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The expression of murine Hox-2 genes is dependent on the differentiation pathway and displays a collinear sensitivity to retinoic acid in F9 cells and *Xenopus* embryos

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ABSTRACT

In this paper we describe experiments that detail the response of murine Hox-2 genes to cellular differentiation and retinoic acid in cell culture. Hox-2 genes are transiently activated in differentiating ES cells even in the absence of retinoic acid (RA), indicating that their induction is a normal aspect of differentiation. Furthermore, in the continuous presence of RA F9 teratocarcinoma cells show a differential ability to maintain Hox-2 expression depending upon whether the cells follow a visceral or parietal endoderm pathway. These data suggest a clear dependence of Hox-2 expression on the degree and type of differentiation in different cells. However, RA also has dramatic differentiation independent effects on Hox-2 regulation. In ES cells the levels of Hox expression are greatly enhanced by exposure to RA, and in F9 cells of the visceral or parietal phenotype the continuous presence of RA is required to maintain these high levels. Nuclear run-on experiments illustrate that Hox-2 genes are active in F9 stem cells and that a large portion of the RA induction is mediated by post-transcriptional mechanisms. Therefore RA exerts its effects on Hox-2 expression by upregulating or modulating genes which are already active, rather than by turning-on silent genes. All nine Hox-2 genes are induced in F9 cells by RA and there is a direct correlation (collinearity) between gene order and the relative dose response of each gene to RA. In *Xenopus* embryos treated with RA, homologues of the Hox-2 genes also displayed a temporal and dose response collinearity with gene organisation. Together these findings suggest that the collinear response to RA is highly conserved in vertebrates and combined with the ability of RA to modify expression during cellular differentiation could be an important feature of the Hox-2 cluster itself used to generate the spatially-restricted patterns of gene expression in embryogenesis.

INTRODUCTION

In vertebrates the four Hox homeobox gene clusters are a highly conserved group of genes evolutionarily related to the *Drosophila Antp* and *Bithorax* complexes (1–3). Extensive analysis of the patterns of gene expression for members from all four of the Hox clusters shows that domains of gene expression are spatially-restricted in different embryonic sites and axes (for reviews see 4, 5). An important feature of these homeobox complexes is that there is a linear correlation between the position of a gene in a Hox cluster and its relative A-P or axial domain of expression in many embryonic tissues (1, 2, 6–13). This property is termed, collinearity (14), and is conserved in arthropods and vertebrates suggesting that regulatory mechanisms for controlling the spatially-restricted domains of Hox expression are an important feature in maintaining the organisation of these gene clusters (2, 3, 15). The Hox genes are believed to function in the specification and interpretation of positional information in the embryo through the particular combination of genes (Hox code) that are expressed at any one regional level (5, 7, 12, 14). This idea is supported by phenotypes arising from experimental perturbation of their expression in vertebrate embryos (16–20).

The conservation in expression and regulation suggests that the signals used to establish and maintain Hox expression patterns may also be conserved. However, in vertebrate embryos the molecular signals that control homeobox genes are largely unknown. Based on a variety of experimental evidence a great deal of interest has focused on possible links between retinoic acid and Hox genes. Many *in vitro* studies have shown that homeobox genes are regulated during RA-induced differentiation of cultured cells (21–30). In embryos RA can affect both the patterning of the limb bud (31–35) and patterns of homeobox expression in the limb (11–13, 36, 37). In a number of vertebrates application of RA results in abnormal growth, differentiation and patterning of the nervous system, neural crest, and branchial arches which in some cases can be correlated with alterations in Hox expression (38–47). In association with inducing growth factors RA can also affect the specification of mesoderm in *Xenopus* (43, 48, 49). Ectopic expression of Hox

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expression in embryos can generate phenotypes that phenocopy those observed when embryos are treated with RA alone (16, 18, 50). Further, evidence suggesting RA has a normal role in embryogenesis comes from the restricted distribution of nuclear RA receptors (51, 52) and cytoplasmic retinoid binding proteins (53–55) which overlap with many of the sites in the embryo sensitive to RA. Together, the response of the Hox homeobox genes to RA in cell culture and embryos, the presence of RA, binding proteins and receptors in embryos (35, 56, 57) and phenotypic links between RA and Hox expression leads to the suggestion that Hox genes may be targets for regulation generated by an RA signalling pathway.

The Hox genes appear to have a role in the specification of regional identity, particularly in the CNS (6, 55, 58, 59), where patterns of Hox expression temporally vary in accordance with the ordered birth of major classes of neurons (60). This suggests that Hox genes may also be linked to cellular differentiation. It is therefore important to have a clear understanding of how RA and differentiation affects Hox expression, and we have used mouse embryonic stem cells and F9 teratocarcinoma cells to examine these processes. We report that the Hox response to RA *in vitro* in mouse F9 cells is rapid and shows tissue-specific kinetics but is also largely dependent on the amount of RA in the culture media. We have compared the RA responsiveness of all members of the murine Hox-2 locus, and have found that a linear correlation between the degree of induction and the position of a gene in the locus occurs in a manner analogous to the human HOX-2 complex (29). We have also treated *Xenopus* embryos with RA and monitored changes in several members of the *Xenopus* Hox-2 cluster indicating that there is an *in vivo* collinear response in embryos as well as tissue culture cells. These findings demonstrate that the nature of the Hox-2 response to RA is conserved in vertebrate evolution and implies it may play a role in the establishment of partially overlapping and graded domains of homeobox expression in the embryo.

MATERIALS AND METHODS

Cell Culture

Tissue culture cells were grown on plastic tissue culture dishes in a 37°C incubator in a 5% CO_2, humidified atmosphere. F9 cells were grown on dishes precoated with 0.1% gelatine. All cells were grown in H-21/PYR media (N.I.M.R. biological services) supplemented with 10% FCS, 2mM glutamine and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Stock solutions (10^{-2}M) of RA, IBMX and dbcAMP were prepared in DMSO, stored aliquoted at −70°C and were appropriately diluted directly from the stock with culture media.

All culture differentiation steps were performed essentially as previously described (61–65). Briefly, parietal endoderm-like cells were obtained by adding RA, dbcAMP and IBMX to F9 stem cells in monolayer culture. The final concentration of RA in the media was 5×10^{-8}M (unless otherwise stated), and the final concentration of IMBX and dbcAMP was always 10^{-4}M. During the differentiation F9 cells were fed with fresh RA containing media every second day. F9 cells were induced to from embryoid bodies (visceral endoderm pathway) by culturing them as small aggregates in bacteriological petri dishes in media containing 5×10^{-8}M RA, and the medium was changed every second day.

The two ES cell lines (ES-HD14 and ES-CCE) were cultured on mitomycin-C treated STO fibroblasts in medium supplemented with 10% FCS. ES cells were differentiated either by aggregation in the absence of a fibroblastic feeder layer (non-chemical differentiation) or by the simultaneous addition of 5×10^{-8} M RA in the culture media (RA-induced differentiation).

RNA isolation

Poly A^+ mRNA from mouse embryonic tissues and tissue culture cells was isolated as described (25, 66). Briefly, mouse tissues and cultured cells were harvested, rinsed in PBS, and homogenised in 3M LiCl, 6M Urea, using a motor driven homogeniser and sonicated for 1 min on ice. The homogenate was stored overnight at 0–4°C, and the RNA precipitate collected by centrifugation and washed by resuspension in LiCl/Urea followed by centrifugation. The pellet was redissolved in 10mM Tris-HCl (pH 7.6), 1mM EDTA, 0.5% SDS then extracted with an equal volume of phenol:chloroform. The aqueous phase was collected, ethanol precipitated and redissolved in 10mM Tris-HCl (pH 7.6), 1mM EDTA, 0.5% SDS. Poly A^+ RNA was selected by oligo-dT cellulose chromatography as described in (66). RNA isolation from *Xenopus* embryos was performed exactly as described in (66).

Nuclear run-on experiments were performed on stem and RA treated F9 cells as previously described (68,69) except that sense RNA for the various genes examined was bound to the filter instead of denature DNA to improve sensitivity. The sense RNA probes were prepared by subcloning the regions into pSP64 and performing SP6 in vitro tanscription reactions. Labelling and hybridisation steps were identical.

Northern blots

RNA samples were denatured at 60°C for 10 min in 70% formamide, 6% formaldehyde-1×MOPS and were separated in a 1.2% agarose, 6.3% formaldehyde gel in 1×MOPS buffer (pH 7.0; 20 mM-MOPS, 5 mM sodium acetate, 1mM EDTA). After electrophoresis the gel was sequentially soaked in 50mM NaOH-0.1M NaCl, 0.1M Tris.HCl (pH 7.6), 2×SSC (each for 20 min), blotted onto a Genescreen (Dupont) nylon membrane, in 20×SSC overnight, and coupled to the filter by UV crosslinking and baking as described (66). Filters were hybridised in 60% formamide, 1×Denhardts, 20mM NaPB pH 6.8, 100μg/ml sheared salmon sperm DNA, 100 μg/ml yeast tRNA, 1%SDS, 10) Dextran sulphate at 65°C for 12 hr. The filters were washed in 2×SSC−0.1% SDS (rt for 1 hr) and 0.2×SSC−1% SDS at 70–80°C for 1–3 hr and exposed to Kodak XAR-5 film at −70°C with an intensifying screen. The bound probe could be removed and the filters reused 5–6 times provided they had not been treated with RNAse. The filters were stripped for re-use by washing in 75% formamide-0.1% SDS at 70°C for 30 min. Treatment of the filters with RNAse was in some cases used to completely eliminate non-specific hybridisation. Filters were treated with 2μg/ml RNAse A (Sigma) in 2×SSC and washed in 2×SSC-0.2% SDS and 0.5% SSC-0.2% SDS both at 50°C for 30 min. All probes used in this work were single stranded P^{32} labelled antisense RNA probes (riboprobes), synthesised according to the polymerase suppliers instructions (promega Biotec) and as previously described (25).

In all cases the intact nature of the mRNA and loading variations were determined by re-hybridising the same or duplicate filters with control probes for mouse actin. The relative levels of expression were quantitatively determined using appropriate exposures of the filters in the linear range which were scanned with a densitometer and the intensity values were

normalised against actin controls. Ideally, it would be best to compare the levels over the time course with the pre-induced stem cell levels. This however, was not possible since the mRNA of Hox 2.5 and 2.6 genes are the only ones detectable in F9 stem cells by northern analysis, prior to RA treatment. As an alternative, the value of expression obtained with 5×10^{-9}M RA at day 1, was equated to 1, and the levels at other times and concentrations compared to it and plotted (Fig. 7B).

Xenopus embryo manipulations

Xenopus eggs and embryos were obtained using standard methods. Briefly, female frogs were injected with human chorionic gonadotropin, 12 hr later eggs were stripped, *in vitro* fertilised and dejellied with 2% cysteine hydrochloride (pH 7.8−8.1). *Xenopus* embryos were cultured in amphibian saline (NAM) but at the midblastula stage were transferred to 10% (v/v) NAM to prevent exogastrulation. *Xenopus* embryos were treated with RA at the late blastula stage (st.9), by transferring dejellied embryos to 1/10 NAM containing RA appropriately diluted from a 10^{-2}M stock solution of RA in DMSO. The embryos were left in the RA solution for 30 min in the dark at 23°C. At the end of this period they were extensively washed with 1/10 NAM (at least 3×) and were allowed to develop in 1/10 NAM at 23°C or 18°C. Control embryos were treated with corresponding dilutions of DMSO in 1/10 NAM and these developed normally in all cases. Staging of the embryos was according to the normal table of Nieuwkoop and Faber (67).

RESULTS

Hox-2 expression during chemical and non-chemical ES cell differentiation

A number of homeobox genes have been shown to respond to RA in different cells lines (see intro) and we have looked at the effect of RA on Hox-2 expression in teratocarcinoma (F9), embryonic stem cells (ES) and fibroblasts (3T3). In initial experiments we examined the effects of RA and culture conditions on Hox-2 gene expression using a few members of the cluster (primarily Hox-2.1) to test for important parameters before extending results to the entire complex. High levels of induced Hox-2.1 expression were observed when ES and F9 cells were differentiated with RA for 24 hr, in contrast to 3T3 cells which exhibited low levels unchanged by RA treatment (Fig. 1A). Low levels of Hox 2.1 expression were observed in untreated ES cells (Fig. 1A), which are more closely related to cells of the early embryo than F9 cells. Unlike F9 cells, ES cells can also differentiate in the absence of chemical inducers to a variety of cell types. We wanted to examine whether a change in Hox-2 expression can normally occur during cellular differentiation without the addition of RA. ES cells were cultured as aggregates and poly A+ RNA was isolated at the time points shown in Figure 1B. In the absence of RA the levels of Hox 2.1 show a moderate induction peaking around 4 hrs and steadily decline during further differentiation. In contrast, ES differentiation in the presence of RA resulted in a rapid and dramatic increase in Hox 2.1 expression and the levels did not decrease during later

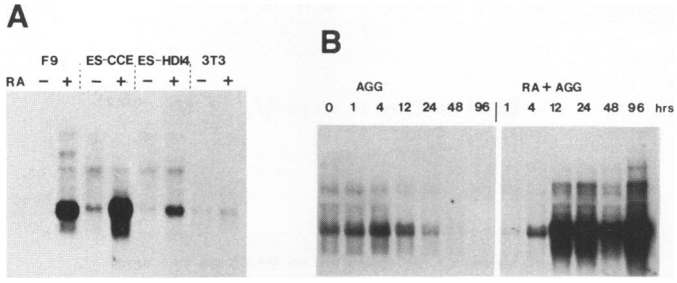

Fig. 1. Induction of Hox 2.1 expression by RA in cultured cells. A. Mouse F9 teratocarcinoma , ES-embryonic stem cell (HD-14 and CCE) and 3T3 fibroblast lines were cultured for 24 hrs in the presence (+) of absence (−) of 5×10^{-8}M RA. 8 μg polyA$^+$ RNA/lane was loaded for ES and F9 cells and 2 μg/lane for 3T3 cells. B. Pattern of expression of Hox 2.1 in ES cells differentiating as aggregates (agg) with or without the addition of 5×10−8M RA. Poly A+ RNA was isolated at the time points (hrs) indicated above each lane and assayed by Northern blotting. 7.5 μg poly A+/ lane was used for the aggregates (agg) and 2.5 μg lane for the aggregates cultured with RA (RA+ agg).

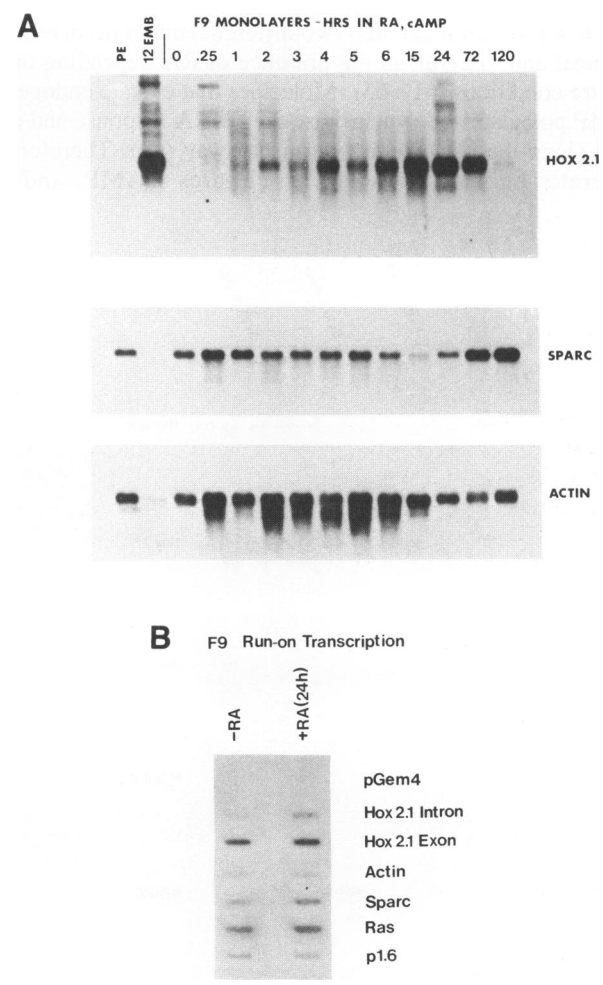

Fig. 2. Transcription rates and time course of Hox 2.1 expression in RA-induced differentiation of F9 cells along the parietal endoderm pathway. A. F9 cells were grown as monolayers in the presence of 5×10−8M RA and 10−4M dbcAMP and IBMX (see methods). Cells were harvested for RNA analysis at the time points indicated as hr of treatment above each lane. Time point 0 corresponds to untreated F9 stem cells. The filter (5 μg RNA/lane) was hybridised with a Hox 2.1 probed and sequentially stripped and hybridised with SPARC and actin probes, to control for differentiation efficiency and loading differences, respectively. 12 EMB: 12.5 d.p.c. embryo (1.5μg/lane), PE: parietal endoderm from 10.5 d.p.c. embryo (0.5 μg/lane). B. Nuclear run-on of relative rates of Hox-2.1 transcription in RA treated and untreated stem cells. In all slots 20 μg.lane of sense RNA generated by SP6 transcription for each of the samples was bound to a filter and hybridised with P^{32}-UTP labelled RNA from isolated nuclei given a 5 min pulse with the UTP. At the right are the respective genes measured and at the top the source of the labelled probe.

stages of differentiation. RA clearly alters the normal timing and levels of Hox-2.1 expression. These results indicate that Hox-2 expression is stimulated by normal differentiation and linked to the differentiation state of the cells, but that the high levels of expression are dependent upon the addition of RA. RA therefore seems to be able to amplify a pre-existing signal and maintains a high level of expression in later stages of differentiation overriding the normal decrease in expression in cells differentiated by aggregation alone.

Hox-2 genes are rapidly and transiently induced by RA in F9 parietal endoderm

The ES cell experiments showed that Hox-2 expression can be linked to differentiation and we wanted to test whether expression can vary in the presence of RA in cells which differentiate along different pathways. To address this point we have used F9 cells which will differentiate into two different endoderm derivatives (parietal and visceral) in the presence of RA depending on the culture conditions (61−65). Molecules that elevate endogenous cAMP potentate and synchronise the F9 RA response and drive cells along the parietal endoderm pathway (62). Therefore, to generate parietal monolayers cultures cAMP and the phosphodiesterase inhibitor IBMX were used at a final concentration of 10^{-4} M, as control experiments demonstrated that they had no effect on Hox-2 expression (data not shown), in the presence of 5×10^{-8} M RA. Poly A+ RNA isolated at various time points was analysed by Northern blotting and hybridisation with a Hox-2.1 probe (Fig. 2A). mRNA is readily detected in RA-induced parietal endoderm cells while there is no detectable expression in untreated F9 stem cells. The detailed time course of induction shows that the Hox 2.1 gene responds very rapidly and large transcripts are already evident within 15 min of RA addition. The main Hox 2.1 transcript appears within 1 hr of RA treatment, maximal levels of transcripts are reached within 6−10 hrs of RA treatment and remain high for 2 days. The amount of Hox 2.1 mRNA begins to decline by day 3 and returns to almost preinduced levels by day 5. To check for appropriate parietal differentiation the filter was reprobed with SPARC (68, 69) and actin served as a loading control. In contrast to Hox-2.1 the induction of SPARC is much slower and reaches peak levels after 5 days when Hox-2.1 is rapidly declining. This reduction during later stages of F9 differentiation, even in the presence of RA, contrasts with the pattern seen in RA treated ES cells (Fig. 1B) and further illustrates a link between Hox-2 expression and differentiation.

One difference between the F9 cells and ES cells was that no expression of Hox-2.1 was observed in the stem cells. The rapid response to RA treatment in F9 cells and the fact that in ES cells RA appeared to upregulate the gene once it was active lead us to investigate whether the stimulation of Hox-2.1 was regulated at the transcriptional level. Nuclear run-on experiments were performed on F9 stem cells and cells treated for 24 hrs with RA to measure the relative rates of transcription (Fig. 2B). Surprisingly, high rates of transcription were observed in the stem cells for Hox-2.1 despite the lack of detectable mRNA by northern analysis. There was only a small increase (2−3X) in transcription upon treatment with RA (Fig. 2B). This experiment indicates that RA is stimulating the Hox-2.1 gene which is already active in stem cells, and further supports the findings in ES cells that RA modulates a gene activity as opposed to inducing *de novo* transcription of an inactive locus. Since the increase in steady-state levels of Hox-2.1 is not accompanied by a similar increase

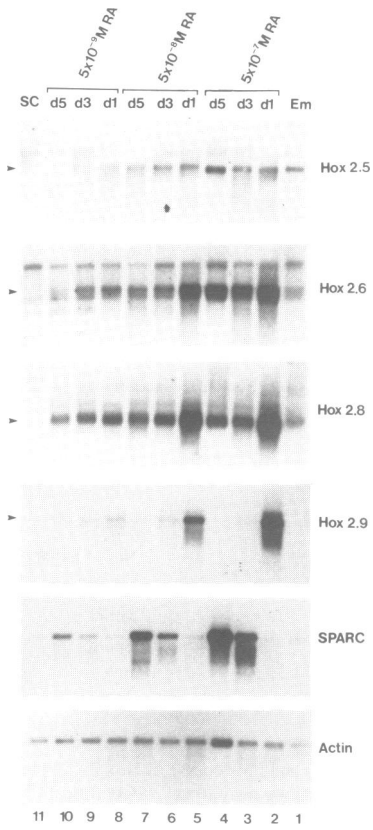

Fig. 3. Time course of RA induction and dose dependence of the Hox 2 genes. Monolayers of F9 cells were treated with three different concentrations of RA (as indicated above the lanes). Poly A^+ RNA was isolated from stem cells and at days 1 (d1), 3 (d3) and 5 (d5) after RA treatment and was analysed by Northern blotting (2.5 μg/lane). The same filter was hybridised sequentially with Hox 2.5, Hox 2.6, Hox 2.8, Hox 2.9, SPARC and actin. 13.5 d.p.c. embryo RNA (Em) serves as a size marker. The Hox 2 genes are arranged in a 5′ to 3′ chromosomal order from the top to the bottom of the panel. Note that at 5×10^{-7}M RA (d1, lane 2) the levels of Hox 2.5, Hox 2.6, Hox 2.8 and Hox 2.9 are progressively higher when compared to each gene's mRNA levels in the 13.5 d.p.c. embryo.

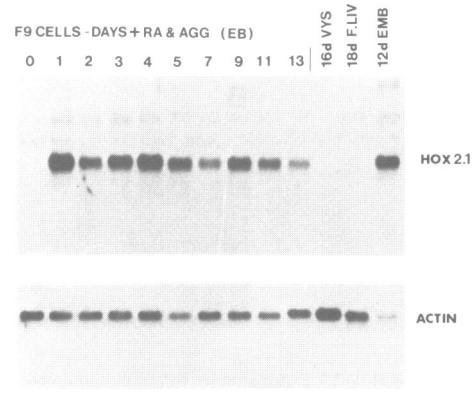

Fig. 4. Hox expression in F9 visceral endoderm aggregates. F9 cells were grown as embryoid bodies (EB) aggregates in the presence of 5×10^{-8}M RA and were harvested at the time points indicated as days of treatment above each lane. After hybridisation with a Hox 2.1 probe the same filter was stripped and hybridised with actin. VYS= visceral yolk sac, f.liv.=fetal liver, EMB=embryo. All lanes contain 2μg RNA/lane except 16 d.p.c. visceral yolk sac that contains 1.2 μg.

in rates of transcription, RA must be regulating expression to a major extent by post-transcriptional mechanisms.

To examine if this pattern was specific only for Hox-2.1 we examined whether other Hox-2 genes showed a similar pattern and if these effects were concentration dependent. F9 stem cells were differentiated along the parietal endoderm pathway with three different concentrations of RA (5×10^{-7}, 5×10^{-8} and 5×10^{-9} M) and harvested for RNA analysis at days 1, 3 and 5 (Fig. 3). The extent of differentiation at all RA concentrations was measured by the induction of SPARC [68,69] (Fig. 3) and by staining with the antibody SSEA-1 (not shown). Some of the Hox-2 genes have detectable levels of RNA in uninduced F9 stem cells. In particular mRNA from Hox-2.5 and Hox-2.6 are observed in untreated F9 cells , but no transcripts from the other Hox-2 genes are detected by northern analysis in uninduced cells (Fig. 3, see also Fig. 6 and 7). However, as shown above in Figure 2B for Hox-2.1, many of these genes are transcriptionally active as determined by nuclear run-on analysis. All Hox-2 genes are induced by RA to peak levels within 12−24 hrs and transcript levels fall during further differentiation, however there are clear differences between the genes which can be seen by comparing Hox-2.5, 2.8 and 2.9 (Fig. 3). The levels of Hox-2.5 are only slightly reduced between days 1−5, while levels of Hox-2.8 and 2.9 rapidly drop between days 1 and 3. In the case of Hox-2.9 RNA transcripts revert to near pre-induced levels by day 3, while the level of Hox-2.8 declines but is maintained significantly above stem cells levels even at day 5. The degree of decline in expression for any given Hox-2 gene depends on the concentration of RA, and this is clearly shown for Hox 2.9 which shows the greatest rate of decline at the highest RA concentration (Fig. 3). Therefore the transient pattern of expression in F9 parietal endoderm is a common feature of all Hox-2 genes. However, both the timing and degree of the decline varies between genes and is dependent on the RA concentration.

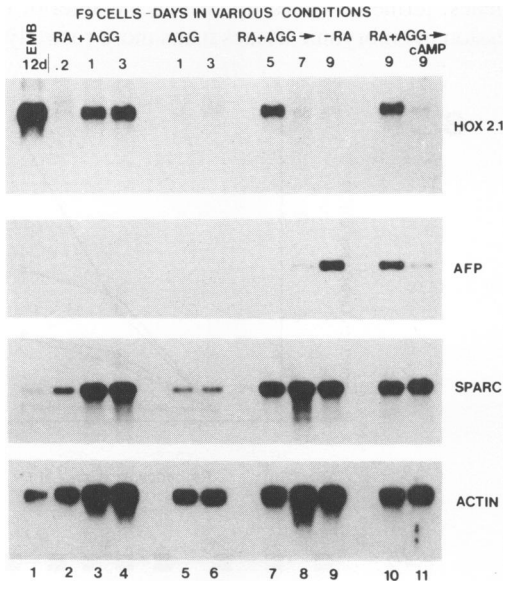

Fig.5. Dependence of Hox 2.1 expression on RA and differentiation in F9 aggregates cultures. F9 aggregates (agg) were grown in varying conditions for the time period indicated as days of culture above each lane. Lanes 2−4 represent F9 cells aggregated in the continuous presence of 5×10−8M RA. Lanes 5 and 6 aggregates without RA. Lanes 7−9 represent the effect of removing RA from the cultures where cells were grown for 5 days in the presence of RA (lane 7) then transferred to RA free medium and cultured for a further 2 days (lane 8) or 4 days (lane 9). Lanes 10 and 11 represent the effects of altering the differentiation pathway of Ra treated aggregates with cAMP. Aggregates were either cultured with RA alone for 9 days (lane 10) or with RA for 7 days and RA+cAMP for 2 additional days (lane 11). Filters were probed first with hox 2.1 then stripped and reprobed successively with AFP, SPARC and a-actin. All lanes contain 8 μg poly A+ RNA except for lane 1 which contains 2.5 μg. EMB is RNA from 12.5 d.p.c. embryos.

Hox-2 expression differs in F9 visceral versus parietal endoderm differentiation

In parallel to the parietal experiments, F9 stem cells were cultured as small aggregates in bacteriological petri dishes in medium containing 5×10^{-7} M RA. This treatment results in the formation of embryoid bodies with an outer layer of visceral endoderm cells surrounding a core of undifferentiated cells (64). In these visceral endoderm-like cells the levels of Hox 2.1 mRNA are also rapidly induced, however the levels remain high for 13−17 days exhibiting only a gradual decline (Fig. 4). The early response and levels of expression are comparable between the visceral and parietal pathways (compare Fig. 2 and 4), which is consistent with the idea that a common first step in both pathways is thought to be the conversion of stem cells into primitive endoderm like cells (65). In the later time points differences in the kinetics of expression are observed as the cells assume a parietal or a visceral endoderm phenotype. We conclude that there is differential regulation of Hox-2 expression along the two pathways.

It has been shown, that the addition of dbcAMP to RA-treated F9 aggregates causes the cells to switch from a visceral to a parietal endoderm differentiation phenotype (65). To further examine the association of Hox-2 gene expression with the differentiation pathway, we have tested the effect of this 'phenotype switch' on Hox 2.1 mRNA accumulation. F9 cells were grown as aggregates for 9 days in the presence of 5×10^{-8}

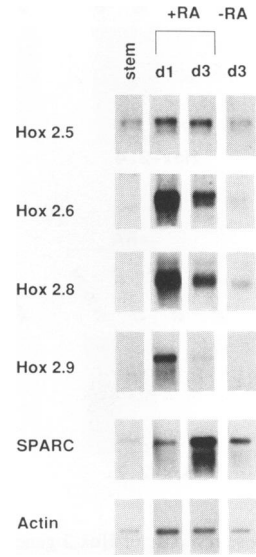

Fig.6. Removal of RA from F9 parietal cultures affects Hox gene expression. F9 cells were treated with RA and some were harvested at day 1 (d1). The remaining cells were cultured for an additional 2 days either in the presence (d3 +RA) or in the absence of RA (d3−RA). After hybridising with a Hox 2.5 probe the same filter was stripped and sequentially hybridised with Hox 2.6, Hox 2.8, Hox 2.9, SPARC and Actin. Hox 2.5 and Hox 2.6 are also expressed at low levels in untreated stem cells (stem). All lanes contain 2.5 μg poly A+ RNA/lane.

M RA. This treatment results in the differentiation of visceral endoderm-like cells that produce large amounts of AFP and express high levels of Hox 2.1 (Fig. 5, lane 10). A subset of these cells received the same treatment until day 7 at which point dbcAMP and RA were added to the culture medium and the cells cultured for a further 2 days (lane 11). As expected, these cells stop expressing visceral endoderm markers (AFP) and increase parietal enriched markers such as SPARC [68,69]. This phenotypic switch is also accompanied by a reduction of the Hox 2.1 mRNA levels, to those comparable to late time points of parietal endoderm differentiation. This clearly shows that even in the presence of RA the levels of Hox-2 expression are dependent on the differentiation pathway.

The continuous presence of RA is required for high levels of Hox-2 expression

Differentiation of F9 cells to visceral endoderm requires both aggregation and RA during the early stages. Figure 5 shows that aggregates in the absence of RA do not upregulate differentiation markers such as SPARC or AFP (compare lanes 3,4 with 5,6), nor do they induce high levels of Hox 2.1. However, a short exposure of F9 cells to RA is sufficient to induce stable differentiation of aggregates, and we examined whether differentiated cells exposed to a transient dose of RA would continue to express Hox genes in the absence of RA. F9 cells were grown for 5 days as aggregates in the presence of RA (Fig. 5, lanes 7–9), and in a sub-set of cells the RA supplemented medium was replaced with normal RA free medium and the cells cultured for a further 2 (lane 8) or 4 (lane 9) days. Removal of RA did not affect the course of differentiation, as shown by the increased expression of AFP, but in contrast, the levels of Hox 2.1 mRNA dramatically declined showing that RA was required to maintain expression.

Similar results in the parietal pathway of differentiation were obtained for a number of other Hox 2 genes (Fig. 6). In this pathway, the experiment was performed earlier in the differentiation course, since at later time points the levels of all genes decline (see Fig. 2 and 3). Control F9 cells were treated with RA for 3 days and RNA isolated at days 0 (stem), 1 and 3. In a subset of F9 cells RA was removed from the cultures 24 hrs before harvesting. In these cultures (d3−RA), the levels of Hox 2 genes are very reduced as compared with the levels in cultures that have been kept continuously in RA (d3 +RA). Levels of SPARC are also slightly lower in these cells, but still higher than stem cells.

These experiments support previous findings that the differentiated phenotype is stable upon removal of RA (61). The decline of Hox-2 expression in both visceral and parietal endoderm in the absence of RA, shows that RNA levels are not only dependent on the differentiation pathway, but they also depend largely on the amount of RA present in the media. It appears that the continuous presence of RA is required but not sufficient for maintenance of highly induced levels of Hox-2 expression.

Collinear sensitivity of the murine Hox-2 genes to RA in F9 cells

The results in Figure 3 illustrated that the timing and response of Hox-2 genes to RA in F9 monolayer cultures was different for various genes. One of the distinguishing features between Hox 2 genes, termed collinearity, is that the anterior boundary of expression in embryonic tissues maps more rostrally for each

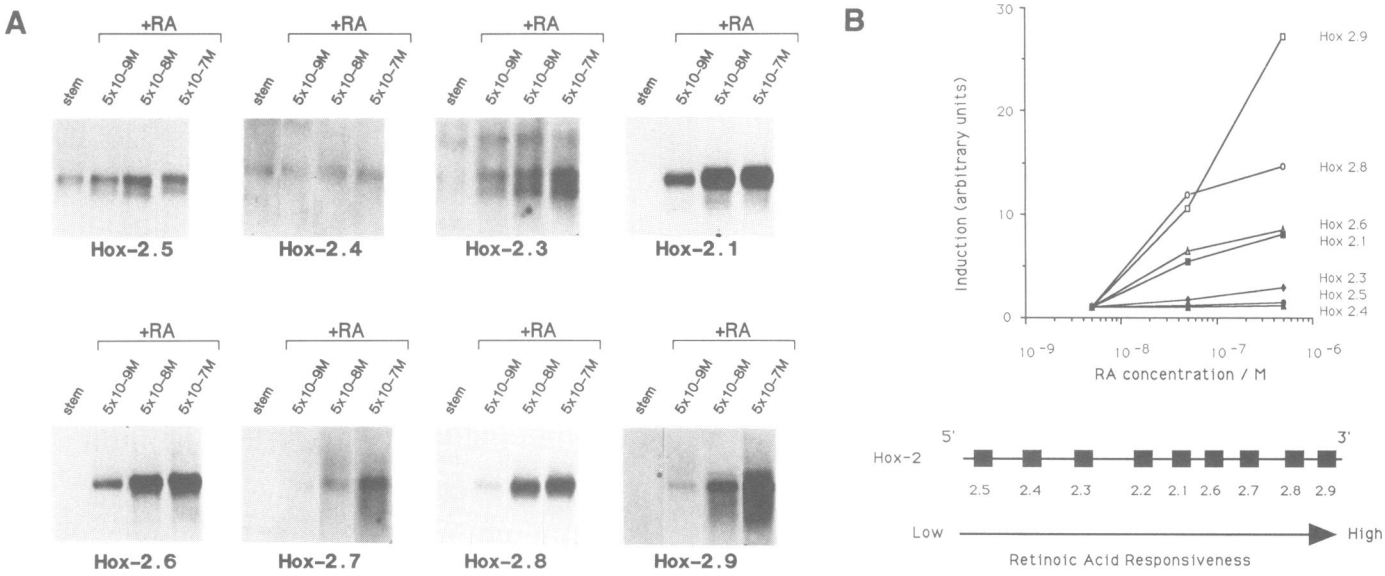

Fig. 7. Collinearity and dose response of Hox 2 genes to RA. A. Northern blot analysis of the responsiveness of Hox 2 genes to RA. Poly A+ RNA was isolated from F9 stem cells and cells treated for 24 hrs with 5×10^{-9}, 5×10^{-8}, or 5×10^{-7} M RA. The order of the genes from left to right, corresponds to the 5′ to 3′ order of the genes on the chromosome (as shown in B). The filters shown (apart from Hox 2.8 and Hox 2.9) have been RNAse treated. Differnt exposure times were used in order to illustrate the response at the lowest RA concentration. Genes such as Hox-2.5 and Hox-2.4 clearly show very little change in response to RA, while the more 3′ genes Hox-2.8 and Hox-2.9 show high levels of sensitivity. B. The response to RA is collinear with the order of the genes on the chromosome. This diagram is based on densitometer scanning of linear exposures of the blots shown in Fig. 7.A and Fig.4. Over the range of concentrations used the levels of Hox 2 genes show a dose response. The level of induction at the lowest RA concentration was equated to 1 and the values at the higher concentrations were set proportionally to this. This calculation measures the relative induction for each gene and this appears to correlate with the position of the gene in the cluster. The relative induction of Hox 2.7 was difficult to measure since even after prolonged exposure it was barely detectable at the lowest RA concentration. The order of the genes in the Hox 2 locus and their collinear responsiveness are diagrammatically illustrated below.

gene as one proceeds in the 5′ to 3′ direction along the chromosome (2). Simeone et al. (29) have demonstrated that the human HOX-2 complex has a collinear response to RA in human teratocarcinoma cells. Thus we were interested in comparing the relative response of the mouse Hox-2 genes to RA in more detail to determine if this collinearity was evolutionarily conserved.

We treated F9 stem cells with three different concentrations of RA (5×10^{-7}M, 5×10^{-8}M or 5×10^{-9}M) and compared the degree of induction of each gene over a five day time course. Figure 7A shows the results at day 1 where the genes of the Hox 2 locus are induced by RA with different efficiencies. The level of induction also varied considerably between the genes and different lengths of exposure are shown to illustrate the response at the lowest RA concentrations. The diagram (Fig. 7B) illustrates both the order of the gene in the Hox-2 cluster and their relative RA sensitivity. Over the range of concentrations used, the most 5′ genes do not show a strong dose response. The difference in dose response between the lowest and the highest RA concentration becomes more pronounced for successively more 3′ genes in the complex.

Due to the variations in basal expression in stem cells and the differences in final induced levels, it was necessary to precisely quantitate the degree of induction for each gene at each RA concentration. Appropriate exposures of the filters were scanned with a densitometer and the intensity values were normalised against actin controls. and plotted (Fig. 7B). There is a clear collinear trend in RA responsiveness. Over the same range of RA concentration, genes that are located at the 3′ end of the locus (Hox 2.8 and Hox 2.9) respond with a greater change in the levels of expression than genes located at the 5′ end (Hox 2.5, Hox 2.4, and Hox 2.3). For example, Hox 2.8 is approximately 15-fold more inducible than Hox 2.5 within the same range of RA concentration. The genes in between show intermediate inducibility. Therefore, overall there is a collinear relationship between the order of the genes in the mouse Hox-2 cluster and their relative sensitivity (dose response) to RA which is conserved in human and mouse teratocarcinoma cells. It must be noted that the absolute levels of different genes vary to a large degree and do not show a collinearity, which could be a consequence of variations in stability or post-transcriptional processing of the mRNAs from different genes.

Differential RA induction of Hox-2 genes in *Xenopus* embryos

A question that arose from the results presented above was whether these findings have any relevance to the *in vivo* situation. Towards this end, the effect of RA on Hox-2 gene expression was studied *in vivo* in *Xenopus* embryos. We are interested in the effects of RA on hindbrain development in *Xenopus* (39, 40) and wanted to determine if phenotypic alterations are paralleled by changes in Hox-2 expression for RA concentrations used in embryo experiments. The expression of two *Xenopus* genes, Xlhbox6 (Hox-2.5) and Xlhbox4 (Hox-2.1) (probes kindly provided by Drs. C. Wright and E. DeRobertis), was examined in RA-treated *Xenopus* embryos. Since Xlhbox6 and Xlhbox4 represent the middle and 5′ end of the *Xenopus* Hox 2 cluster respectively (Fig. 8B), by comparison to the mouse Xlhbox4 should be more responsive to RA than Xlhbox6 in treated embryos.

To test this hypothesis, *Xenopus* embryos were treated with 10^{-5}M RA at late blastula (stage 9), for 30 min. Control embryos were either untreated or treated with 1% DMSO, since the stock RA solution is made in DMSO, and developed normally in all cases. Embryos were harvested for RNA isolation at 30 and 60 min and at stages 11.5 (mid-gastrula) and 24 (tailbud). Results are shown in Figure 8A. No signal was obtained from either gene at 30 or 60 min after RA administration in either control or RA treated embryos. At mid-gastrula stage (st. 11.5), transcription from the Xlhbox4 gene is highly induced in RA-treated embryos. In contrast, transcripts from Xlhbox6 were not found in either control or RA-treated embryos at stage 11.5. At tailbud stage (st. 24), both homeobox genes are readily detected in control embryos and both genes are induced in RA-treated embryos. However, Xlhbox4 is induced by RA to a higher degree than Xlhbox6.

We do not observe the activation of the *Xenopus* genes immediately upon RA application. The onset of transcription in normal development has been estimated to occur at late gastrula (st. 12) for Xlhbox4 and early neurula (st. 13) for Xlhbox6 suggesting that there is a temporal collinearity in the order that the genes are activated in embryogenesis. RA does not appear to alter the onset of expression but upregulates the levels of expression once the genes are activated in normal embryogenesis, which is consistent with the findings in mouse ES and F9 cells. The degree of responsiveness to RA of these two *Xenopus* genes is also in agreement with the responsiveness in tissue culture of the cognate mouse and human Hox-2 genes. This suggests that the collinear relationship between Hox-2 expression and RA induction maybe a highly conserved normal feature of *in vivo* Hox-2 regulation in vertebrates.

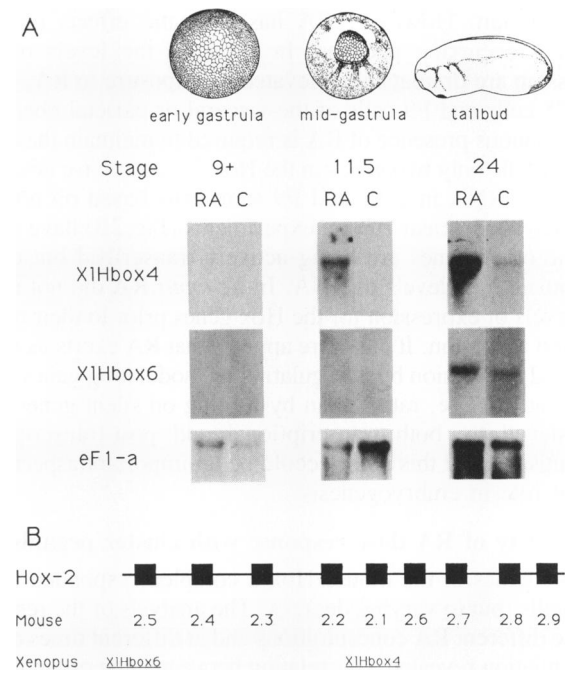

Fig.8. *Xenopus* Hox 2 gene expression in RA-treated embryos. A. *Xenopus* embryos have been treated with a 30-min pulse of RA at stage 9, as described in methods. Total RNA was analysed by Northern blotting after 60-min (st.9+), and when control embryos have reached stage 11.5 and stage 24. The filter was sequentially hybridised with RNA probes corresponding to Xlhbox4, Xlhbox6 and eFI-a. Five embryo equivalents of total RNA were loaded for stages 9+ and 11.5 and approximately 3.5 embryo equivalents for stage 24. 'RA' indicates the samples from RA-treated embryos and 'C' indicates samples from control untreated embryos. The morphology of control embryos at the corresponding stages is shown above the lanes. B. Alignment of the mouse Hox 2 cluster and cognate *Xenopus* Hox 2 genes.

DISCUSSION

Hox homeobox-containing genes must be responding to a variety of molecular signals in vertebrate development which are required to establish their spatially-restricted domains of expression. Experiments presented in this paper provide further support for the idea that at least one of these signals could be retinoic acid (RA). We have screened a large number of potential signalling molecules for Hox-2 genes using cell lines and with the exception of moderate effects by serum and insulin, RA has generated the most pronounced changes in gene expression. We presented a detailed analysis of the properties of the RA response, it relationship to differentiation, its collinear relationship to chromosomal organisation and the conservation of the response in human and mouse teratocarcinoma cells and *Xenopus* embryos.

Relationship of Hox expression to differentiation and RA

It has been argued that induced Hox expression is not a normal feature of the differentiation of cells in culture but a property of their ability to respond to RA (22, 23, 29, 30). Our experiments show that Hox-2 genes are transiently activated in non-RA induced differentiation of ES cells, indicating that it is a normal aspect differentiation at least in mouse cells. Furthermore, even in the continuous presence of RA F9 cells show a differential ability to maintain Hox-2 expression depending upon whether the visceral or parietal pathway is utilised, and cells shifted from the visceral to the parietal phenotype will down-regulate expression. These findings together demonstrate a clear dependence of Hox-2 expression on the degree and type of differentiation. However, RA has dramatic effects on these normally occurring patterns. In ES cells the levels of Hox expression are dramatically elevated by exposure to RA, and in both ES cells and F9 cells of the visceral or parietal phenotype the continuous presence of RA is required to maintain these high levels. While only two genes in the Hox-2 cluster have detectable levels of mRNA in untreated F9 stem cells based on northern blot analysis, nuclear run-on experiments (Fig.2B) have shown that the other genes are being actively transcribed but do not accumulate high levels of RNA. In *Xenopus* RA did not induce high levels of expression for the Hox genes prior to their normal temporal activation. It therefore appears that RA exerts its effects on Hox-2 expression by upregulating or modulating genes which are already active, rather than by turning-on silent genes. This is achieved by both transcriptional and post-transcriptional mechanisms, and this ability could be an important aspect of its normal role in embryogenesis.

Collinearity of RA dose response with cluster organisation

All nine genes in the mouse Hox-2 complex responded to RA in F9 cells, but to varying degrees. The analysis of the response at three different RA concentrations and at different times during differentiation revealed a correlation between gene order and the degree of dose response (Fig. 3 and 7). Because the Hox-2 genes are transiently induced, we focused our analysis on these patterns at the early time points to eliminate late effects dependent on differentiation. Two general classes in responsiveness can be observed. One representing genes in the 5′ part of the cluster (Hox-2.5, 2.4, 2.3 and 2.2) show relatively small degrees of induction. It is possible that this group is responsive, but requires higher concentrations of RA to be fully activated. We have not tested this in F9 cells since RA concentrations higher than used in this study are toxic for these cells. The second class comprises the 3′genes (Hox-2.1, 2.6−2.9) which are very sensitive to RA.

These two classes are interesting in relation to the patterns of expression of the genes during mouse development. The first class have boundaries of expression which map in the trunk, while the second group are segmentally-restricted in the rhombomeres, branchial arches and neural crest of the head. The head and the trunk appear to utilise different segmental mechanisms for patterning in that the somites play a critical role in the trunk and the rhombomeres and neural crest the primary role in the head (7, 70−73). The different sensitivity of the two classes to RA in F9 cells may therefore reflect normal *in vivo* differences in how these groups of genes are established and maintained in the embryo and what regions they regulate. Several studies have suggested that RA has an important role in patterning the head and nervous system (16, 38, 40, 43−45, 54, 55) and it is interesting to note that the Hox genes expressed in this regions are most sensitive to RA.

The data presented here on Hox-2 show that the collinear relationship between the chromosomal order of the genes and A-P boundaries of expression in the mouse embryos (2, 6, 7, 70)can be extended to include differential sensitivity to changes in RA concentration.. These findings confirm and extend the results of Simeone et al (29, 30) who have shown that the human HOX-2 cluster has a collinear RA response in N-TERA2 cells. However, there are differences between the human and mouse Hox-2 response. In the human cells the HOX-2 genes take upto six days to complete the induction of the least responsive 5′ group, which suggests that the induction may be directly linked to cellular differentiation rather that activation by RA. The human genes not only have a graded RA dose response but they display a temporal order of activation spanning many days, collinear with the order of the genes in the cluster. Our data do not allow us to speculate whether similar temporally ordered activation takes place in F9 cells for the mouse Hox-2 genes. We note that all mouse Hox-2 genes were fully activated within 15−24 hrs. In particular, a centrally located gene (Hox 2.1) has induced transcripts 15 min after exposure to RA and reaches maximal levels in 6−12 hrs, while its human counterpart requires 120 hrs of exposure to RA before it is induced. There could be a temporal order to the mouse Hox-2 induction in F9 cells with respect to initial activation or peak accumulation, but because the genes respond so rapidly this must occur over an order of a few minutes to hours and is therefore difficult to quantitate. These differences may reflect basic changes in properties or differentiation states of the human versus mouse cell lines, not alterations in Hox regulation. However, despite these differences there is a striking similarity in the RA response which implies that the differential response to RA may be an important parameter in the generation of the partially overlapping domains of expression observed *in vivo*.

Conservation of the graded response of Hox-2 genes in Xenopus embryos

The chick limb bud has provided the best support for *in vivo* regulation of Hox genes by retinoic acid in vertebrate embryos (12, 13) and our findings that Hox-2 gene expression is upregulated, in a specific differential manner, in RA-treated *Xenopus* embryos further supports the link betwen RA and Hox genes. The *Xenopus* related Hox 2.5 gene (XLHbox6) was very low in RA sensitivity and the Hox 2.1 related (Xlhbox4) had a higher level of response. The more 3′Xlhbox4 gene also appeared earlier in development suggesting a temporal collinearity in the embryo, as observed in the human HOX-2 experiments. We have

recently verified that the *Xenopus* homologue of Hox-2.6 (Xho1A) fits this collinear pattern. More members of the *Xenopus* cluster must be tested, but our *in vivo* data support the idea that the collinear response to RA could be a feature of the Hox-2 complex itself much like the ordered A-P boundaries of expression, conserved in all vertebrates during evolution.

The *in vivo* role of RA is a difficult problem. In light of the many potential sources of retinoids in the embryo assayed by ZPA grafting experiments (35), and the absence of information on the *in vivo* spatial distribution of RA and related active compounds it is not possible to reliably predict locations of RA gradients that could pattern Hox genes in the embryo. Our experiments also demonstrate that RA can exert post-transcriptional effects on Hox expression which could be one means of rapidly altering the distributions of RNA in the embryo independent of the location of nuclear receptors and transcription. It will be of great interest to examine potential changes in the levels and/or domains of expression of Hox genes by *in situ* hybridisation in embryos, and to correlate these with the restricted RA induced phenotypic alterations such as those observed in the anterior hindbrain (39, 40, 45). The *Xenopus* Krox-20 gene shows changes in segmental patterning in RA treated embryos (40, 45) and it seems equally likely that these are accompanied by changess in Hox expression. It appears increasingly likely that Hox genes are direct and/or indirect downstream targets for the action of RA. and play a role in cellular differentiation.

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