The LIM domain-containing homeo box gene Xlim-1 is expressed specifically in the organizer region of Xenopus gastrula embryos

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A novel cysteine-rich motif, named LIM, has been identified in the homeo box genes lin-11, Isl-1, and mec-3; the mec-3 and lin-11 genes determine cell lineages in Caenorhabditis elegans. We isolated LIM class homeo box genes from Xenopus laevis that are closely related to lin-11 and mec-3 in the LIM and homeo domains. This paper deals with one of these genes, Xlim-1. Xlim-1 mRNA is found at low abundance in the unfertilized egg, has a major expression phase at the gastrula stage, decreases, and rises again during the tadpole stage. In adult tissues the brain shows the highest abundance, by far, of Xlim-1 mRNA. The maternal and late expression phases of the Xlim-1 gene suggest that it has multiple functions at different stages of the Xenopus life cycle. In the gastrula embryo, Xlim-1 mRNA is localized in the dorsal lip and the dorsal mesoderm, that is, in the region of Spemann’s organizer. Explant experiments showed that Xlim-1 mRNA is induced by the mesoderm-inducer activin A and by retinoic acid, which is not a mesoderm inducer but affects patterning during Xenopus embryogenesis; application of activin A and retinoic acid together results in synergistic induction. The structure, inducibility, and localized expression in the organizer of the Xlim-1 gene suggest that it has a role in establishing body pattern during gastrulation.

[Key Words: Xenopus laevis; homeo box genes; LIM domain; Xlim-1; organizer; activin A; retinoic acid]

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The basic body plan of the vertebrate embryo is established in early development as a result of a series of cell interactions that lead to the differentiation of the mesoderm and the elaboration of the dorsoventral and antero-posterior axes. As studied in the amphibian embryo, two early inductive events can be distinguished that are responsible for mesoderm and neural differentiation [for review, see Smith 1989; Dawid et al. 1990; Melton 1991]. During cleavage and blastula stages, a signal or set of signals from the vegetal hemisphere specifies the equatorial or marginal zone as mesoderm, simultaneously establishing dorsoventral polarity in the embryo. Overt dorsal development is first signaled by the appearance of the dorsal lip, a region known as the organizer because of its profound inducing ability that generates a complete secondary axis when transplanted to the ventral side of a host embryo [Spemann and Mangold 1924; see reviews cited above]. During gastrulation, dorsal mesoderm moving from the region of the dorsal lip along the blastocoel roof toward the animal pole induces the neural plate, with its anteroposterior polarity being established in this process. Multiple signals from the mesoderm are believed to be involved in patterning the neural plate along the anteroposterior axis [Mangold 1933; Sharpe and Gurdon 1990].

The molecular basis of embryonic induction and pattern formation in the amphibian embryo is not fully understood, but at least two peptide growth factors appear to be involved in mesoderm induction, fibroblast growth factor [FGF] [Kimelman and Kirschner 1987; Slack et al. 1987] and activin [Asashima et al. 1990; Smith et al. 1990; Thomsen et al. 1990]. These factors exert multiple effects on responsive cells, one important effect being the control of gene expression. In particular, the very rapid activation of regulatory genes by mesoderm-inducing factors is likely to be an important step in the hierarchy of events that leads from factor action to tissue differentiation and pattern formation [for review, see Dawid et al. 1990; De Robertis 1990, 1991; Melton 1991]. The earliest known response gene for activin in frog embryo explants is the homeo box gene Mix.1, during normal embryogenesis, this gene is expressed only for a few hours in late blastula and gastrula stages, and its mRNA is localized in the vegetal hemisphere, the region of the
future endoderm and mesoderm [Rosa 1989]. Two other homeo box genes, \textit{Xbox} 3 and \textit{XIHB}ox 6, which are induced somewhat later in animal explants by inducing factors [Ruiz i Altaba and Melton 1989a; Cho and De Robertis 1990], have been shown to be involved in posterior development of the embryo [Ruiz i Altaba and Melton 1989b, Cho et al. 1991].

The work mentioned above, which implicated homeo box genes as potential regulators of pattern formation in amphibian development, complements extensive studies in other organisms including vertebrates [De Robertis et al. 1990, Lonai and Orr-Urtreger 1990, Kessel and Gruss 1990, Wilkinson and Krumlauf 1990]. More than 80 homeo box genes have been isolated and classified into several subfamilies by amino acid sequence relationships (Scott et al. 1989). In some subclasses of homeo box genes a second conserved domain is associated with the homeo domain. It has been shown for both the POU class [Herr et al. 1988] and the paired class [Bopp et al. 1986] that the second conserved domain participates directly in DNA binding [Ruvkun and Finney 1991; Treisman et al. 1991]. Recently, a new class of homeo box genes was identified, which consists of the \textit{Caenorhabditis elegans} genes \textit{mec}-3 [Way and Chalfie 1988] and \textit{lin}-11 [Freyd et al. 1990], and a rat gene, \textit{Isl}-1 [Karlsson et al. 1990]. These three genes have two tandemly repeated \textit{Caenorhabditis} genes that consists of the \textit{homeo box} genes, \textit{Xlim-1} and \textit{Xlim-2}, which are involved in vertebrate embryogenesis, we classified group” (Scott et al. 1989). In some subclasses of homeo box genes a second conserved domain is associated with the homeo domain. Thus, three distinct clones were identified as candidate of LIM-containing homeo box genes in Xenopus. The properties of \textit{Xlim}-2 and \textit{Xlim}-3 will be described elsewhere.

To examine whether members of this class of homeo box genes are involved in vertebrate embryogenesis, we isolated LIM domain-containing homeo box genes from \textit{Xenopus laevis}. In this paper we describe some properties of the \textit{Xlim}-1 gene that is similar in the homeo box and LIM domains to \textit{lin}-11. \textit{Xlim-1} is activated by the mesoderm-inducer activin A and by retinoic acid in animal explants from blastula embryos. In the gastrula embryo, \textit{Xlim-1} RNA is specifically expressed in the dorsal lip and in the dorsal mesoderm, as it extends anteriorly. These properties suggest that \textit{Xlim-1} is involved in the establishment of the body plan during gastrulation in \textit{X. laevis} embryos.

**Results**

**Isolation and sequence of the \textit{Xlim}-1 gene**

Isolation of LIM class homeo box genes from \textit{Xenopus} was attempted by the polymerase chain reaction (PCR) with a variety of primer pairs and DNA from embryonic cDNA libraries as a template. Degenerate oligonucleotide primer pairs were designed on the basis of sequences conserved among the LIM class homeo domains of \textit{mec}-3 [Way and Chalfie 1988], \textit{lin}-11 [Freyd et al. 1990], \textit{Isl}-1 [Karlsson et al. 1990], and another \textit{Caenorhabditis} LIM-containing homeo box gene, \textit{ceh}-14 [see Materials and methods; Bürglin et al. 1989; T.R. Bürglin and G. Ruvkun, pers. comm.]. We did not succeed in generating the desired PCR products when using primer pairs based on conserved residues of the LIM domains, or with LIM domain primers together with a homeo box primer, presumably because the LIM motif represents rather few and dispersed conserved amino acids. The two primers corresponding to the RGPRTTI and WFQNRRSKE sequences in the homeo domain [primers A and B in Fig. 1C] yielded the expected band of 0.18 kb. This band was isolated and cloned into a plasmid vector, and 200 clones were sorted into groups by their hybridization intensity with the PCR product, and by cross-hybridization [see Materials and methods]. One sequence class, including PCR clone 28, accounted for >90% of all clones and was named \textit{Xlim}-1 (Fig. 1A). Sequence analysis revealed that \textit{Xlim}-1 and a minor class named \textit{Xlim}-2 (clones 30 and 152) are closely related to \textit{lin}-11. \textit{Xlim}-2A and \textit{Xlim}-2B, which show 97% nucleotide sequence identity, probably correspond to the two copies of the same gene occurring because of the apparent pseudotetraploid nature of \textit{X. laevis} [Thiébaut and Fischberg 1977]. Although the deduced 39-amino-acid sequence [between the PCR primers] of \textit{Xlim}-1 is the same as that of \textit{Xlim}-2B and shows only one difference to \textit{Xlim}-2A, the nucleotide sequences show only 72% identity, suggesting that \textit{Xlim}-1 and \textit{Xlim}-2 are different genes. A third sequence class [clone 171], named \textit{Xlim}-3, is more homologous to \textit{lin}-11 and \textit{ceh}-14 than to other reported homeo domain proteins. Thus, three distinct clones were identified as candidates of LIM-containing homeo box genes in Xenopus.

The properties of \textit{Xlim}-2 and \textit{Xlim}-3 will be described elsewhere.

The sequence of long \textit{Xlim}-1 cDNA clones predicts a protein of 403 amino acids that contains LIM1 and LIM2 domains in addition to the homeo domain [Fig. 1B]. \textit{Xlim}-1 has a very short protein sequence before the LIM1 domain, and linkers between LIM1 and LIM2 and between LIM2 and the homeo domain that are similar in length, but not in sequence, to those of \textit{mec}-3, \textit{lin}-11, and \textit{Isl}-1 [Way and Chalfie 1988, Freyd et al. 1990; Karlsson et al. 1990]. The carboxy-terminal region of \textit{Xlim}-1 is longer than that of other known LIM domain genes and is rich in proline (16%), glycine (10%), and serine (13%). This proline-rich carboxy-terminal region of \textit{Xlim}-1 is similar in composition but not in sequence to that of \textit{lin}-11 [17% proline].

The amino acid sequences of the LIM and homeo domains of \textit{Xlim}-1 were aligned with those of other LIM class homeo box genes and with \textit{rhombotin} [McGuire et al. 1989; Boehm et al. 1990], a gene that contains two LIM domains but no homeo domain. Figure 1C shows that the LIM1 domain has two and the LIM2 domain has four conserved residues in addition to the consensus cysteine and histidine residues that define the LIM motif. The LIM domains of \textit{Xlim}-1 show 35–54% sequence identities to the LIM domains of the other genes listed in Figure 1C. Two additional LIM domain-containing genes, \textit{CRIP} [Birkenmeier and Gordon 1986] and \textit{hCRP}
**Figure 1.** (Continued on facing page.)
[Liebhaber et al. 1990], show lower sequence similarity with Xlim-1. In the homeo domain, homology between Xlim-1 and lin-11 is high (59 of 61 residues identical). As homeo domains are known to be involved in sequence-specific DNA binding and gene regulation, the target sequences of Xlim-1 may be closely related to those of lin-11.

Developmental expression of the Xlim-1 gene

The developmental expression of the Xlim-1 gene is shown in Figure 2A. Minor RNA bands that appear at different stages are likely to be encoded by the Xlim-1 gene, as the Xlim-2 gene shows only a 72% sequence identity in the homeo box region, which is likely to be the most similar region of the two genes. At this level of homology, no cross-hybridization would be expected under the conditions used. A low-abundance maternal transcript of 2.7 kb persists to the gastrula stage and then disappears. We do not know how this transcript relates to the major zygotic RNA represented by the sequence in Figure 1B. The major embryonic transcript of 3.4 kb appears at the early gastrula stage, implying that the Xlim-1 gene is activated during embryogenesis at or very shortly after the midblastula transition [Newport and Kirschner 1982]. Xlim-1 RNA accumulation peaks at late gastrula to early neurula stages, and decreases and rises again in the tadpole. As is discussed below, these two periods of RNA accumulation correspond to periods of distinct spatial regulation of Xlim-1 expression.

In adult frog tissues Xlim-1 RNA is present primarily in the brain in the form of the major zygotic 3.4-kb species (Fig. 2B). Lower levels of this RNA are seen in the eye and the kidney, and the ovary contains the 2.7-kb maternal RNA. Testis appears to express a more complex set of smaller RNAs. Although we have not analyzed further the Xlim-1 transcripts in the testis we know that this RNA sample was intact based on its hybridization with a different gene, the β2-subunit of Na,K-ATPase [see Good et al. 1990]. Lung, muscle, and skin (Fig. 2B), and heart, liver, stomach, pancreas, and spleen (data not shown) contained no detectable Xlim-1 mRNA.

Spatial distribution of Xlim-1 expression in the embryo

To examine the localization of Xlim-1 RNA, embryos at stage 11 (gastrula) were dissected and analyzed by Northern blots. Figure 3 shows that Xlim-1 mRNA was present predominantly in the vegetal [endoderm and mesoderm] and dorsal halves (lanes 2,4), and at low levels in animal [ectodermal] and ventral halves [lanes 1,3]. For compar-

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**Figure 1.** [A] Nucleotide sequences of PCR products obtained as described in Materials and methods. The predicted amino acid sequence of Xlim-1 and Xlim-2B, which is identical, is shown at the top; that of Xlim-3 is at the bottom. Underlining indicates amino acids that are different from Xlim-1, Xlim-2A has a single-amino-acid difference, isoucine [ATT codon], which is also underlined. Dots indicate identical nucleotides. The GenBank/EMBL accession numbers for sequences of PCR clones 30, 152, and 171 are Z11587, Z11588, and Z11589, respectively. [B] Nucleotide and predicted amino acid sequence of Xlim-1. Amino acids are in the single-letter code. Asterisks denote in-frame stop codons. Below the sequence the general structure of the cDNA clone, pXH32, is illustrated. The positions of helix 1–3 and the turn are indicated in the homeo domain [Scott et al. 1989]. PCR primers A and B are shown by arrows.
from a dorsovegetal viewpoint shows that the domain of
was supported by detailed inspection of several stained
this low level of staining is significant, it may corre­
erally from the dorsal lip and may form a belt around the
Xlim-1 expression is narrow vegetally at the dor­
Xlim-1 expression is absent from
the dorsal ectoderm (superficial layer); this conclusion
Figure 4C) show that
RNA appears to extend lat­
Xlim-1 RNA is concentrated at
Xlim-1 mRNA was expressed
EF-la
Mix.1
EF-la
Mix.1
Mix.1 gene (Rosa 1989) and the elongation factor gene
were
ison (see text).
Figure 3. Spatial distribution of
Xlim-1 mRNA in gastrula em­
and dorsal (Dor) halves. Ten micrograms of total RNA was ap­
(En + M), or into ventral (Ven) and dorsal (Dor) halves. Ten micrograms of total RNA was ap­
and the future anterior end of the embryo; concomi­
tantly, the expression of Xlim-1 moves anteriorly [Fig. 4C,D]. Although Xlim-1 mRNA is still present at the region of the dorsal lip, its highest level is reached in an area close to the anterior tip of the advancing mesoderm, as seen most clearly in Figure 4D. Expression is limited to a narrow region at the dorsal side of the embryo, and very little Xlim-1 RNA is present elsewhere in the em­bryo at the mid- to late gastrula stage (Fig. 4C,D). During later development the Xlim-1 gene is expressed in sev­eral tissues, for example, in adult frogs where brain and, to a lesser extent, kidney and eye, contain Xlim-1 mRNA [see Fig. 2B]. These tissues also express Xlim-1 mRNA during tadpole stages of development [M. Taira, M. Jam­rich, H. Otari, and I.B. Dawid, unpubl.]. This later ex­pression in different tissues, especially the nervous sys­tem, represents a distinct phase of expression from that during gastrulation, as reflected in the rise, fall, and se­cond rise in the quantity of Xlim-1 RNA [see Fig. 2A]. It is likely that different regulatory influences control the early and late phases of Xlim-1 gene expression in Xeno­pus.

Activation of Xlim-1 gene expression by activin A and retinoic acid
Because Xlim-1 RNA is not significantly expressed in the ectodermal region of the gastrula but is expressed in

Figure 2. (A] Developmental expression of Xlim-1. Total RNA samples (7.5 |xg) from stages 0 [unfertilized egg], 8 [early blast­
Figure 3. Spatial distribution of Xlim-1 mRNA in gastrula em­bryos. Stage 11 Xenopus embryos were dissected into ectoderm [Ec] and endoderm plus mesoderm [En + M], or into ventral [Ven] and dorsal [Dor] halves. Ten micrograms of total RNA was ap­plied on each lane. Mix.1 and EF-la were analyzed for compar­ison (see text).
dorsal mesoderm, we tested whether this gene could be activated in animal explants by mesoderm inducers. Activin A, known to be a strong inducer of dorsal mesoderm [Asashima et al. 1990; Smith et al. 1990; Thomsen et al. 1990], elicited the accumulation of Xlim-1 RNA in such explants, whereas TGF-β2 and TGF-β3, less powerful mesoderm inducers [Rosa et al. 1988; Roberts et al. 1990], did not [Fig. 5A]. Retinoic acid (RA) is not a mesoderm inducer but has various effects on Xenopus embryos as is explored in the Discussion. There has been no report of gene activation by RA alone in animal explants of blastula embryos, but RA treatment of whole embryos enhances the expression of several homeo box genes [Cho and De Robertis 1990; Sive and Cheng 1991]. As shown in Figure 5A, RA elicited the accumulation of Xlim-1 RNA in such explants, and RA plus activin A had a definite synergistic effect. For comparison we hybridized the same RNA samples with a Mix.1 probe. Mix.1 is strongly induced by activin A in agreement with earlier work that used XTC-MIF [Rosa 1989], which contains activin A as its major effective component [Smith et al. 1990]. In contrast to Xlim-1, Mix.1 is not affected by RA either alone or in combination with activin. Figure 5B shows that FGF does not induce the expression of Xlim-1 and does not affect the induction by either activin A or RA. The FGF-responsive gene 1A11 [Rosa and Dawid 1990] was used as a positive control in this experiment.

Mix.1 was identified as a gene rapidly induced by activin even in the presence of the protein synthesis inhibitor cycloheximide [Rosa 1989]. Figure 5C shows that Xlim-1 is likewise inducible by activin A in the presence of this inhibitor. In Figure 6A we compare the induction kinetics of Mix.1 and Xlim-1 by activin A: Both mRNAs accumulate rapidly, but Mix.1 appears to lead and peaks at 3 hr after induction, whereas Xlim-1 RNA lags slightly, reaching its highest level after 4 hr. RA induces Xlim-1 less rapidly than activin, with peak RNA accumulation after 7 hr [Fig. 6B]. When exposed to both factors, animal explants respond with kinetics that correspond to those for RA with a 7-hr peak but at a substantially higher level. The rise of Xlim-1 RNA in activin-induced animal explants corresponds to that in the whole embryo [see Fig. 2A] and in vegetal halves [data not shown], but the reduction of the RNA in explants treated with activin alone is faster than in the embryo. Eleven hours after induction animal explants do not contain detectable levels of Xlim-1 RNA [Fig. 6A] in contrast with whole embryos of comparable age, that is, about stage 17 [see Fig. 2A]. Thus, Xlim-1 expression in the embryo after the midgastrula stage may depend on RA.

Exposure of whole Xenopus embryos to RA leads in a concentration-dependent manner to anterior malformations as in microcephaly [Durston et al. 1989]. Although no gross effects on development are apparent in the blastula and gastrula stages, a ventralizing effect on explanted mesoderm has been observed [Ruiz i Altaba and Jessel 1991a]. RA-treated gastrula embryos, as with activin-induced animal caps, respond to RA by enhanced...
Figure 5. Induction of Xlim-1 expression in animal explants. (A) Stage 8–8.5 animal explants were cultured in the presence of different factors for 4 hr. (B) Stage 8–8.5 explants were cultured for 5 hr until siblings reached stage 10.5. (C) Stage 9–9.5 animal explants were cultured for 30 min in CHX, 30 min in CHX plus activin A, and 2 hr in activin A; siblings reached stage 10–10.5. Five micrograms of total RNA from each sample was analyzed for expression of different genes, as indicated. In A, hybridization with EF-la, and in B and C, staining with ethidium bromide [not shown] verified that closely similar levels of RNA had been loaded. The following factor concentrations were used: activin A, 50 pM (1.4 ng/ml); RA, 10 μM; TGF-β2, 200 ng/ml; TGF-β3, 50 ng/ml; bFGF, 100 ng/ml; cycloheximide (CHX), 5 μg/ml.

expression of Xlim-1 RNA (Fig. 7). The enhancement is the result of overexpression in both the dorsal and ventral regions of the embryo (Fig. 7, bottom). At later stages of development the total levels of Xlim-1 RNA in RA-treated embryos are not very different from those in control embryos.

Discussion

The LIM class of homeo box genes in vertebrate development

We have identified three LIM class homeo box genes in a cDNA library derived from Xenopus embryo RNA, implying that multiple members of this subclass of genes are expressed in vertebrate embryos and have a role in developmental regulation in these animals. In this paper we describe some properties of one of these genes, named Xlim-1. Although the Xlim-1 gene is highly homologous to the lin-11 gene in the homeo domain, homology between the LIM domains of the two genes is only partial (Fig. 1C). In contrast, the rhombotin genes of human and Drosophila are highly conserved (Boehm et al. 1990), suggesting precise structural requirements for execution of the biological function of LIM domains; therefore, Xlim-1 may not be the Xenopus homolog of lin-11. The LIM domain binds both zinc and iron (Li et al. 1991), but its function is not understood at present, it may have a role in protein–protein or in protein–DNA interactions. Both mec-3 and lin-11 function in C. elegans in determining cell lineage during asymmetric cell divisions (Way and Chalfie 1988; Freyd et al. 1990), and a similar type of function could be fulfilled by the Xlim-1 and additional LIM class homeo box genes in amphibian embryogenesis.

The role of the dorsal lip and the dorsal mesoderm in gastrulation

The ability of the dorsal lip to induce a secondary axis when transplanted into a host embryo has earned it the name “organizer” and much attention since the original work of Spemann and Mangold (1924). Yet it has become clear that organizer induction is not the first cell interaction in amphibian embryogenesis. During the cleavage and blastula stages, mesoderm induction establishes the dorsoventral polarity of the future mesoderm and the entire embryo along an axis that is set initially by cytoplasmic movements shortly after fertilization (for re-
are first localized in the dorsal lip and the mesoderm immediately above it (Fig. 4A,B), but a short time later peak expression has moved to the anterior region of the advancing mesoderm (Fig. 4C,D).

How could the regionally distinct expression of a homeo box gene, whose protein product may be expected to be localized in the nucleus, affect the differentiation of a neighboring tissue? Clearly, the effect must be indirect. An excellent example for such interactions has been reported by Reuter et al. (1990) and Panganiban et al. (1990). In the formation of the midgut in Drosophila the expression of the dpp gene, which encodes a growth factor related to TGF-β, is regulated by the homeo box genes Ubx and abdA. In the mesoderm of the middle region, Ubx activates dpp, whose product translocates to the endoderm where it activates another homeo box gene, labial. In more posterior mesoderm the activity of abdA represses both Ubx and dpp, and labial is not expressed in the endoderm. Genetic evidence clearly demonstrated that appropriate expression of these genes (and others, omitted here) is required for midgut morphogenesis. A similar network of regulatory interactions may operate in the amphibian gastrula during neural induction; the Xlim-1 gene is expected to be one of the participants in this network.

The role of RA in patterning during gastrulation

RA has profound effects on a variety of biological systems. When added to Xenopus embryos it inhibits head development (Durston et al. 1989), implying a role as a posterior determinant in embryogenesis. This idea has received support from different experiments. Animal explants induced to form dorsal mesoderm by activin become more ventral if also treated with RA (Ruiz i Altaba and Jessel 1991a). Likewise, gastrula ectoderm induced to form anterior structures (e.g., cement gland) is converted to more posterior cell types by exposure to RA (Sive et al. 1990). Finally, XHbox 6, a posteriorly expressed homeo box gene, is not inducible by RA and weakly inducible by activin but synergistically induced by activin plus RA (Cho and De Robertis 1990). The overall conclusion of these studies has been that RA affects cells that are specified as mesoderm by turning them into a ventral direction that, in subsequent differentiation, corresponds to enhancing posterior or suppressing anterior development, or both. To exert this ventralizing effect in normal embryogenesis, a ventrally localized source of RA in the gastrula has been postulated (Ruiz i Altaba and Jessel 1991a).

Our results raise some questions regarding the above interpretation. The Xlim-1 gene is expressed in an extreme dorsal pattern in the gastrula (Fig. 4), and it is nevertheless induced in animal explants by RA, either alone or in synergy with activin (Fig. 5A). Furthermore, RA-treated embryos overexpress Xlim-1 RNA at the gastrula stage (Fig. 7). Thus, RA enhances the expression of at least one dorsal marker in the frog gastrula, just as it enhances some (XHbox 6; Cho and De Robertis 1990) but not all (Xbox 3; Ruiz i Altaba and Jessel 1991a) pos-

**Figure 7.** Expression of Xlim-1 in RA-treated embryos. Embryos at stage 8.5–9 were exposed to 10 μM RA for 30 min, as described [Durston et al. 1989]. Total RNA was extracted at various stages and analyzed for Xlim-1 RNA. At stage 11 some of the embryos were dissected into dorsal and ventral halves and 5 μg of total RNA was analyzed. Blotting with EF-1α probe showed that equal amounts of dorsal and ventral RNA were loaded onto the gel.
terior markers. Nevertheless, the biological effects of RA in the amphibian embryo are certainly ventralizing and posterior enhancing, both when applied early as in our experiments [Durston et al. 1989; Ruiz i Altaba and Jessel 1991a], or later when the effect is mostly on brain development [Ruiz i Altaba and Jessel 1991b; Sharpe 1991]. A resolution of this apparent contradiction might come from determining whether RA effects seen in explant or whole embryo experiments represent in vivo functions of this molecule. Also, it will be important to determine which RA effects are direct. Finally, although the presence of RA in the embryo is established [Durston et al. 1989], its distribution is not. Direct measurements of RA localization are technically difficult; an indirect approach is to use an indicator gene that responds to RA. It is possible that Xlim-1 is such an indicator gene. If the localization of Xlim-1 and Mix I in vivo in the gastrula and their responses in explant experiments were taken as guides to the distribution of activin and RA in the embryo, it would follow that activin is present in the entire vegetal hemisphere, and RA in the dorsovegetal region. If RA were present in the ventrovegetal region, the Xlim-1 gene should be activated there because it does respond in this region to exogenously added RA [Fig. 7]. At least one isoform of the RA receptor is expressed during Xenopus embryogenesis [Ellinger-Ziegelbauer and Dreyer 1991], but its distribution does not give any direct indication to the possible role of RA in early development.

Materials and methods

PCR cloning of fragments from Xlim homeo box genes

PCR amplification with degenerate primers [Saiki et al. 1988; Gould et al. 1989] was performed in 100 μl of 10 mM Tris-Cl [pH 8.3], 50 mM KCl, 2 mM MgCl₂, 0.01% gelatin, 0.2 μM each of dATP, dGTP, dCTP, and dTTP, 10 ng/μl each of oligonucleotides, 2.5 ng/μl DNA from cDNA libraries prepared from stage 13/14 and stage 24/25 Xenopus embryos [Richter et al. 1988], and 0.025 U/μl of Taq polymerase. The reaction cycles were [94°C, 1 min; 37°C, 2 min; 65°C, 1 min] x 2, and [94°C, 1 min; 50°C, 1 min; 72°C, 1 min] x 30. The following oligonucleotide primers were used [see also Fig. 1C]: CCAGATCC(C/A)GA/G(GGACCCT/AT)GC/AG(A/G)ACNACNAT (primer A, sense strand corresponding to RGRPTTI plus BamHI linker), and CCGTGCAGCT/CTT/CTT/TTAGGC/CCGGC/AG/TTT/CTTG- (A/G)IAACCA (primer B, antisense strand corresponding to WFQRRSKE plus SalI linker). The amplified 0.18-kb band was purified from an agarose gel, and the ends were filled with Klenow fragment, digested with BamHI and SalI, and cloned into the BamHI and SalI sites of pBluescript KS[ +] [Stratagene]. Recombinant clones were screened by colony hybridization using the purified 0.18-kb fragment labeled with [α-32P]dCTP and primers A and B. Hybridization was performed in 0.5 M sodium phosphate buffer (pH 7.0), 1 mM EDTA, 1% bovine serum albumin, 7% SDS [Church and Gilbert 1984], at 65°C for 12–16 hr. Filters were washed in 2× SSPE [1× SSPE is 0.1 M sodium phosphate buffer (pH 7.0), 0.15 M NaCl, 0.6 M EDTA] and 0.1% SDS at 65°C. Some recombinant clones were subjected to PCR reaction using primers A and B to obtain DNA fragments to be used as hybridization probes after labeling with [α-32P]dCTP and primers A and B.

Embryonic cDNA libraries in λgt11 [Richter et al. 1988] were screened with inserts from different clones labeled as described above. Twenty clones of Xlim-1 were isolated from ~1.4 x 10⁶ plaques of the stage 13/14 and 24/25 libraries, and the inserts were recloned into the EcoRI site of pBluescript KS[ +]. Nucleotide sequencing was performed on both strands by the using Sequenase kit with or without 7-deaza-dGTP or dITP [U.S. Biochemical]. The longest of eight Xlim-1 clones that were characterized, pXH32 (3.1 kb), was used for further analysis.

RNA blotting

Probe DNAs for hybridization analysis were generated by PCR amplification using recombinant plasmid and KS/SK primers [Stratagene]. The entire Xlim-1 cDNA clone [Fig. 1B] was used as a probe. DNA was labeled by multiprimed labeling with [α-32P]dCTP.

Total RNA was extracted from tissues of adult frogs and embryos by the acid guanidinium thiocyanate/phenol/chloroform method [Chomczynski and Sacchi 1987] or the guanidinium thiocyanate/phenol method [Sargent et al. 1986]. RNA samples [5–10 μg] were electrophoresed in 1% agarose/formaldehyde or 1.1% agarose/glyoxal gels [Thomas 1983], blotted onto nylon filters, and hybridized as described by Church and Gilbert [1984].

Induction experiments

Induction experiments were carried out as described previously [Rosa et al. 1988]. Animal explants were dissected at stage 8–9 [Nieuwkoop and Faber 1967] and cultured in the presence of different factors for times given in the figure legends. The following growth factors were used: human recombinant activin A, [provided by Genentech] TGF-β2 [from R&D Systems], recombinant TGF-β3 [provided by A. Roberts] [Roberts et al. 1990]; and basic FGF [from AMGEN Biochemicals].

Whole-mount in situ hybridization

Hybridization experiments followed the method described by Hemmati-Brivanlou et al. [1990] and Harland [1991]. Embryos derived from albino females were used. Digoxigenin-labeled RNAs were prepared by in vitro transcription of Xhol- or BamHI-digested pXH32 for antisense or sense strand probes, respectively, with the use of an RNA-labeling kit (Boehringer Mannheim). Antidigoxigenin antibodies [Fab fragment] conjugated with alkaline phosphatase were from Boehringer Mannheim.

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