypes of RA-treated embryos injected with *v-erbA* mRNA.

1992). We therefore asked whether v-erbA would affect the RA-induced expression of this gene. As shown in Fig. 2A, v-erbA repressed the RA-induced expression of *Xlim-1* in animal pole cell explants below levels observed in control explants injected with 82t mRNA (approx. 80% reduction; Fig. 2A, lanes 4,6). This appeared to be a specific effect of v-erbA on RA-induced expression of *Xlim-1* since v-erbA did not alter the induction of *Xlim-1* by activin in animal pole cell explants (Fig. 2B, lanes 2, 3). Therefore, similar to results seen in numerous cell lines using various reporter genes, v-erbA was able to interfere with RA-inducible gene expression in *Xenopus*, which likely accounts for its ability to rescue embryos from the teratogenic effects of RA.

v-erbA alters normal *Xenopus* development

Since v-erbA was able to interfere with the response of *Xenopus* embryos to exogenous RA, it was anticipated that v-erbA would also repress endogenous RA function and thereby perturb normal *Xenopus* development if RA was critical during the early embryonic stages. The earliest observed effects of injection of 1.6-3.2 ng of *v-erbA* RNA were seen in late neurula stage embryos, in which cement gland development appeared reduced or delayed (not shown). This result, as well as those described below, were not due to an RNA injection artifact since embryos injected with an equivalent or greater amount of mRNA encoding the *v-erbA* null mutant 82t developed normally. Also noted in the v-erbA embryo at this stage was a less distinct head fold in the neural plate and somewhat reduced elevation of the neural folds along the neural tube. These developmental aberrations were somewhat more apparent in embryos injected in only one of the blastomeres at the two-cell stage, in which the uninjected side served as an internal control (Fig. 3A). Embryos injected with *v-erbA* mRNA were consistently 'stubbier' in appearance or shorter than their tailbud stage sibling controls (Fig. 3B). Though not apparent in these photographs, *v-erbA* mRNA-injected tailbud embryos often demonstrated an external bulging of tissue in the region just posterior and ventral relative to the eye, the region of the developing branchial arches.

The phenotype of tadpole stage embryos previously injected with *v-erbA* mRNA was distinct from that of uninjected or 82t mRNA-injected siblings (Fig. 3C-E). Head morphology was clearly altered in a high percentage of *v-erbA* mRNA-injected embryos (Fig. 3C). Additional defects noted by visual inspection in many of these embryos included (a) dorsal fin defects, (b) heart and blood defects, in which blood pools were found in various regions of the embryo and/or blood never reached the heart, which itself showed structural alterations in many cases, (c) alterations in gut structure, (d) changes in pigmentation, including hypo- and hyper-pigmentation (e) microphthalmia. Interestingly, in a number of the *v-erbA* mRNA injection experiments a large percentage of the embryos showed a sharp dorsal bend (approx. 30°-120°) in the primary embryonic axis just posterior to the hindbrain (Fig. 3D). Higher levels of injected *v-erbA* mRNA (4-6 ng) commonly resulted in more extreme embryonic defects (see Fig. 3E for a range of phenotypes); however, since many of these embryos failed to gastrulate properly (a common defect in *Xenopus* development) and only a low percentage of these embryos

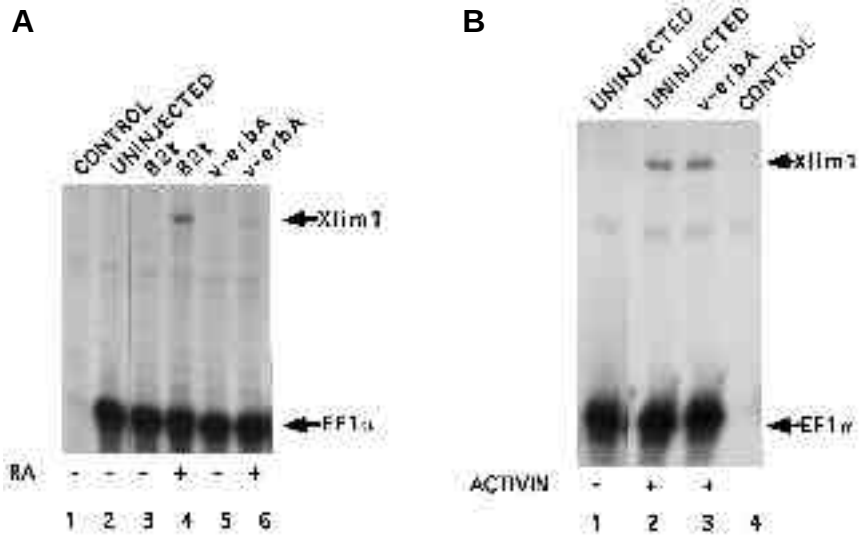

Fig. 2. Effects of v-erbA on RA- or activin-induced *Xlim-1* expression in animal pole cell explants. Animal pole cell explants from uninjected, 82t and *v-erbA* mRNA-injected embryos were treated with (A) RA (5 μM), or (B) activin (4 ng/ml) for 6-7 hours. Induction of *Xlim-1* expression was assessed by RNAse protection assays. An *EF-1*α probe was included as a control to assess relative RNA loading. Control samples contained only tRNA and probes. The results from one representative experiment are shown.

survived to the tadpole stage, they were not examined in detail.

Further analysis of *v-erbA* mRNA-injected embryos demonstrated that production of abnormal tadpoles, such as those seen in Fig. 3C, required the presence of v-erbA in the dorsal region of the embryo (Fig. 4A). Ventral *v-erbA* mRNA injections produced only a small percentage of abnormal embryos; the abnormal embryos produced by ventral injections of *v-erbA* mRNA were probably due to injection mistakes in which one dorsal and one ventral blas-

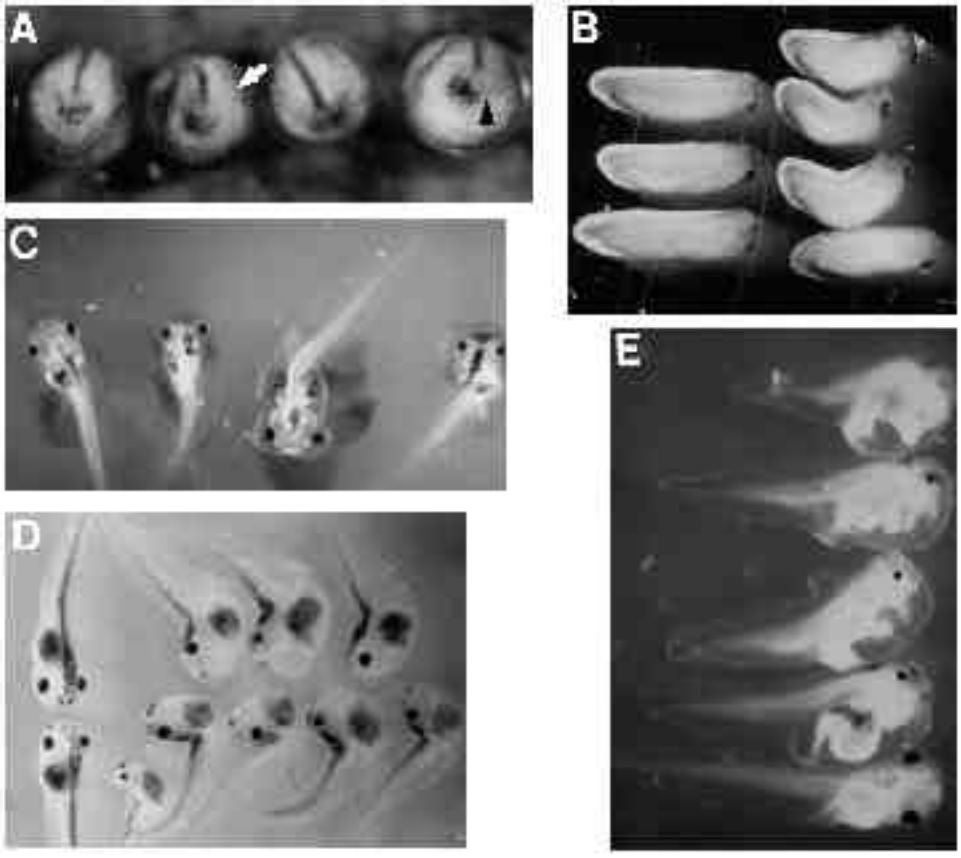

Fig. 3. Effects of v-erbA on *Xenopus* development. Uninjected embryos and embryos injected with 1.6-4 ng of *v-erbA* or 82t mRNA were examined at various stages of development. (A) Anterior view of late neurula stage embryos unilaterally injected with *v-erbA* mRNA. Arrowhead indicates reduced cement gland formation and arrow indicates reduced head fold on side of the embryo that received the *v-erbA* mRNA injection. Dorsal is at the top. (B) Lateral view of uninjected (left) and *v-erbA* mRNA-injected (right) tailbud stage embryos. Anterior is right, dorsal is at top. 82t mRNA-injected embryos appeared to be identical to uninjected embryos. (C) Dorsal view of one uninjected (left) and three *v-erbA* mRNA-injected tadpole stage embryos. Anterior is at the top, except for the third embryo which is in the reverse orientation. 82t mRNA-injected embryos appeared to be identical to the uninjected embryo shown. (D) Dorsal-lateral view of eight tadpole stage embryos injected with *v-erbA* mRNA (right) and two 82t mRNA-injected controls (left). Note the sharp dorsal bend in the primary axes of the *v-erbA* mRNA-injected embryos. (E) Lateral view of tadpole stage embryos (top four) depicting the range of effects of a high level (4-6 ng) of *v-erbA* mRNA injection. An uninjected control embryo is shown at the bottom. Anterior is at the right. Injection of equivalent amounts of 82t mRNA had no apparent effects on embryonic development.

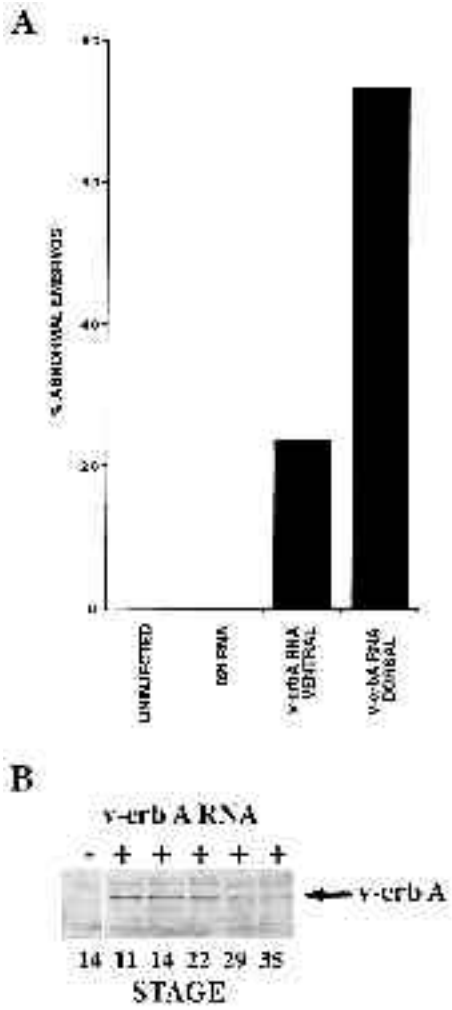

Fig. 4. Effects of dorsal versus ventral *v-erbA* mRNA injection on *Xenopus* development. (A) Four-cell embryos were injected with one of the indicated RNAs in two dorsal or two ventral blastomeres and embryos were scored at the tadpole stage (see Fig. 3C). Data presented are the combined results from two experiments. Number of embryos n=55 (uninjected), n=45 (82t), n=17 (ventral *v-erbA*), n=30 (dorsal *v-erbA*). (B) Embryos were injected with *v-erbA* mRNA at the two-cell stage and the levels of *v-erbA* protein present at the indicated stages was assessed by immunoblotting. Arrow indicates the *v-erbA* protein (M_r 75×10^3) which was not present in uninjected controls. Similar results were obtained using an antiserum (gift of R.N. Eisenman) that recognizes the gag-portion of v-erbA.

tomere were injected. This result indicates that the effects of v-erbA on normal development and rescue of RA teratogenicity occur within the same region of the embryo, and suggests that the effects of v-erbA in normal development may be due to repression of the RA/RAR signalling pathway. Further support for this idea comes from studies of embryos injected with the S61G v-*erbA* mutant RNA. In this v-erbA mutant, the serine residue at position 61 was changed to glycine, the amino acid normally found at this position in c-erbA (Sharif and Privalsky, 1991). The S61G mutant has been shown to retain its ability to strongly repress thyroid hormone and estrogen induced transcriptional activation, however, its ability to repress RA-induced transcription was somewhat compromised. Microinjection of S61G *v-erbA* mRNA resulted in the v-erbA phenotype at approx. 50% of the efficiency of an equivalent amount of wild-type *v-erbA* mRNA (not shown). This result is in agreement with the reduced level of the S61G v-erbA repression of RA-induced transcription in cell culture reporter gene assays (Hall et al., 1992), and supports the contention that the effects of v-erbA on normal *Xenopus* development were due to disruption of RA mediated events. In addition, v-erbA-mediated effects on *Xenopus* development most likely occur prior to the tadpole stages, since the level of *v-erbA* protein detected on immunoblots containing extracts of embryos injected with *v-erbA* mRNA appear to decline very rapidly during the tailbud stages (Fig. 4B).

Histological sections reveal early defects in *v-erbA* mRNA-injected embryos

Stained histological sections of tailbud stage embryos were examined in order to supplement the gross morphological observations, as well as search for clues as to the underlying early developmental alterations that result in the defects found in tadpoles previously injected with *v-erbA* mRNA. Lateral sagittal sections of tailbud stage embryos revealed a disruption of normal branchial arch/visceral pouch development. The outpockets of the foregut, the visceral pouches, were seen as a striped pattern in control embryos that was not present in *v-erbA* mRNA-injected embryos of the same age (Fig. 5A,B). Likewise, ventral coronal sections showed an alteration of branchial arch/visceral pouch structure in *v-erbA* mRNA-injected versus 82t mRNA-injected controls (Fig. 5C,D). The embryos injected with *v-erbA* developed a reduced number of branchial arches/visceral pouches and enlargement of the arches that were present.

Further defects were noted in mid-sagittal sections of tailbud stage embryos which revealed an altered morphology of foregut structure, as well as an increase in head mesenchyme cells just below the anterior portion of the notochord in embryos containing v-erbA (Fig. 5E,F). Transverse sections of tailbud embryos clearly showed that development of the heart was abnormal in the *v-erbA* mRNA-injected embryos. v-erbA embryos had hearts approximately one-half the size of sibling controls at this stage, and the walls of the heart were uniformly thick, whereas sibling control hearts showed regional differences in the thickness of the heart wall (Fig. 5G,H). Interestingly, it has been reported that rat embryos obtained from mothers that were reared and bred on a vitamin A deficient diet had heart muscles that were thick and spongy in appearance (Warkany et al., 1948).

The outcome of some of these early developmental defects are depicted in coronal sections taken from 5-day old tadpoles injected with 82t control mRNA or *v-erbA* mRNA (Fig. 5I,J). These sections were at the level of the most ventral portion of the eyes and highlight the disruptive effects of v-erbA on head structures and gut development; such defects were likely the result of the alterations in branchial arch/visceral pouch and foregut development noted above in sections from tailbud stage embryos. Additional observations of embryo sections suggested that embryos containing v-erbA had enlarged notochords and

reduced thickness of neural tissue in the CNS. However, further studies will be necessary to substantiate and quantitate these effects. In summary, histological sections from tailbud stage embryos injected with *v-erbA* mRNA revealed a number of distinct developmental alterations that likely contribute to the gross morphological defects observed in swimming tadpoles.

v-erbA alters the pattern of expression of *Xtwi* and *XAP-2* in cranial neural crest cells

Whole-mount in situ hybridization analyses were performed on *v-erbA* mRNA-injected embryos using molecular markers expressed in the cranial neural crest and head of late neurula/early tailbud stage embryos, to delineate further the early developmental abnormalities. For analysis of cranial neural crest cells, anti-sense probes complementary to *Xtwi* and *XAP-2* were utilized (Hopwood et al., 1989; Winning et al., 1991; Bradley et al., 1992). Whereas expression of *Xtwi* and *XAP-2* appeared to be normal in neural crest cells of the mandibular arch, expression of these two genes in hyoid arch crest cells was clearly altered in *v-erbA* mRNA-injected embryos relative to control (82t mRNA-injected) embryos (Fig. 6). Notably, expression of *Xtwi* and *XAP-2* in the hyoid arch appeared to be reduced and was found in a more dorsal position relative to expression in the neural crest cells of the more posterior branchial arches (Fig. 6B,D). In contrast, *Xtwi* and *XAP-2* expression in hyoid crest cells of control embryos extended to a more ventral position relative to the expression of these same genes in the branchial arch crest cells (Fig. 6A,C). In addition, the expression pattern of *Xtwi*, and to a lesser extent, *XAP-2*, in the branchial neural crest cells also appeared to be affected by injection of *v-erbA* mRNA (Fig. 6B). Other markers of early head development such as *En-2*, *Krox-20* and *Xlim-1* were also examined by whole-mount in situ hybridization in *v-erbA* mRNA-injected embryos. No alterations in the spatial expression patterns of these genes was noted, although minor differences in staining intensity between *v-erbA* mRNA-injected and control embryos were sometimes observed (not shown). In summary, the analysis of expression patterns of molecular markers in conjunction with the histological analysis, suggest that v-erbA likely affects cranial neural crest cell development, which probably contributes to the morphological defects observed in later stage embryos.

Citral rescues *Xenopus* embryos from the teratogenic effects of retinol

A second independent approach was utilized in an effort to block the endogenous RA signalling pathway. Retinol (Vitamin A) serves as a precursor in the formation of RA in biological systems and it has recently been reported that citral (3,7-dimethyl-2,6-octadienal) was able to partially block the conversion of retinol to retinoic acid and that this block resulted in a reduced ability of retinol to induce hyperplasia and inhibit phorbol ester-induced gene expression in mouse epidermis (Connor and Smit, 1987; Connor, 1988). Therefore, it was of interest to determine if citral could be used to block RA production via metabolic conversion from retinol in *Xenopus* embryos and thereby protect these embryos from retinol teratogenicity.

In accord with the observations of others (Durston et al., 1989), it was found that retinol concentrations greater than 20-fold that of RA were required to observe teratogenic effects in *Xenopus* embryos, and that morphologically these effects were identical to those produced by administration of RA at the late blastula/early gastrula stage (Fig. 7A and data not shown). Addition of an equimolar amount of citral at the time of retinol administration (late blastula/early gastrula stage) resulted in a significant reduction in the teratogenic effects of retinol in *Xenopus* embryos (Fig. 7A). However, as anticipated, citral was unable to rescue embryos from the teratogenic effects of exogenous RA (not shown). The ability of citral to rescue embryos from the teratogenic effects of retinol was quantitated by scoring for the presence or absence of anterior structures such as cement gland and eyes, tissues frequently lost upon retinoid treatment of embryos (Fig. 7B). The results demonstrate that embryos treated with retinol in the presence of citral retained the ability to produce cement glands and two distinct, complete eyes in contrast to siblings treated with retinol alone. The ability to produce some eye tissue was not clearly affected by citral in this experiment, however, at higher levels of retinol resulting in increased teratogenic effects, significant rescue of the ability to produce eye tissue was observed (not shown). The ability of citral to rescue embryos from retinol teratogenicity was highly dose dependent; equimolor or slightly greater than equimolar amounts of citral to retinol resulted in the highest percentage of rescue (not shown).

The effects of citral in reducing retinol teratogenicity appeared to be specific, since related compounds in which the aldehyde group of citral was replaced by an alcohol (nerol) or nitrile (3,7-dimethyl-2,6-octadienenitrile) had no effects (Fig. 7B). Furthermore, formaldehyde, an unrelated compound containing an aldehyde group, at similar concentrations also had no ability to prevent retinol teratogenicity, indicating that an aldehyde functional group alone was not sufficient for rescue (not shown). Thus, it seems likely that in *Xenopus*, as in the mouse skin model, citral was able to block the metabolic conversion of exogenous retinol to RA.

Citral alters normal *Xenopus* development

The ability of citral to interfere with retinol induced teratogenicity suggested that citral was blocking the conversion of exogenous retinol to RA. Thus, it was anticipated that citral would reduce the formation of RA from endogenous retinol in *Xenopus* embryos. Direct measurement of endogenous retinoid levels by HPLC analysis (Creech Kraft and Juchau, 1992) demonstrated that treatment of gastrula stage embryos with 60 μM citral resulted in a 62% decrease in RA, 2.5 hours after treatment (Fig. 8). After 16 hours of citral treatment there was no difference in RA levels of control and citral-treated embryos (not shown). Therefore citral was able to reduce the endogenous levels of RA in *Xenopus* embryos, but only during the initial stages of treatment.

Since citral was able to reduce retinol teratogenicity and decreased endogenous RA levels, we examined the effects of citral treatment on *Xenopus* development. Embryos were treated with micromolar concentrations of citral overnight, beginning at the late blastula/early gastrula stage. Early

Fig. 5. Sections of control and *v-erbA* mRNA-injected embryos. Uninjected embryos and embryos injected with *v-erbA* or 82t mRNA at the two-cell stage were fixed, sectioned and stained. In all sections examined, 82t mRNA-injected controls appeared identical to uninjected controls and showed no obvious developmental defects. (Magnification = 62.5×). (A,B) Lateral sagittal sections of control (uninjected) (A), and *v-erbA* mRNA-injected (B) embryos at the tailbud stage. Arrowheads indicate the visceral pouches that partition the intervening branchial arches in the control embryo, which are absent or present in a rudimentary form in the *v-erbA* mRNA-injected embryo. Dorsal is at the top, anterior is to the right. (C,D) Ventral coronal sections of control (82t mRNA-injected; C) and *v-erbA* mRNA-injected (D) embryos at the tailbud stage. Arrowheads indicate branchial arches. Anterior is to the right. (E,F) Mid-sagittal sections of control (uninjected; E) and *v-erbA* mRNA-injected (F) embryos at the tailbud stage. The foregut is designated by f and the arrow indicates the region ventral to the notochord discussed in the text. Dorsal is at top, anterior is to the left. (G,H) Transverse sections of control (uninjected; G) and *v-erbA* mRNA-injected (H) embryos at the tailbud stage. Arrows indicate developing heart. Dorsal is at the top. (I,J) Coronal sections of control (82t mRNA-injected) (I) and *v-erbA* mRNA-injected (J) embryos at the tadpole stage. The ventral portion of the eye is designated by e; arrow indicates gut structures. Anterior is to the left.

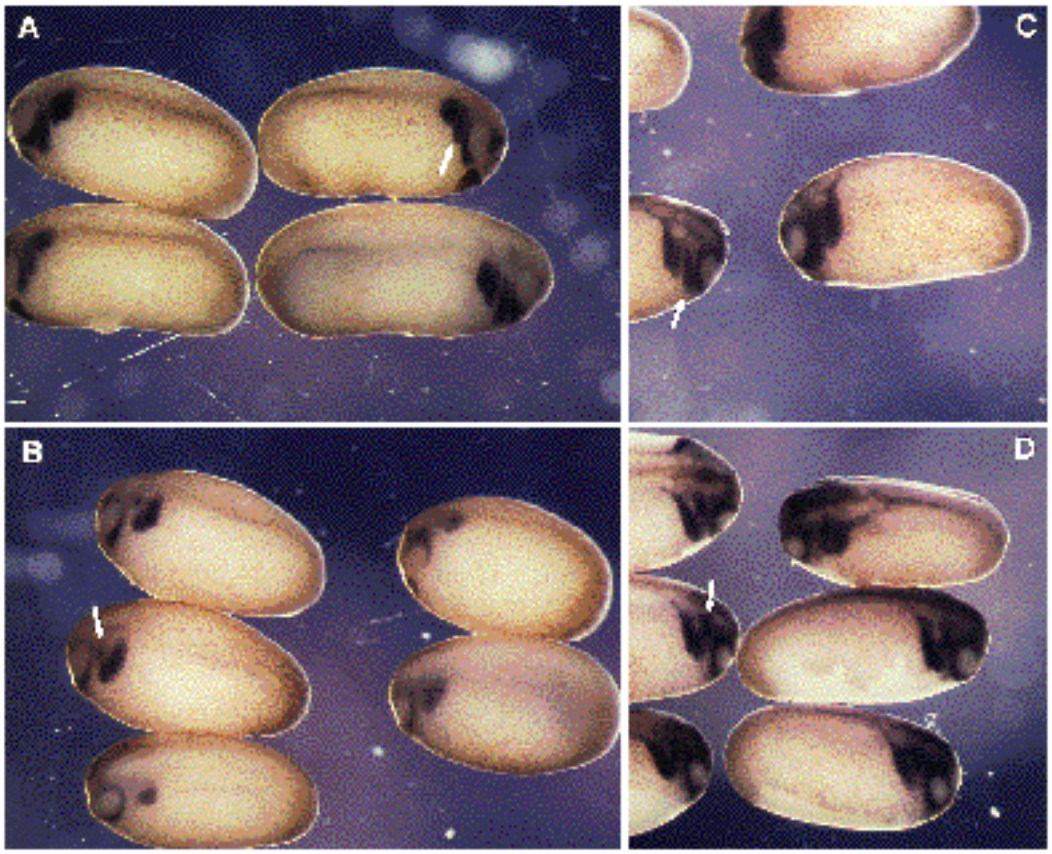

Fig. 6. Effects of v-erbA on *Xtwi* and *XAP-2* expression. Whole-mount in situ hybridization analysis of *Xtwi* (A,B) and *XAP-2* (C,D) expression in control (82t mRNA-injected; A,C) and *v-erbA* mRNA-injected (B,D) embryos (stage 23). Arrows indicate staining of hyoid crest cells, which are found just posterior to mandibular crest cells (surrounding the future eye) and anterior to the anterior and posterior components of the branchial arch crest. Dorsal is at the top in all panels.

tailbud embryos previously treated with citral showed a dose-dependent reduction in embryo length (Fig. 9A), taking on a 'stubby' appearance that was reminiscent of *v-erbA* mRNA-injected embryos. In addition, the citral-treated embryos appeared to have an excessive bulging of tissue in the region of the developing branchial arches, just ventral and posterior to the eyes (not shown). These effects were not observed in embryos treated with the closely related compounds nerol, 3,7-dimethyl-2,6-octadienenitrile, or with formaldehyde (not shown).

Embryos treated with citral and allowed to develop to the tadpole stage revealed a phenotype clearly distinct from control sibling embryos, that was in many respects similar to that seen previously in tadpoles injected with *v-erbA* mRNA (Fig. 9B). Head abnormalities, alterations in gut development, changes in pigmentation levels, microphthalmia, blood pools and heart defects were noted in these embryos. In contrast to the *v-erbA* mRNA-injected embryos, the citral-treated embryos rarely showed dorsal fin defects. Treatment of embryos with compounds related to citral but without the aldehyde functional group, or treatment with formaldehyde, had no effect on *Xenopus* development up to the tadpole stage (not shown). However, treatment with the alcohol dehydrogenase inhibitor 4-methylpyrazole elicited a phenotype similar to the citral phenotype in these embryos (not shown), although much less efficiently than citral. The observation that these two chemically unrelated compounds, citral and 4-methylpyrazole, were able to produce similar developmental alterations in *Xenopus* embryos and their reported ability to inhibit metabolic conversion of retinol to RA in certain systems (Connor and Smit, 1987), suggests that inhibition of RA production at sufficient levels or at the proper time, was a likely cause of the observed developmental defects.

Gastrula and neurula stage embryos are sensitive to citral treatment

Xenopus embryos appear to be most sensitive to the teratogenic effects of exogenous RA when it is present during the late blastula to mid-neurula stages (Durston et al., 1989). These results have been interpreted to indicate that endogenous RA function is critical at this point in development. It was therefore of interest to determine the temporal course of sensitivity to inhibition of RA production by citral in *Xenopus* embryos. Embryos at various stages of development were treated with 60 μM citral and were allowed to develop to the tadpole stage, at which time they were scored for abnormal head morphology, in which the heads appear enlarged and expanded laterally ventral to the eyes (see Fig. 9B). A dose of 60 μM citral was used, since this concentration resulted in little apparent toxicity in most embryo batches treated at stage 9 (late blastula) or later, yet gave a high frequency of defective embryos. Interestingly, treatment of embryos with citral prior to stage 9, often resulted in embryo death and alterations in gastrulation. This result could be due to toxic effects of citral at early stages or might possibly point to a role for RA in developmental events prior to gastrulation. Treatment of gastrula stage or older embryos with 60 μM citral rarely resulted in embryo death, however a very high percentage (90%) of embryos

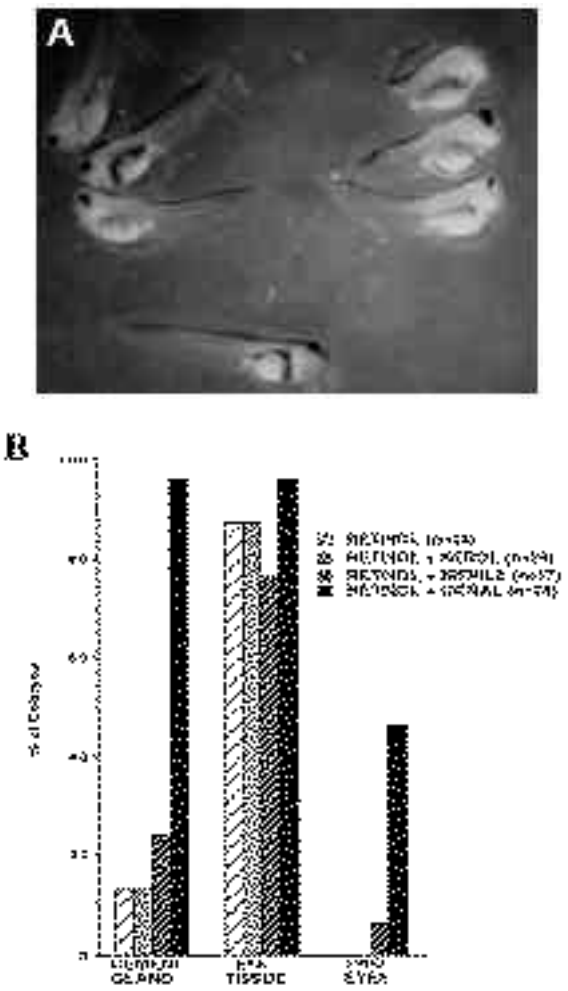

Fig. 7. Rescue of retinol-treated embryos by citral. Stage 9.5-10.5 embryos were treated with 25 μM retinol +/− 30 μM citral or related compounds overnight (approx. 16 hours). (A) Lateral view of three retinol-treated embryos (right), three embryos treated with retinol and citral (left), and one untreated embryo (bottom) at the tadpole stage. (B) Embryos treated with retinol +/− citral or related compounds were scored on day 4 for the presence of cement gland, eye tissue or two complete eyes. The results from one of two representative experiments is presented. *n* = number of embryos scored. citral: 3,7-dimethyl-2,6-octadienal; nerol: 3,7-dimethyl-2,6-octadien-1-ol; nitrile: 3,7-dimethyl-2,6-octadienenitrile.

treated during gastrulation (stages 10-12) showed the developmental alterations described above (Fig. 9B,C). Early to mid-neurula stage embryos (stage 13-16) treated with citral also showed similar developmental deficiencies, but at a reduced frequency (~50%). There was a precipitous drop in sensitivity to citral treatment when embryos reached the late neurula stage, such that no abnormal tadpoles were ever observed when embryos were treated with citral after stage 17. These results suggest that endogenous RA or other retinol metabolites are likely to be necessary during the gastrula and neurula stages for normal development. This result coupled with the results demonstrating that exogenous RA has its most severe teratogenic effects during this same period of development (Durston et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991a), indicates that the levels of retinoids, particularly retinoic acid, must be strictly regulated at these stages to ensure proper development of *Xenopus* embryos.

DISCUSSION

Two independent approaches have been utilized to disrupt the functions of RA in early *Xenopus* development. In one approach, embryos were treated with citral in an effort to reduce the amount of RA produced from retinol in these embryos, as has been done previously in the mouse skin model (Connor and Smit, 1987; Connor, 1988). The success of this approach was demonstrated by citral's ability to rescue embryos from the teratogenic effects of retinol, but not RA, and by direct measurements of endogenous RA levels, which were reduced by approx. 60% after a short treatment with citral. Citral also affected the levels of other endogenous retinoids in *Xenopus* embryos (Fig. 8), which could potentially contribute to the observed developmental aberrations and to the inhibition of retinol teratogenicity. Compounds very similar to citral were unable to rescue *Xenopus* from the teratogenic effects of retinol, and they did not cause the developmental defects that occurred after treatment with citral. In the second approach, *v-erbA* mRNA was microinjected into *Xenopus* embryos in an attempt to block RA function at the level of the nuclear receptor. Previous reports have shown that v-erbA represses the transcriptional response to RA-activated RARs and that this property of v-erbA correlates with its function in neoplasia (Sharif and Privalsky, 1991). The success of this approach in *Xenopus* was demonstrated by the ability of v-erbA to reduce the teratogenic effects of RA in early development and by its ability to repress the RA-inducible expression of the *Xlim-1* gene in animal pole cell explants.

A number of lines of evidence suggest that the developmental abnormalities resulting from *v-erbA* mRNA injection were likely to be chiefly due to interference with the normal function of endogenous RA in *Xenopus* development. (1) Two completely independent approaches (citral treatment and *v-erbA* mRNA microinjection) that would be expected to affect RA signalling at two different levels (production of RA versus RAR function), resulted in embryos with a similar set of developmental abnormalities. Thus, although one cannot be completely certain about the specificity of each interfering agent, the fact that these two very different approaches led to a subset of similar phenotypic defects, suggests that inhibition of retinoid signalling is likely to be at least partially responsible for the common developmental defects caused by these agents. (2) The *v-erbA* mutant S61G, which has a reduced capacity to repress RA-induced transcription yet retains the full capacity of wild-type *v-erbA* in repressing thyroid hormone- and estrogen-induced transcription (Sharif and Privalsky, 1991), demonstrated a similar reduced efficiency at producing defective embryos. (3) Although v-erbA can also repress transcriptional activation mediated by thyroid hormone and estrogen receptors, the presence in *Xenopus* embryos of functional receptors for either thyroid hormone or estrogens has not been conclu-

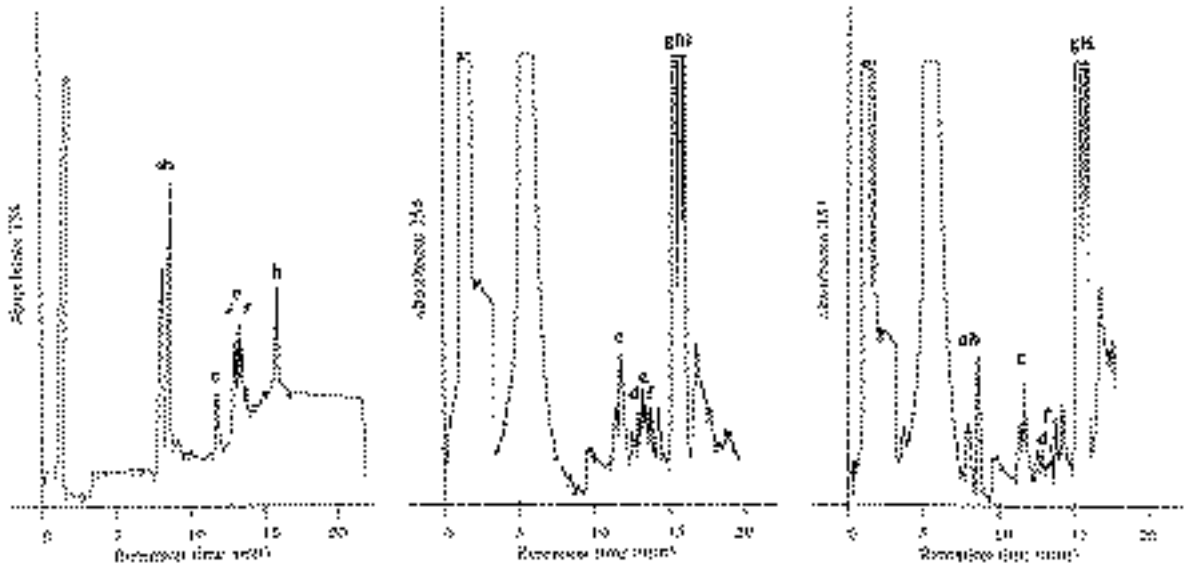

Fig. 8. HPLC analysis of the effects of citral on endogenous RA levels in *Xenopus* embryos. (Left) Chromatogram of reversed phase HPLC separation of a mixture of authentic retinoid standards. Peaks: a, 4-oxo-all-*trans* RA (810 ng/ml); b, 4-oxo-13-*cis* RA (1050 ng/ml); c, all-*trans*-RAG (300 ng/ml); d, 13-*cis*-RA (265 ng/ml); e, 9-*cis*-RA (283 ng/ml); f, all-*trans* RA (216 ng/ml); h, retinol (700 ng/ml). Sensitivity: 0.04 AUFS. (Middle) Sample chromatogram of HPLC analysis of retinoids extracted from 100 stage 12 *Xenopus* embryos. Peak c coeluted with the authentic all-*trans*-RAG (242 ng/g); peak d coeluted with the authentic 13-*cis*-RA (102 ng/g); peak e coeluted with the authentic 9-*cis*-RA (160 ng/g); peak f coeluted with the authetic all-*trans*-RA (100 ng/g); peak g coeluted with the authentic didehydro-retinol (3104 ng/g); peak h coeluted with the authentic retinol (2748 ng/g) and peak i coeluted with the authentic retinal (240 ng/g). Sensitivity: 0.005 AUFS. (Right) Sample chromatogram of HPLC analysis of retinoids extracted from 100 stage 12 *Xenopus* embryos treated with 60 μM citral for 2.5 hours. Peak a coeluted with the authentic 4-oxo-all-*trans*-RA (120 ng/g); peak b coeluted with the authentic 4-oxo-13-*cis*-RA (240 ng/g); peak c coeluted with the authentic all-*trans*-RAG (238 ng/g); peak d coeluted with the authentic 13-*cis*-RA (44 ng/g); peak f coeluted with the authentic all-*trans*-RA (38 ng/g); peak g coeluted with the authentic didehydro-retinol (3442 ng/g); peak h coeluted with the authentic retinol (3200 ng/g); peak i coeluted with the authentic retinal (840 ng/g). Sensitivity: 0.005 AUFS.

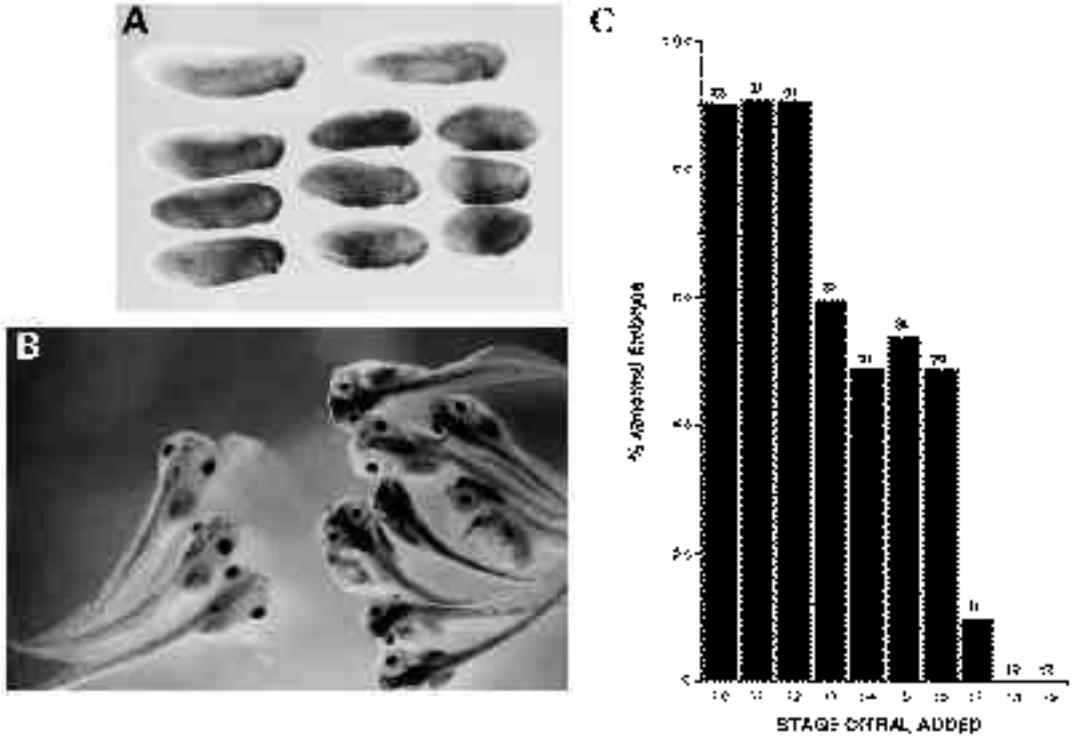

Fig. 9. Effects of citral on early *Xenopus* development. (A) Lateral view of tailbud stage embryos untreated or treated for 16 hours with increasing concentrations of citral, beginning at stage 10. Top two embryos are controls; bottom, left to right, embryos treated with 20 μM, 40 μM, and 60 μM citral, respectively. Anterior is at right. (B) Dorsal view of control (left) and citral-treated (60 μM, 16 hours beginning at stage 10) embryos at the tadpole stage. Anterior is at right for control embryos and left for citral-treated embryos. (C) Embryos treated with 60 μM citral for 16 hours beginning at the indicated stages were scored for abnormal head morphology at the tadpole stage. Numbers above bars indicate the total number of embryos scored in two independent experiments.

sively demonstrated until developmental stages much later than those studied here. Additionally, thyroid hormone and estrogens have not been detected in early *Xenopus* embryos and are believed to be initially present just prior to metamorphosis or at the post-metamorphic stages, respectively. The recently proposed ligand-independent role of thyroid hormone receptor α in early *Xenopus* development (Banker and Eisenman, 1993) would presumably not be disrupted by v-erbA. (4) The effects of v-erbA (and citral treatment) on *Xenopus* embryos were similar to those noted in embryos from other species that were obtained under vitamin A-deficient conditions (Maden and Holder, 1991). Reported effects include head, face, ear, and eye defects, and cardiovascular malformations.

The developmental defects consistently present in a high percentage of the treated embryos included head region defects, alterations in gut development, defects in the circulatory system, including heart abnormalities and blood pools, dorsal fin defects and alterations in pigmentation. Microphthalmia was also observed in a large fraction of treated embryos, however, eye formation is easily disrupted and therefore it is not known if this was a specific effect of the v-erbA or citral treatments. The structures affected by v-erbA or citral treatment were structures also often affected by teratogenic doses of RA in a number of species (Shenefelt, 1972), suggesting that the RA signalling pathway must be tightly regulated, since apparently either increased (or early) or reduced (or delayed) RA signalling leads to developmental defects. Notably, the majority of the structures affected by v-erbA or citral treatment receive a contribution of cells from the neural crest, indicating that patterning, differentiation or migration of neural crest cells might be affected by reduced levels of RA signalling. Indeed it has been reported that retinoid treatment of mouse or quail embryos interferes with the development and migration of neural crest cells (Thorogood et al., 1982; Webster et al., 1986; Pratt et al., 1987). The fact that dorsal injection of v-erbA was more efficient at producing these developmental aberrations and that formation of the branchial arches, which receive a large contribution of cells from the neural crest (a dorsal structure), was disrupted by *v-erbA* mRNA injections also support a role for RA signalling in normal development involving neural crest cells. Additionally, dorsal injection of *v-erbA* mRNA was shown to affect the expression pattern of two molecular markers of cranial neural crest cells, *Xtwi* and *XAP-2*. It is not clear at this time whether the perturbation of expression of these two genes by v-erbA was due to a change in the positional identity, differentiation status, or migratory capacity of the crest cells. A potential role for RA signalling in neural crest cell function has been previously proposed, principally derived from studies performed in mammals (Maden and Holder, 1991). Interestingly, regional specification in the branchial portion of the vertebrate head is believed to involve *HOX* genes (Hunt et al., 1991), whose expression can be modulated by RA (Simeone et al., 1990).

Although RA has been proposed to play a role in the development of the primary body axis, we infrequently observed defects in the anterior-posterior axis of treated embryos. In certain rare batches of embryos we observed a very sharp dorsal kink in the primary body axis of *v-erbA* mRNA-injected embryos. In other cases high levels of v-erbA appeared to cause a truncation of axial structures, although this latter effect could have been due to gastrulation defects produced by the high levels of RNA injected. However, since injection of equal or greater amounts of the 82t mRNA did not cause axial defects similar to those observed with high level *v-erbA* mRNA injection, this result could be interpreted to suggest that low level RA signalling is necessary for normal primary axis formation. In any case, primary axis formation in *Xenopus* was not extremely sensitive to reduced RA signalling as compared to the other morphogenetic events described above.

Two very recent reports have described studies similar to those presented here in which the effects of *c-erbA* mRNA microinjection were studied (Old et al., 1990; Banker and Eisenman, 1993). The results from these two studies using c-erbA are in good agreement with the present results utilizing v-erbA. However, *c-erbA* mRNA microinjection either did not cause or infrequently resulted in the gross developmental defects observed at high frequency when v-erbA mRNA was injected. This difference was not likely to be due to the presence of the gag portion of *v-erbA*, since production of gag alone (82t m RNA injections) had no effects on development. Rather, the differential effects of *c-erbA* and *v-erbA* mRNA microinjection may have been caused by differences in the amounts and/or stability of the two proteins produced in the injected embryos. We were clearly able to observe high levels of *v-erbA* protein up to early tailbud stages, and v-erbA was still detectable at the early tadpole stages, time points when c-erbA was only detectable at low levels or not at all in these other studies. The apparent differences in *c-erbA* and *v-erbA* protein levels in these studies could be due to differences in translational efficiency of the respective mRNAs or to differences in protein stability. The *v-erbA* mRNA was transcribed from a vector containing globin 5′ and 3′ sequences that have been reported to increase the translational efficiency of mRNAs (Krieg and Melton, 1984) whereas the *c-erbA* transcripts injected in the other studies did not have these sequences. An increased stability of v-erbA versus c-erbA could derive from selected mutations in the viral erbA structure that might enhance the propagation or proliferation of the avian erythroblastosis virus. Alternatively, v-erbA and c-erbA have been proposed to differ in their capacity to interact with RARs or RXRs, possibly involving specific RA-responsive DNA elements (Privalsky, 1992).

The recent proliferation of reports describing heterodimerization among members of the nuclear receptor super-family (Leid et al., 1992) suggests that v-erbA may interfere with RA signalling in *Xenopus* embryos by interacting with another nuclear receptor. The simplest model is one in which v-erbA heterodimerizes with RARs (all subtypes) and decreases RA-induced transcription. Alternatively, since c-erbA (and presumably v-erbA) and RARs appear to readily form stable dimers with RXRs (Hermann et al., 1992), the decrease in response to all-*trans* RA could result from the loss of RXRs able to form transcriptionally active heterodimers with RAR due to v-erbA/RXR dimer formation. Either mechanism would presumably result in reduced transcriptional activity mediated by RARs. It is also possible that the high levels of v-erbA present in the injected embryos would allow interactions with both RXRs and

RARs. We have recently obtained evidence that v-erbA can also reduce the teratogenic effects of 9-*cis* retinoic acid [a high affinity RXR and RAR ligand, (Allenby et al., 1993)] in *Xenopus* development, albeit at a somewhat reduced efficiency relative to the rescue of RA-treated embryos (unpublished observations). Thus it seems likely that the observed effects of v-erbA must directly or indirectly affect RAR function, and cannot be due solely to effects on RXR transcriptional activity. Although we only infrequently observed effects of reduced RA signalling on primary axis formation in *Xenopus*, these results do not rule out the possibility that either low level RA signalling is sufficient for primary axis formation or that another retinoid such as 9-*cis* retinoic acid, functioning via the RXR, could be utilized to pattern the primary embryonic axis. The discovery of RXR homologs in *Drosophila* (Oro et al., 1990) and the absence of RAR homologs in this species, suggests that the RXR signalling pathway is more ancient in evolutionary terms. Thus it could be imagined that 9-*cis* retinoic acid working through RXRs, could function in primary axis formation or other early patterning events, while all-*trans* RA, working through the more recently evolved RARs, would play a role in patterning an evolutionarily more recent addition such as the neural crest. It is our expectation that tools such as v-erbA and citral will be useful in further elucidating the complex role of retinoids in early development.

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