Xenopus Hox-2 genes are expressed sequentially after the onset of gastrulation and are differentially inducible by retinoic acid

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Summary

In this paper, we review experiments to characterise the developmental expression and the responses to all-trans retinoic acid (RA) of six members of the Hox-2 complex of homeobox-containing genes, during the early development of *Xenopus laevis*. We showed that the six genes are expressed in a spatial sequence which is colinear with their putative 3' to 5' chromosomal sequence and that five of them are also expressed rapidly after the beginning of gastrulation, in a 3' to 5' colinear temporal sequence. The sixth gene (*Xhox2.9*) has an exceptional spatial and temporal expression pattern. The six genes all respond to RA by showing altered spatiotemporal expression patterns, and are also RA-inducible, the sequence of the magnitudes of their RA responses being colinear with their 3' to 5' chromosomal sequence, and with their spatial and temporal expression sequences. Our data also reveal that there is a pre-existing anteroposterior polarity in the embryo's competence for a response to RA. These results complement and extend previous findings made using murine and avian embryos and mammalian cell lines. They suggest that an endogenous retinoid could contribute to positional information in the early *Xenopus* embryo.

Key words: Hox genes, retinoic acid, *Xenopus laevis*.

Introduction

The main anteroposterior (A-P) axis of the amphibian embryo is specified via intercellular signals. Signals acting during gastrulation certainly specify A-P differences in the developing neural plate (Spemann, 1931; Mangold, 1933). There is a historical controversy (still unresolved) as to whether these signals are transacting (emitted by underlying mesoderm; Holtfreter, 1933; Mangold, 1933; Sharpe and Gurdon, 1990) or homeogenetic (emitted by the organiser and spreading from cell to cell through the neural plate; Spemann, 1931, 1938; Ruiz i Altaba and Melton, 1990) or both. It is clear, in any event, that the organiser (i.e., the dorsal lip of the blastopore) is an important signal source (Spemann and Mangold, 1924; Spemann, 1931). There is also evidence that specifying the A-P axis of the axial mesoderm requires signals during gastrulation (Nieuwkoop et al., 1985; Eyal Giladi, 1954; Kaneda and Hama, 1979), as well as possibly earlier (Ruiz i Altaba and Melton, 1989).

There is, presently, interest in the idea that an active form of vitamin A (an active retinoid) is one of the intercellular signals that specifies the vertebrate A-P axis. A first specific basis for this idea is the finding that treating early amphibian embryos with a pulse of the well-known active retinoid, all-trans retinoic acid (RA) can cause various A-P transformations in the main body axis (Durston et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991a; Papalopulu et al., 1991). These effects work directly both on the developing neural plate (Durston et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991a), as well as on, axial mesoderm (Ruiz i Altaba and Jessell, 1991b), and there is a sensitivity peak for posteriorisation during gastrulation (Durston et al., 1989; Sive et al., 1990). A second, general basis for suspecting that a retinoid is important is the general evidence, via characterisation of retinoid receptors, that retinoids are bona fide signal molecules (Dolle et al., 1989; Petkovich et al., 1987; Brand et al., 1988; Mangelsdorf et al., 1990) and specifically (from the suggestive expression patterns of transcripts for retinoid receptors and binding proteins) that retinoids are signals in early development (Ruberte et al., 1990, 1991; Vaessen et al., 1990). A third point is that studies on the chicken limb bud indicate interesting parallels with A-P specification of the main body axis as well as suggesting that a retinoid specifies A-P values in the limb bud. Here, the ZPA (zone of polarizing activity; a posterior organiser region which specifies the posterior side of the limb), can be replaced by Hensen's node; the gastrula stage organiser of the chicken embryo (Hornbruch and Wolpert, 1986). It can also be replaced, specifically by a local RA source (Tickle et al., 1982) and measurements of RA in the limb bud also show a posterior to anterior gra-
dient of endogenous RA in an effective concentration range (Thaller and Eichele, 1987).

The endogenous signals that specify the vertebrate A-P axis are likely to work by regulating the expression of class 1 homeobox-containing genes (Hox genes). These vertebrate homologues of Drosophila homeotic (HOM) genes, in the Antennapedia and Bithorax complexes, are strongly suspected (from their HOM gene homologies, their expression patterns and the results of gene manipulation experiments) to provide A-P positional information in early vertebrate development (reviewed by McGinnis and Krumlauf, 1992). It is important to characterise their endogenous expression, as well as their responses to putative positional signals. An important aspect of the functioning of Hox genes is that they are organised in chromosomal complexes (four in mammals, each being a partial or total homologue of the Drosophila Antennapedia or Bithorax complexes together; Acampora et al., 1989; Boncinielli et al., 1991; Duboule and Dolle, 1989; Akam et al., 1989; Graham et al., 1989); and that these complexes act as functional units. In a recent study (Dekker et al., 1992, and unpublished observations), we have characterised the endogenous expression patterns and RA responses of six genes in one Hox gene complex (the Hox-2 complex), in the early embryo of the amphibian Xenopus laevis. We found that the Xenopus Hox-2 complex resembles mouse Hox complexes that have been studied in showing colinearity between its putative 3' to 5' Hox gene sequence, and the spatial and temporal sequences in which these genes are expressed in the early embryo. We found, further, that the early Xenopus embryo, like human and murine teratocarcinoma cell lines, shows 3' to 5' colinearity in the response of Hox-2 genes to RA (Simeone et al., 1990; Papalopulu et al., 1991). RA also interacts with an endogenous A-P gradient in competence to regulate Hox-2 gene expression patterns in the early embryo. These findings are discussed in relation to a potential role for an endogenous retinoid in specifying the vertebrate A-P axis, during gastrulation and neurulation.

Materials and methods

RNA isolation

Xenopus eggs were fertilized in vitro and cultured at room temperature (19-21°C) in tap water. Synchronous development allowed the collection of many embryos of defined stages (Nieuwkoop and Faber, 1967). Embryos were dejellosed using 2% cysteine at pH 7.8, and then either used directly to extract RNA (Fig. 5) or else dissected, to generate embryo fragments for RNA extraction, (Figs 4, 6 and 7). The embryos were dissected in 10% Flickinger's medium (Flickinger, 1949; 58 mM NaCl, 1 mM KCl, 0.24 mM NaHCO3, 1 mM Na2HPO4, 0.2 mM KH2PO4, 0.5 mM CaCl2, 1 mM MgSO4, pH 7.5) using tungsten needles. The embryo fragments, or whole embryos were frozen in liquid nitrogen, then kept in a −80°C freezer before being homogenised in guanidine isothiocyanate buffer, and pelleted through an 5 M CsCl cushion, to extract total RNA. 25 µg samples of this RNA were then used to perform RNase protection assays.

Probes and RNase protection assay

For our experiments, we cloned two new Xenopus Hox-2 genes, Xhox2.7 and Xhox2.9 (Dekker et al., 1992). DNA templates were made for each of the six Hox-2 genes, as well as for the Xenopus laevis S8 (Xom62/9; Mariotti et al., 1988) ribosomal protein transcript. The last template was used to generate an internal standard, to quantify the amount of RNA in each sample. All of the templates were made using subcloned fragments of less than 300 bp in length (Xhox2.7 147 bp, Xhox2.7 211 bp, Xhox-1A 153 bp, Xhox-1B 150 bp, XHbox2 109 bp, XHbox2 227 bp, S8 (Xom62/9) 89 bp). These were inserted into CsCl gradient-purified pGEM3Zf(−) (Promega) recombinants. Sp6 RNA polymerase was then used to make antisense RNA probes (labelled via (α-32P) GTP), as required for the RNase protection assays. The probes were first treated with DNase-I, and then purified on a 7% urea-acrylamide gel. In the RNase protection assays, mixtures of five labelled antisense probes (Hox-2 genes, plus the internal standard) were used (at 45°C) to hybridise to RNA extracted from embryos or fragments thereby protecting their homologous transcripts from digestion by a mixture of 2 µg ml−1 RNase T1 (Pharmacia) and 40 µg ml−1 RNase A (Gibco BRL). Because all six Hox genes were assayed in the same sample together with an internal standard, it was possible to make unambiguous comparisons of the expression levels of these six genes. Autoradiograms from these gels were then analysed densitometrically, used to yield normalised values for expression of the six Hox genes (as described in a phosphorimager Molecular Dynamics, Compaq, Image Quant Desk 386/25e), and these are set out as percentages of the maximum expression. All other procedures were basically as described by Melton et al. (1984), with slight modifications as described by Simeone et al. (1990). The expression patterns were quantified by using densitometry to measure the autoradiogram exposures corresponding to each Hox gene messenger and to the internal standard (Xom62/9). The exposure corresponding to each messenger was then normalised (for each gel lane) to that corresponding to the internal standard, and the normalised expression was then plotted, or diagrammed.

In situ hybridisation

The procedure for whole-mount in situ hybridisation was essentially as described by Tautz and Pfeifle (1989), and Kintner and Melton (1987) and as modified by Hemmati-Bri梵lou et al. (1990). A linearised template of XHbox6 (the same template as was used for the RNase protection experiments) was used for in vitro transcription reactions in the presence of digoxigenin-11-UTP (Boehringer Mannheim 1209 256) with SP6 RNA polymerase (as described by Melton et al., 1984). The whole-mount embryos were examined using a Zeiss Axiovert 35 microscope.

In situ hybridisation on sections was performed essentially as described by Wilkinson et al. (1987). Sections were treated with the XHbox6 35S-UTP-labelled antisense RNA probe in hybridisation mix at 55°C overnight. The slides were washed under stringent conditions and treated with RNase A and RNase T1 to remove unhybridised, non-specifically bound probe. Autoradiography was performed.
with Ilford K5 emulsion, with exposures between 2 and 4 weeks. Sections were examined under an Olympus BH-2 microscope using dark-field illumination and photographed.

Results

Spatial colinearity

We determined the spatial order in which the six Hox-2 genes were expressed along the embryo’s A-P axis (Fig. 1). We dissected tailbud-stage embryos (stage 26) into seven pieces along their A-P axes (fragment one: most anterior, to seven: most posterior), and examined expression of all six Hox-2 genes in each piece. The results (Fig. 2) substantiate and extend previous studies of the expression of Xhox-IA (Harvey et al., 1986; Harvey and Melton, 1988) and XlHbox6 (Sharpe et al., 1987; Wright et al., 1987; Cho and de Robertis, 1990). The spatial expression patterns of Xhox-IB and XlHbox2 expression and of our two newly isolated Hox-2 genes (Xhox2.7 and Xhox2.9) have not previously been described. Our results showed, for the first time, that the spatial sequence in which these members of a Hox gene complex are expressed along the main A-P axis of the Xenopus embryo is colinear with their putative chromosomal sequence, thus confirming Xenopus resembles Drosophila and the mouse (Akam, 1989; Duboule and Dolle, 1989; Graham et al., 1989; Wilkinson et al., 1989), in this respect. The maximal expression of the most 3′ gene, Xhox2.9, was localised in fragment 2, but some expression was also observed in fragment 3. The next most 3′ located genes (Xhox2.7, Xhox-IA and Xhox-IB (Harvey and Melton, 1988; Harvey et al., 1986), were expressed maximally in fragment three, Xhox2.7 expression being slightly anterior to that of Xhox-IA and Xhox-IB. XlHbox2 (Wright et al., 1987; Muller et al., 1984) was expressed maximally in fragment 5, and showed some expression in fragments 4 and 6, and the most 5′ located gene (XlHbox6; Sharpe et al., 1987) was expressed in fragments 5 to 7 (Fig. 4). The A-P expression sequence for these six genes is thus Xhox2.9 (most anterior), Xhox2.7, Xhox-IA = Xhox-IB, XlHbox2, XlHbox6 (most posterior). Further experiments, with earlier developmental stages (Fig. 3, see below) showed that Xhox-IA expression is, at least transiently, anterior to Xhox-IB expression.

Of the six Hox-2 genes used in this study, Xhox2.9 formed an exception in its spatial expression pattern at stage 26. Its expression is strongly localised to a relatively small region (fragment 2), in the anterior part of the embryo. The mouse homologue Hox-2.9 and a chicken labial homologue Ghox.lab, also show a similarly restricted expression pattern at a comparable developmental stage (Wilkinson et al., 1989; Hunt et al., 1991; Sundin and Eichele, 1992).

Temporal colinearity

We also looked at the timing of Hox-2 gene expression. Fig. 3 shows that all six Hox-2 genes were expressed during gastrulation and neuration (expression rising after the beginning of gastrulation (stage 10), maximum around stage 15-20 (neurula), decreasing to low levels by stage 35 (tadpole)). These results confirm and extend previous studies of the expression of Xhox-IA and Xhox-IB (Fritz and de Robertis, 1988; Harvey and Melton, 1988; Harvey et al., 1986; Wright et al., 1987; Sharpe et al., 1987; Fritz et al., 1989; Muller et al., 1984). We found that five of the six Hox-2 genes show temporal colinearity. They are expressed sequentially, in a temporal sequence which is strictly colinear with their 3′ to 5′ chromosomal sequence. This result parallels previous findings showing temporal colinearity in the expression of four murine Hox-4 complex genes during early development of the mouse (Izpisua-Belmonte et al., 1991b) and of the chicken limb (Dolle et al., 1989). Previous studies (eg. Gaunt, 1988; Baron et al., 1987; Kessel and Gruss, 1990), also indicate that generally more 3′ located mouse Hox genes tend to be expressed earlier during embryogenesis than more 5′ localised Hox genes, but temporal colinearity has not been demonstrated explicitly in vivo for other Hox clusters. A biphasic expression pattern was observed for five of the six Hox-2 genes examined (Xhox2.9 is an exception). This pattern could reflect different phases of the expression of these genes (see below).

Temporal colinearity was also found in experiments with embryo fragments. We looked at the spatiotemporal expression of five genes by harvesting (normal and RA-treated) embryos at sequential developmental stages, cutting into consecutive A-P fragments, and measuring Hox gene expression in each fragment at each stage. Embryos were dissected into three fragments: an anterior fragment (A) a middle fragment (M) and a posterior fragment (P), and this dissection was done at three stages; stage 13 (early neurula), stage 15 (neurula) and stage 20 (late neurula). RNA was isolated and RNase protections were done on the fragments using Xhox2.7, Xhox-IA, Xhox-IB, XlHbox2 and XlHbox6 as probes. Fig. 5 (solid bars) shows that Xhox2.7, Xhox-IA, Xhox-IB and XlHbox2 are each initially expressed in untreated embryos at a low level, and that each then appears to show a wave of increased expression, which begins posteriorly and proceeds anteriorly. The waves are sequential (Xhox2.7, Xhox-IA, Xhox-IB, XlHbox2 and XlHbox6 is expressed last, and its expression begins and remains posterior. These results thus confirm our suspicion, already raised by the measurements on whole embryos, that Hox-2 complex gene expression occurs in the whole embryo, just as does Hox-4 complex expression in the developing limb (Dolle et al., 1989; Nohno et al., 1991; Izpisua-Belmonte et al., 1991a), genitalia (Dolle et al., 1991) and in the early mouse embryo (Izpisua-Belmonte et al., 1991b), in sequential posterior-to-anterior waves. These findings were repeated in two experiments.

The exceptional expression pattern of Xhox2.9

Xhox2.9 formed an exception to the temporal colinearity of the Hox-2 complex, since its expression is low during early development (gastrula and early neurula), and only reaches its maximum at the end of neuration (stage 20). We can conclude from these data that, although five of the six Hox-2 genes (Xhox2.7, Xhox-IA, Xhox-IB, XlHbox2 and XlHbox6) are expressed 3′ to 5′ sequentially during the early development of Xenopus laevis, Xhox2.9, the most 3′ located gene in the Xenopus Hox-2 gene complex, is an exception, which does not fit in with the 3′ to 5′ temporal
Xhox-2.9

bithorax complexes (ANT-C and BX-C), shown, from left to right, in their 3'-5' genomic sequence and their anterior to posterior (A-P) expression sequence in the early embryo. Middle: the murine Hox-2 complex, showing homologies between particular murine Hox-2 genes and particular Drosophila HOM genes, as deduced by Duboule and Dolle (1989), on the basis of conserved variations in the ß-helical subdomains of the homeodomain region of the protein products of these genes. Below: the six Xenopus Hox-2 genes used in this study (coloured squares and named). These are ordered (from left to right) according to their specific (vertical) DNA and amino acid sequence homologies with particular groups of Drosophila homeotic and murine Hox genes, as revealed by conserved variations in the homeodomain; Kappen et al., 1989; Scott et al., 1989; Fritz and De Robertis, 1988; Fritz et al., 1989; Regulski et al., 1987). Xhox-2.7 and Xhox-2.9 are new genes, recently cloned in one of our labs (Pannese and Boncinelli, unpublished). They also each show particularly high sequence homology (both within the homeobox, and in flanking sequences) with particular mammalian Hox-2 complex genes, and have therefore tentatively been assigned to the Xhox-2 complex (Kappen et al., 1989; Fritz and De Robertis, 1988; Fritz et al., 1989, this study). It was already known, for XlHbox2 and XlHbox6 (Fritz et al., 1989), and for Xhox-1A and Xhox-1B (Harvey and Melton, 1988) respectively, that these two pairs of genes are closely genetically linked, as indicated in the upper copy of the complex by lines connecting these genes. We used pulsed-field gel electrophoresis to examine the linkage of these genes (Dekker et al., 1992) and thus now show that Xhox-2.9, Xhox-2.7, Xhox-1A, and XlHbox6 are also closely linked. We can therefore conclude that all six of the putative Xenopus Hox-2 genes used in this study are, indeed, closely linked in the same chromosomal complex.

Fig. 2. The spatial expression patterns of the Xenopus Hox-2 genes. Stage-26 Xenopus (tailbud) embryos were dissected into seven fragments, as indicated in the diagram, and the expression of each of the six Xenopus Hox-2 genes was determined in each fragment, using quantitative RNAse protection. RNAse protection gels were made, using RNA prepared from each fragment. The gels were set up to assay expression of each of the six Hox genes, and of an internal standard (Xom62/9). The gels were then analysed quantitatively, using a phosphorimager (Molecular Dynamics, Compaq, Image Quant (Desk 386725e)) to yield normalised values for expression of each of the six Hox genes. These values were calculated as percentages of the maximum expression for each gene and are diagrammed by the thickness of each appropriately coloured bar. See the materials and methods for further details. The figure shows that the spatial expression sequence of these six genes is at least approximately colinear with their putative 3'-5' chromosomal sequence. Two genes (Xhox-1A and Xhox-1B) appear to be expressed at a similar A-P level.

Fig. 3. The temporal expression patterns of the Xenopus Hox-2 genes. RNA was extracted from Xenopus embryos harvested at the sequential developmental stages shown and analysed for expression of the six Hox genes, using quantitative RNAse protection as in Fig. 2. The data (set out as percentages of the maximum expression of each gene) show that the temporal expression sequence of five of the six genes is colinear with their putative chromosomal sequence in the Hox-2 complex. Xhox-2.9 is an exception (see text for details).

Fig. 4. The effect of RA on the expression of the Xenopus Hox-2 genes. Xenopus embryos were treated with RA (10^-6 M RA, continuous treatment from stage 10 onwards), and were harvested at sequential developmental stages, and analysed for Hox-2 gene expression, as in Fig. 2. The RA treatment caused overexpression of each of the six Hox-2 genes examined (see text for details). The figure shows the maximum expression level reached by each gene in RA-treated embryos (expressed as a percentage of the maximum expression for each gene in untreated embryos). It will be seen that the most 3' Hox-2 gene (Xhox-2.9) is the most affected by RA, and that successively more 5' genes are successively less affected.

The experiments with embryo fragments (Fig. 5) show that RA alters the normal Hox gene expression patterns, since no posterior-to-anterior waves are now observed. Expression of Xhox-2.7, Xhox-1A and Xhox-1B now starts and remains anterior, while the XlHbox2 and XlHbox6 expression patterns are abnormal, but end up posterior. It is thus notable that the normal final spatially colinear expression sequence of these genes is at least approximately preserved after RA treatment. The expression sequence of these genes simply spreads out, so that 3' genes are now expressed at more anterior positions. The more 3' genes also tend to be induced more rapidly by RA than the more 5' genes. This finding suggests that the early embryo contains a pre-existing, RA-insensitive, anteroposterior polarity, a point which we will take up below.

Pre-existing A-P polarity

The idea that early Xenopus embryos contain a pre-existing anteroposterior polarity was tested directly by exposing anterior axial tissue to RA. We dissected out the presumptive forebrain and underlying prechondal plate mesoderm from the most anterior part of a stage-12.5 early neurula embryo (see Fig. 6), cultured these explants up to stage 20 without RA and in the presence of 10^-8 M, 10^-7 M and 10^-6 M RA (continuously) and examined the expression of four Hox-2 genes. The untreated explants showed no Hox-2 gene expression, as expected, since Hox gene expression
Fig. 7. Expression of XIPHbox6 in stage-28 Xenopus laevis embryos. (A) Detection of specific XIPHbox6 mRNAs in a normal (non-RA-treated) stage-28 embryo by whole-mount in situ hybridisation using digoxigenin-11-UTP-labelled antisense mRNA and alkaline phosphatase with nitro blue tetrazolium (NBT) as a substrate for the label. XIPHbox6 expression is localised posteriorly in the spinal cord of the embryo (blue staining; NBT). The dark color of the head is due to pigment. (B, C) Detection of XlHbox6 mRNAs by in situ hybridisation using antisense [35S]UTP-labelled mRNAs on transverse sections of the trunk region of the embryo. (B) Control embryo (non-RA treated): the mRNA is expressed abundantly in the posterior CNS (N), and also expressed at a low level in the lateral mesoderm (LM). (C) RA-treated embryo (10^{-6} M RA continuously). RA enhances XlHbox6 expression, which now becomes very evident in the posterior mesoderm, as well as in the (open) neural tube. The anteroposterior localisation boundary of XlHbox6 expression is not strongly affected (this remains posterior: not shown). Note: the sections show some nonspecific signal in the epidermis (E) due to pigment.
has not been described in the forebrain or in anterior axial mesoderm in early embryos. Treatment with a low RA concentration (10^{-8} \text{ M}) resulted in the expression of two 3' located genes, Xhox-1A and Xhox-1B. Treatment with 10^{-7} \text{ M} RA gave somewhat more expression of Xhox-1A and Xhox-1B and also induced XlHbox2 expression. With 10^{-6} \text{ M} RA, Xhox-1A and Xhox-1B were expressed still more, but XlHbox2 was expressed less strongly than after treatment with 10^{-7} \text{ M} RA. No XlHbox6 expression was found at any of the RA concentrations tested. These results were found in two separate experiments. They suggest that RA can transform anterior embryonic axial tissue to a more posterior specification, but that a pre-existing A-P polarity also regulates Hox-2 gene expression at stage 12.5, since XlHbox6 is not expressed after treating head tissue with RA. The expression of this gene cannot be enhanced in domains that are in this case too anterior. Similar results have been found using anterior late gastrula ectoderm cultured up to stage 28 (tailbud stage). This also showed no XlHbox6 expression (Sharpe et al., 1987; Sharpe and Gurdon, 1990). The embryo thus appears to be polarized with respect to its competence to express Hox genes in response to RA.

**Discussion**

There were five main conclusions from this study.

First, we showed for the first time in a *Xenopus* embryo, that the six Hox genes studied show spatial colinearity between the sequence of A-P levels at which they are expressed along the main A-P axis of the embryo and their putative 3' to 5' sequence in the *Xenopus* Hox-2 chromosomal complex.

Second, we showed that five of the six Hox genes examined show temporal colinearity. They are expressed sequentially, after the beginning of gastrulation in a sequence that is colinear with their putative 3' to 5' sequence in the *Xenopus* Hox-2 complex. Four of the five genes (Xhox2.7, Xhox1A, Xhox-1B and XlHbox2) seem to be expressed in sequential waves, which travel from posterior to anterior along the main A-P axis of the embryo. The fifth (XlHbox6) is expressed last, and its expression begins and remains posterior. The situation apparently parallels that for the Hox-4 complex in the developing mouse embryo (Izpisua-Belmonte et al., 1991b). One possible explanation would be that the opening of the Hox-2 gene complex (Gaunt and Singh, 1990) is regulated by the increasing production of a morphogen, which spreads from the posterior end of the embryo (blastopore region) during gastrulation and neurulation. We note, also, that a biphasic expression pattern was evident for five of the six Hox-2 genes. We suspect that there are two specific phases of Hox-2 gene expression. (1) A coordinated phase, characterised by sequential activation of these genes, in a temporal sequence that is colinear with their 3' to 5' location in the Hox-2 complex. (2) A phase in which expression is individually modulated, such that each gene in the Hox complex is finally expressed in a specific domain along the A-P axis, with a relatively sharp anterior boundary (Wilkinson et al., 1989). RA seems to affect both phases (see below).

Third, we observed that Xhox2.9 has an exceptional tem-
poral expression pattern when compared with the other five *Xenopus* Hox-2 genes studied in this paper. This gene is expressed at a low level during gastrulation (stage 10) and neurulation (up to stage 15) and only reaches peak expression at a late stage (20; late neurula). No biphasic expression comparable to that of the other Hox-2 genes is evident, the *Xhoz2.9* expression peak coincides with the second expression phase of the other Hox-2 genes. We conclude that individual modulation of Hox gene expression dominates the expression pattern of *Xhoz2.9*, a conclusion that is also strongly indicated in other studies of the expression of *labial*-like Hox genes (Sundin and Eichele, 1992; Murphy et al., 1989; Wilkinson et al., 1989; Frohman et al., 1990; Hunt et al., 1991).

Fourth, we found that 10⁻⁶ M RA (non-localised treatment, applied from the beginning of gastrulation) destroys the wave patterns, causing Hox gene expression that is at least as high and rapid, anteriorly as posteriorly. The sequence of the magnitudes with which RA treatment affects expression of these six genes is colinear with the A-P sequence of axial positions at which they are normally expressed. It is possible that RA mimics an endogenous morphogen that contributes to regulating the expression of the Hox-2 complex during the gastrula and neurula stages of development. It could be that RA mimics the posterior morphogen postulated above and regulates opening of the Hox-2 complex, but our finding of an RA-competence gradient (below) raises the possibility that RA only regulates transcription of the complex, not its opening.

Fifth, we presented evidence for a pre-existing RA-insensitive A-P polarity in the early *Xenopus* embryo. We found that the normal spatial expression sequence of expression of the Hox-2 complex was at least partly conserved after treatment with a high RA concentration. We found further that anterior neuroectoderm and axial mesoderm, which express no Hox genes without RA treatment, expressed only the more anterior genes (and not *XlHbox6*), when treated with RA. The embryo is thus polarized with respect to its competence to express Hox genes in response to RA. These findings are compatible with the idea that an endogenous retinoid is a morphogen, but they make it unlikely that the spatial expression sequence of Hox-2 genes in the early embryo is regulated simply by a retinoid gradient. They provide an interesting puzzle concerning the nature of polarised RA competence, and the relationship of this phenomenon to the expression of retinoid receptors and to opening and transcription of the Hox-2 complex. The phenomenon of prepatterned or of a pre-existent A-P polarity in the early frog embryo has already been suggested by others (Sokol and Melton, 1991; Ruiz i Altaba and Jessell, 1991; Gerhart et al., 1989) and is confirmed by our findings.

It is clear that the findings above raise many questions that can only be answered by in situ data. We have obtained in situ hybridisation data for *XlHbox6* (Fig. 7), and these confirm that *XlHbox6* mRNA, like its protein product (Wright et al., 1987) is localised posteriorly, as from stage 16, when its expression is first detectable, until the latest stage examined (stage 28). The mRNA is strongly expressed in the posterior CNS, and there is also a low level of expression in the lateral mesoderm. RA enhances the expression of *XlHbox6* mRNA such that expression now becomes very evident in the posterior mesoderm, but RA treatment does not strongly affect the anteroposterior localisation of *XlHbox6* expression (which remains posterior as was already indicated by our dissection studies).

Our findings thus revealed details of the endogenous expression, and the inducibility by RA of the Hox-2 complex of class 1 homeobox-containing genes during early *Xenopus laevis* embryogenesis. These investigations are a first step towards investigating the regulation of the expression of this Hox complex by different embryonic inducers during specification of the *Xenopus* A-P axis. They already give some clues as to the nature of the regulatory mechanism.

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