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The Role of Growth Factors in Embryonic Induction in *Xenopus laevis*

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ABSTRACT Establishment of the body pattern in all animals, and especially in vertebrate embryos, depends on cell interactions. During the cleavage and blastula stages in amphibians, signal(s) from the vegetal region induce the equatorial region to become mesoderm. Two types of peptide growth factors have been shown by explant culture experiments to be active in mesoderm induction. First, there are several isoforms of fibroblast growth factor (FGF), including aFGF, bFGF, and hst/kFGF. FGF induces ventral, but not the most dorsal, levels of mesodermal tissue; bFGF and its mRNA, and an FGF receptor and its mRNA, are present in the embryo. Thus, FGF probably has a role in mesoderm induction, but is unlikely to be the sole inducing agent in vivo. Second, members of the transforming growth factor-β (TGF-β) family. TGF-β2 and TGF-β3 are active in induction, but the most powerful inducing factors are the distant relatives of TGF-β named activin A and activin B, which are capable of inducing all types of mesoderm. An important question relates to the establishment of polarity during the induction of mesoderm. While all regions of the animal hemisphere of frog embryos are competent to respond to activins by mesoderm differentiation, only explants that include cells close to the equator form structures with some organization along dorsoventral and anteroposterior axes. These observations suggest that cells in the blastula animal hemisphere are already polarized to some extent, although inducers are required to make this polarity explicit.

How do inducing factors affect the differentiation of the responsive tissue? One approach to this question has been to look for gene expression in response to induction, especially the activation of regulatory loci like homeobox genes. Several homeobox-containing genes including *Mix.1, Xhox3, X1Hbox1,* and *X1Hbox6, goosecoid* and members of a new class of genes named *Xlim,* are activated by inducing factors with different patterns of expression in the embryo. Differential expression of regulatory genes probably controls the formation of distinct tissues in an orderly pattern during embryogenesis.

Key Words: Growth factors, Induction, Embryogenesis

INTRODUCTION

We are interested in the general developmental question of how the fertilized egg, a single cell, develops into an animal with many differentiated cell types arranged in a particular and well-organized pattern. This is a very old question and is as yet unanswered. In our studies of the South African clawed frog *(Xenopus laevis),* we have obtained answers to some specific questions on the way to resolving the larger issue of pattern formation in vertebrates in general.

MESODERM INDUCTION IN THE FROG EMBRYO

The differentiation of the zygote into the tadpole is controlled to a large extent by interactions between cells. In all animals, particularly in all vertebrates, cell interactions early in the process are critical for development; this early class of cell interactions is usually called embryonic induction. In the frog, the first inductive interaction takes place very soon after fertilization, after cleavage has started, and continues through the midblastula stage (about 6 hr), in which the zygote contains a few thousand cells. The top of the early embryo, referred to as the animal region, will give rise to ectoderm and the bottom, referred to as the vegetal region, will give rise to endoderm. The mesoderm comes from the middle region, which is also called the marginal zone. The earliest induction is a set of signals that come from the vegetal region and move sideways and upward toward the marginal zone; in this induction the cells in the marginal zone are specified for subsequent differentiation into mesoderm (Fig. 1). This early induction of the mesoderm is essential to the establishment of the dorsal–ventral axis of the embryo. The inductive signal originating in the vegetal region cannot be transmitted through the blastocoel; therefore the cells in the animal cap which are capable of responding to the signal do not receive it during normal development because it doesn't penetrate that far from its source in the vegetal region of the embryo (for reviews of mesoderm induction, see Smith, 1989; Dawid et al., 1990; Melton, 1991).

The process of gastrulation begins at about 10 hr, as cells in the marginal zone move inward. This invagination starts at the dorsal side, forming the dorsal lip which is also called Spemann's Organizer (Fig. 2). During gastrulation, these dorsal cells begin to migrate toward the animal pole along the roof of the blastocoel.

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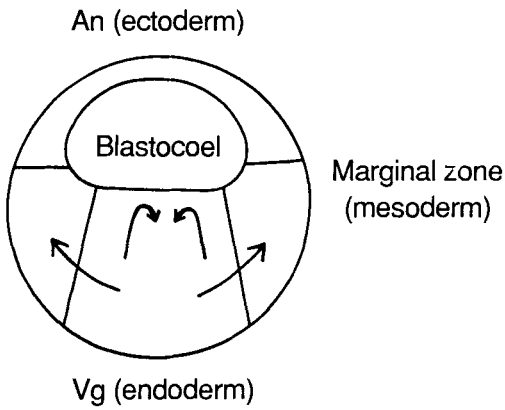

Fig. 1. Mesoderm induction occurs in early amphibian development. During blastula stages, a set of signals emanates from the vegetal region (future endoderm) and impinges on the marginal zone, specifying it as future mesoderm. The animal region retains its inherent fate as future ectoderm because the vegetal signal does not reach this region during normal embryogenesis.

By initiating its migration on one side, the movement of the mesoderm sets up the dorsal–ventral axis. Dorsal mesoderm moves into the blastocoel up to the animal pole, and during this process induces the neural plate in the overlying ectoderm. As the leading edge of the mesoderm forms anterior structures and the overlying neural plate develops into brain, while the trailing edge and overlying neural plate become posterior structures, the migration of mesoderm during gastrulation and its interaction with the ectoderm establish the anterior–posterior axis. In this way the basic shape of the embryo is set up during the gastrula stage. A little later in development the embryo begins to elongate and eventually takes on the features of a tadpole with a head and tail (for the embryology of *Xenopus*, see Nieuwkoop and Faber, 1967; Hausen and Riebesell, 1991; Kay and Peng, 1991; and references therein).

To sum up these events, mesoderm induction is responsible for the formation of the mesodermal tissues and for specifying the dorsal–ventral axis. During the complicated rearrangements that occur during gastrulation the anterior–posterior axis is formed primarily through the behavior and influence of dorsal mesoderm. With these two axes set, the general animal pattern emerges. Therefore, pattern formation in the early embryo is very closely linked to mesoderm induction. In other vertebrates, the dorsoventral and anteroposterior polarities are established by variations of these types of processes.

How can we know that mesoderm induction occurs? Experimental study of this phenomenon is based primarily on work of Nieuwkoop and others about 25 years ago, who showed that animal pole (pre-ectodermal) tissue cultured in saline will differentiate into epidermis. However, when the animal pole tissue was combined with a piece of vegetal tissue it differentiated into a variety of mesodermal structures (Fig. 3; reviewed in Nieuwkoop, 1973; Smith, 1989; Dawid et al., 1990).

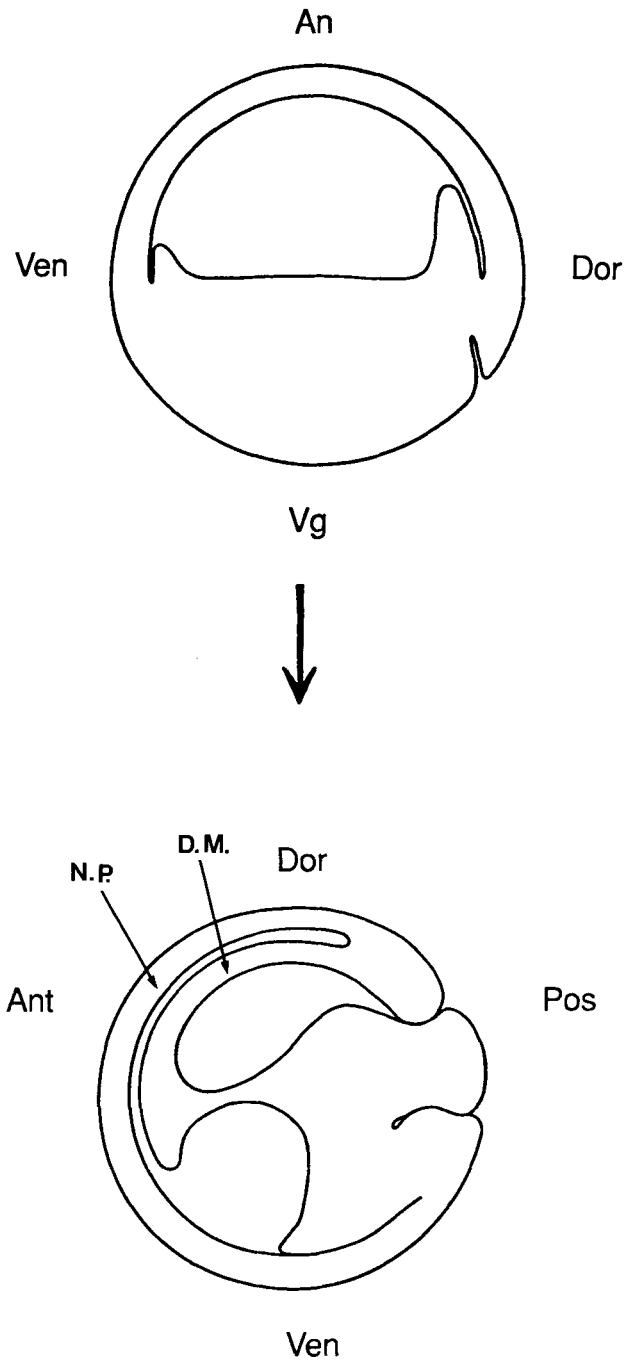

Fig. 2. Gastrulation in *Xenopus*. Cell invagination at the dorsal side of the embryo creates the dorsal blastopore lip, and dorsal mesoderm begins its movement along the blastocoel roof (upper drawing). At the late gastrula stage (lower drawing), with the embryo having turned by 90°, the dorsal mesoderm (D.M.) has advanced to the original animal pole. During this period the dorsal ectoderm has been induced to form the neural plate (N.P.); together with the underlying mesoderm, it forms the dorsal axis of the embryo, assuming an anteroposterior pattern during the gastrulation process.

Normally the animal pole cells do not form mesoderm—the marginal cells do. The fact that the animal pole

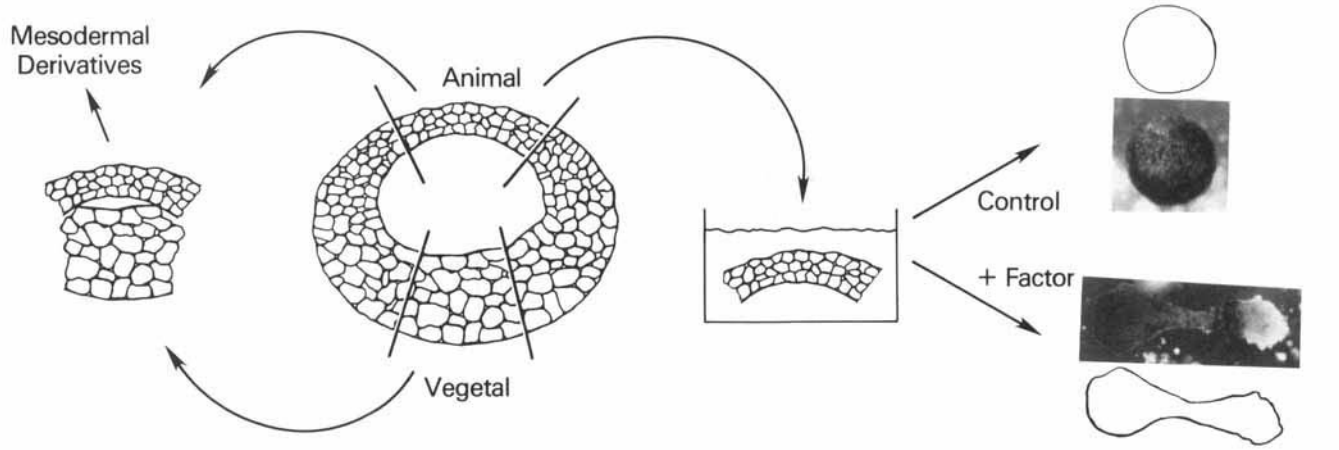

Fig. 3. Mesoderm induction assay. To the left, a recombinant assay is shown (Nieuwkoop, 1973). Animal explants, which will form atypical epidermis in control culture, form mesodermal tissues when cultured in contact with vegetal cells. To the right, the growth factor assay is shown. Animal explants cultured in the presence of mesoderm inducing factors elongate and form mesodermal tissues as seen by histological examination. The activation and repression of specific genes can be observed in such induced explants.

cells can be induced to form mesoderm by incubation with vegetal tissue does not in itself prove that the vegetal tissue normally induces marginal cells to differentiate into mesoderm in vivo. But there are also other published data (Sargent et al., 1986) that support this conclusion strongly, and there is good reason to believe that these tissue recombination experiments that demonstrate mesoderm-inducing activity, do recreate what happens normally in vivo (Dawid et al., 1990).

In recent years, the assay illustrated on the right side of Figure 3 has been used by many laboratories to identify and investigate soluble mesoderm-inducing factors. When animal tissue is placed in culture with a mesoderm-inducing factor, the tissue elongates and eventually differentiates into mesodermal derivatives, while control explants form epidermal structures.

Induction of mesoderm in this system has nothing to do with proliferation. There is no difference in the rate of cell division between a control tissue and an induced tissue, and many of the morphological and biochemical consequences of induction still occur when proliferation is inhibited by, for example, aphidicolin (Gurdon and Fairman, 1986; Symes and Smith, 1987).

THE ROLE OF GROWTH FACTORS IN MESODERMAL INDUCTION

Mesoderm inducers include molecules that belong to the TGF-β and FGF families of peptide growth factors. In a long-standing collaboration with Roberts, Sporn, and their colleagues, we showed that TGF-β2 and TGF-β3 isoforms, but not TGF-β1 and TGF-β5, could induce mesoderm (Rosa et al., 1988; Roberts et al., 1990). Recently, it has been shown by a number of laboratories that activin, a member of the TGF-β family, is the most potent mesoderm inducing substance, being effective at lower concentrations and producing strong biological effects (Asashima et al., 1990; Smith et al., 1990; Thomsen et al., 1990). Thus, activin A and/or B, rather than the TGF-betas, is likely to be a natural mesoderm inducer. Although direct evidence to identify the natural inducer is still incomplete, the fact that TGF-β is so much less effective than activin makes it much less likely that TGF-β is responsible for mesoderm induction in the embryo. TGF-β2 mRNA exists in the embryo at a low concentration, but TGF-β3 has not been found in the frog embryo.

FGF isoforms also induce mesoderm, but the types of mesodermal differentiation induced by FGF are limited (Slack et al., 1987). FGF mostly induces ventral types of tissues, such as muscle and mesenchyme, but usually not the very dorsal tissues, such as notochord. Thus it appears that FGF alone cannot carry out all the in vivo functions of mesoderm induction, but it almost certainly has a role in the process.

One test of whether a molecule identified as a mesoderm inducer in an artificial system actually functions in vivo is whether it is expressed in vivo at the right time and place. Activin mRNA can only be detected in the late blastula stages (Thompson et al., 1990). However, there is a lot of biological evidence that induction starts in early cleavage and is largely completed by the late blastula stage. So it appears doubtful that the synthesis of activin mRNA at this relatively late stage affects mesoderm induction in the embryo. However, recently an activin-like protein has been identified in the egg (Asashima et al., 1991). We do not know whether this activin-like activity is either A or B for which molecular probes exist, or whether the egg might contain a novel activin isoform whose mRNA cannot be detected with any of the available probes. It is also possible that the activin detected in the egg was made in early oogenesis and stored for later use in the fertilized egg. The RNA may have been synthesized and degraded earlier in oogenesis and thus not be detectable in the egg. Whatever the explanation, there is reason to believe that activin-like proteins are present

in the early embryo despite our inability to detect activin mRNA.

Both FGF mRNA and the FGF protein, which has only some of the functions needed for mesoderm induction, are present in the egg and early embryo (Kimelman and Kirschner, 1987; Slack and Isaacs, 1989). Also, a *flg*-like form of the FGF receptor is present in the early embryo (Gillespie et al., 1989; Musci et al., 1990; Friesel and Dawid, 1991). The FGF receptor has also been shown to function in early embryogenesis (Amaya et al., 1991). Thus it is very likely that FGF is one, but not the only, factor involved in mesoderm induction.

There are many reports of other members of the TGF-β family that might have a role in early embryogenesis. One interesting molecule is Vg1, which is related in sequence to TGF-β and expressed in the early embryo (Rebagliati et al., 1985; Weeks and Melton, 1987), but it has so far not been ascribed any biological function. TGF-β5 is also present in the egg and early embryo (Kondaiah et al., 1990), but, when isolated from frog cell cultures and tested in the animal cap mesoderm-inducing assay, it had no activity (Roberts et al., 1990). The bone morphogenetic protein 4 (BMP4) mRNA and protein are present in the egg and early embryo, and it has been found recently that a crude culture fluid from BMP4-expressing COS cells has some mesoderm-inducing activity (Köster et al., 1991). However, in collaboration with H. Reddi we were unable to find any evidence for mesoderm-inducing activity of purified BMP4, whether alone or in combination with TGF-β1, TGF-β2, or FGF. There are various possible explanations for the apparent inducing ability of the BMP4-containing culture medium.

BIOLOGICAL CONSEQUENCES OF MESODERM INDUCTION

There are a number of biological and biochemical consequences of mesoderm induction. In our studies of the biological responses, we have used highly purified human recombinant activin A at concentrations of 50–200 pM. When animal cap explants are incubated with activin, a number of different mesodermal derivatives are induced. The differentiated tissues include muscle and notochord, but under the conditions used in the past no organized, polar structures reminiscent of an embryo are formed. Recently it was shown that a factor isolated from mouse P388D1 cells, originally called PIF, can induce the differentiation of animal explants into a structure that has been called an "embryoid" (Sokol et al., 1990). Although the embryoid is not a perfect frog embryo, it has an anterior–posterior polarity and frequently differentiates one or two eyes. Eyes are very anterior structures that require several induction events for their formation; eyes had not been observed in the usual mesoderm inducing assay previously used. The explanation for the differences between these recent results and earlier experience probably resides in the technique rather than in the properties of the culture medium used by Sokol et al. as source of the mesoderm-inducing activity since PIF medium has been shown to contain activin A (Thomsen et al., 1990). We found that the same type of structured embryoids can be induced using purified recombinant human activin or the culture fluid from XTC cells (called MIF) (see Smith, 1987) if a large segment of the animal cap is used in the assay. The conventional segment used for this assay is a piece removed from the center of the blastula embryo cut with a 60° angle to the center of the embryo; such explants do not organize when induced. In contrast, large animal explants form organized embryoids in 20% of the cases when induced with purified activin, or with crude PIF, or MIF culture media (Figs. 4, 5). These observations suggest that the animal tissue in a mid-blastula has already set up a prepattern—a differentiated state in which some of the cells closer to the equator are different from those at the animal pole. This latent pattern can be developed in vitro by inducing factor (activin). Therefore, it appears that in normal development the formation of polarity is the result of an interaction between an inducing signal and a variable competence to react of different cells in the responding tissue. This point of view is in agreement with the recent conclusions of Sokol and Melton (1991).

GROWTH FACTOR REGULATION OF HOMEOBOX GENE EXPRESSION IN EARLY FROG DEVELOPMENT

There are many genes that are activated and repressed when responsive tissues are induced to differentiate. Some of the genes that have been studied for some time are tissue-specific genes, such as the muscle-specific α-actin gene, first introduced into the field by Gurdon et al. (1985). Some epidermal keratin genes are turned off when the tissues are induced into mesoderm, whereas they are expressed in the control cultures that differentiate into ectoderm (Dawid et al., 1988). Beyond these genes that represent the terminal differentiation of various tissues, we have focused our attention on some rapidly responding genes that might be expected to have regulatory functions. Such early response genes have been isolated in different ways and shown to respond differentially to FGF and to activin (Rosa and Dawid, 1990). Two early genes that we have studied are called *Mix*.1 and *Xlim*-1 and encode two homeodomain proteins with distinct expression patterns in early embryogenesis.

The *Mix*.1 homeobox gene was discovered in a screen for genes that are rapidly activated during mesoderm induction (Rosa, 1989). *Mix*.1 is not induced by FGF but is turned on dramatically within 15 minutes of adding activin. The gene is turned on even in the presence of cycloheximide, and so it presumably does not need protein synthesis for its activation. It is what one might call an immediate–early response. The gene is transiently expressed in the gastrula stage during normal embryogenesis. In the late blastula to early gastrula *Mix*.1 RNA is distributed only in the vegetal half of the embryo in the region in which mesoderm and possibly also endoderm will form during later development.

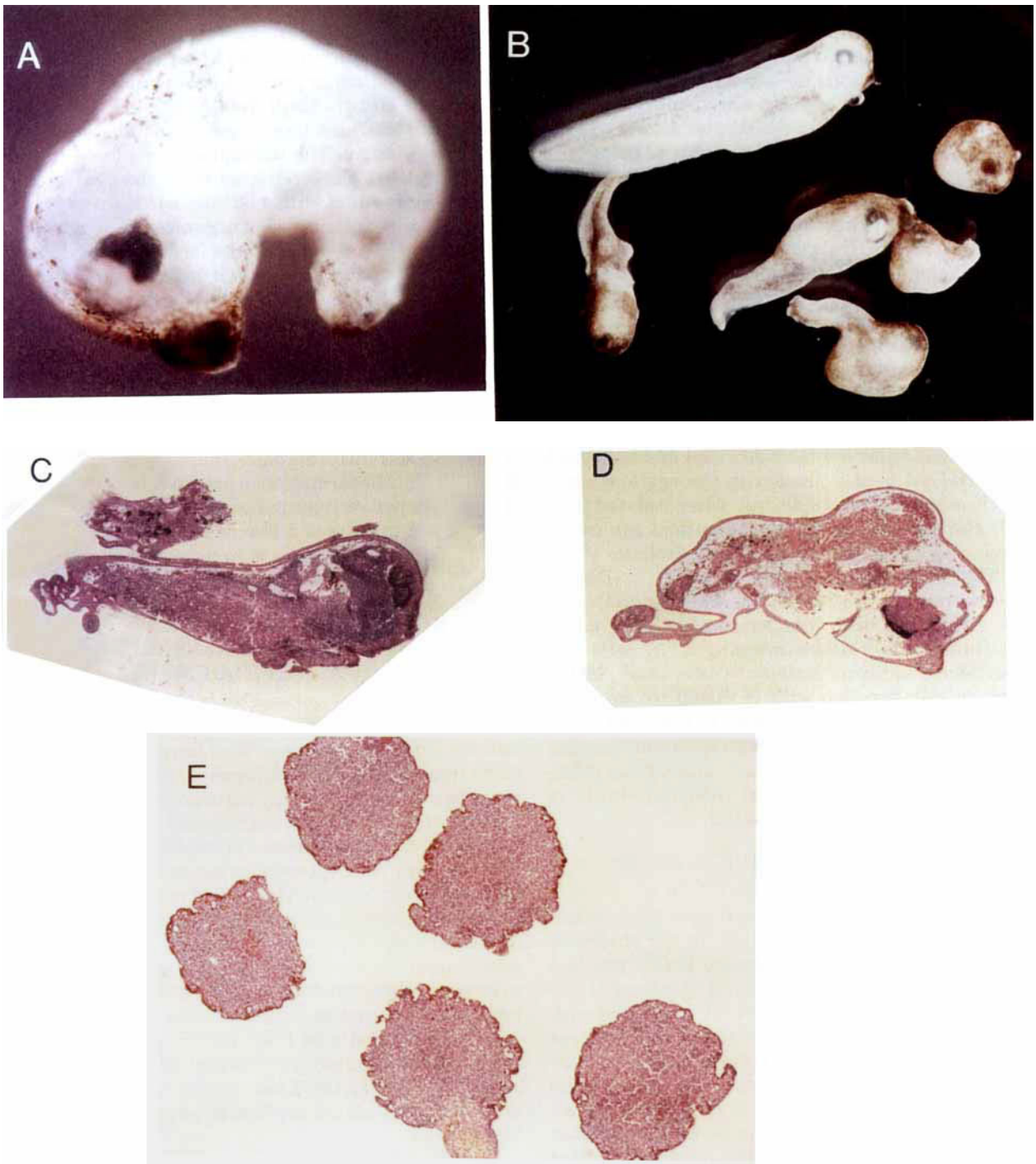

Fig. 4. Embryoids formed by the induction of large animal explants with activin A. Embryoids are structures with eyes and apparent anteroposterior polarity. **A, B:** Embryoids produced in 50 pM human recombinant activin A. In B, several embryoids are shown together with a normal tadpole of the same age (3 days after fertilization). **C, D:** Sections of embryoids. The embryoid in C was induced with crude culture fluid of XTC cells (MIF), the embryoid in D with crude medium from P388D1 cell (PIF). **E**, large animal explants cultured in control medium.

The *Xlim*-1 gene is a homeobox gene belonging to a newly discovered subclass of homeobox genes. The first three genes known in this subclass are characterized by having a homeodomain that is rather divergent from the *Antennapaedia* homeodomain; associated with the homeodomain are two copies of a distinct conserved sequence characterized by a cysteine repeat. The name LIM came from the first letters of the names of the first three genes identified in this subclass: *lin*-11, *Isl*-1, and *mec*-3 (Way and Chalfie, 1988; Freyd et al., 1990;

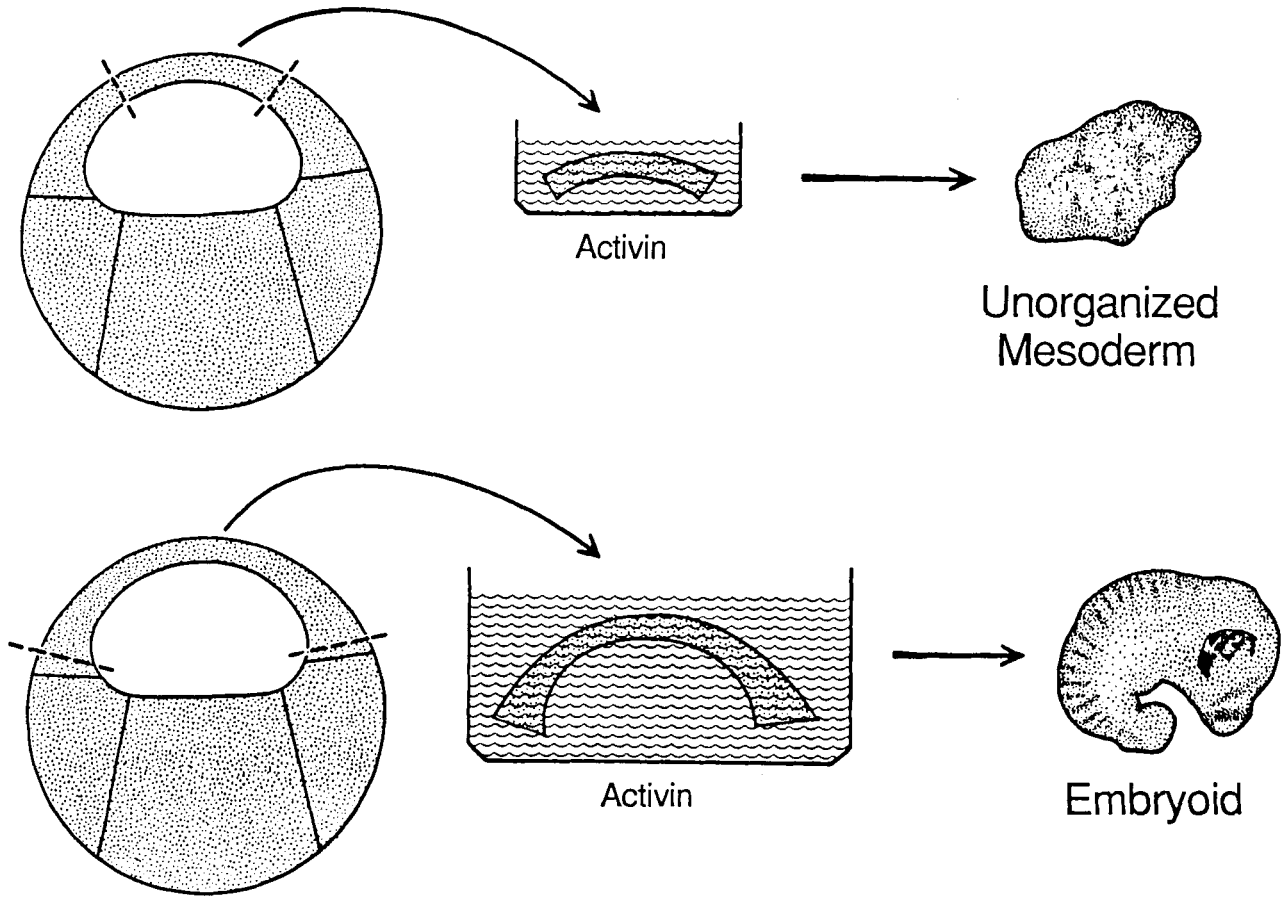

Fig. 5. Conclusion of induction experiments. See text for further discussion.

Karlsson et al., 1990). *Lin*-11 and *mec*-3 are lineage determining genes in *C. elegans*, and *Isl*-1 binds to the upstream region of the insulin gene.

Additional proteins containing the LIM motif have been reported. The *rhombotin* or *Ttg* gene contains the cysteine-rich LIM domain, but no homeodomain (Boehm et al., 1991). This gene is a possible oncogene because it was found next to chromosomal breakpoints in T-cell tumors. It is expressed prominently in the hindbrain during normal embryogenesis; its function is not known.

We looked for LIM class genes in Xenopus and found two of them, named *Xlim*-1 and *Xlim*-3 (Fig. 6). They contain a LIM class-specific homeodomain and two LIM domains. The homeodomains are more highly conserved than the LIM domains, but the latter are quite recognizable by the invariant positions of all cysteine residues. The C-terminal tails are quite different in length and sequence. Figure 7 shows a comparison of all known LIM domains. CRIP and hCRP, two LIM proteins without homeodomains, form a separate group and seem to be rather distantly related to the other proteins. Among the other proteins, the first and the second LIM domains form separate groups, suggesting the possibility that the duplication of the LIM domain is a very ancient event.

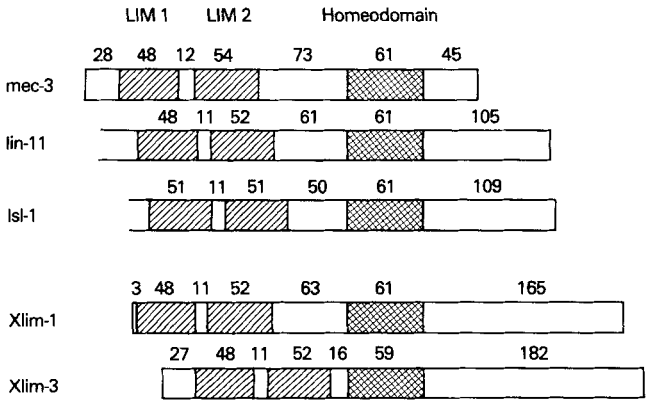

Fig. 6. Domain structure of LIM class homeodomain proteins. The LIM and homeodomains are indicated by different hatching. Numbers above each domain indicate length in amino acid residues. Open bars indicate incomplete sequences. See Figure 7 for references to published genes.

We have studied the *Xlim*-1 gene in more detail because of its potential role in pattern formation. In the adult frog, *Xlim*-1 mRNA is most expressed in the brain but there is also some mRNA in the kidney and the eye. During embryogenesis, the mRNA is expressed during the gastrula stage, then its level dips during neurula-

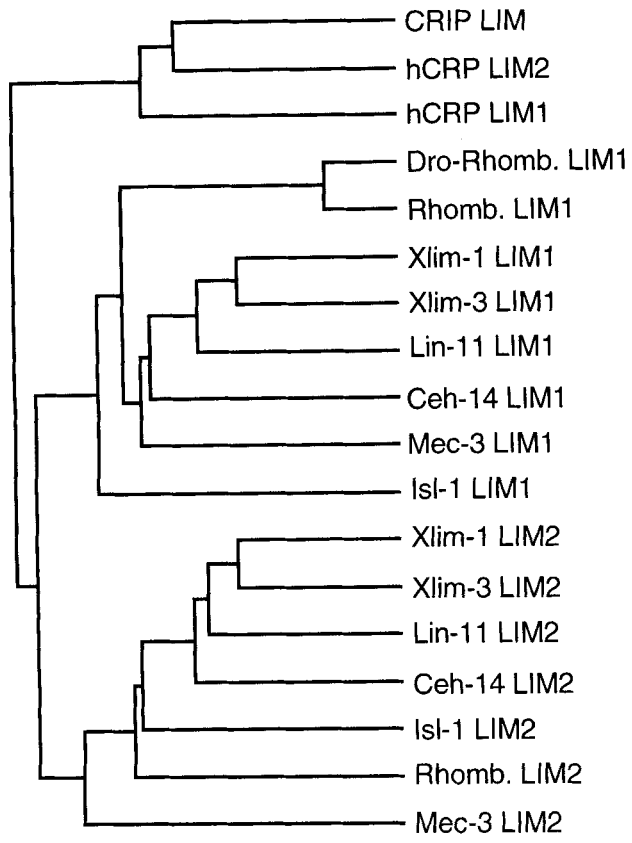

Fig. 7. Sequence comparison of known LIM domains. The first and second domain from the N-terminus are identified as LIM1 and LIM2, and were considered separately. Pairwise comparisons of amino acid sequences were carried out by the PILEUP program of the GCG package. The horizontal distance between any two sequences is a measure of their relatedness. References to sequences are as follows: CRIP, Birkenmeier and Gordon, 1986; hCRP, Liebhaber et al., 1990; rhombotin and *Drosophila* rhombotin, Boehm et al., 1991, and refs. therein; lin-11, Freyd et al., 1990; Ceh-14, Bürglin and Ruvkun, unpublished, see also Bürglin et al., 1989; mec-3, Way and Chalfie, 1988; Isl-1, Karlsson et al., 1990.

tion, and later rises again. There is also a low abundance maternal mRNA that we have not studied in detail. When we examined the distribution of *Xlim*-1 mRNA by dissection we found very little mRNA in the ectoderm, with most of it present in the dorsovegetal quadrant of the gastrula embryo. By contrast, *Mix*.1 mRNA is localized in the vegetal half with no differential distribution between dorsal and ventral regions.

Dorsovegetal localization of *Xlim*-1 is interesting because this is the area in which Spemann's organizer forms at the beginning of the gastrula stage (for review, see Dawid et al., 1990; Melton, 1991; De Robertis et al., 1991). Recent whole-mount in situ hybridization experiments (Taira et al., 1992) have demonstrated that *Xlim*-1 mRNA is expressed precisely in the organizer region during the gastrula stages. Thus, expression of this gene provides a molecular marker for the organizer, and may be a promising tool in the molecular analysis of the remarkable biological properties of this region of the embryo. Other homeobox genes expressed in the organizer region of frog embryos have recently been isolated by Blumberg et al. (1991). One of these genes, named *goosecoid,* is of particular interest because of its structure and expression in the organizer region of the gastrula.

Since the *Xlim*-1 gene is not normally expressed in the animal (ectodermal) region of the embryo we could test whether it is induced by activin in an explant experiment. The result is positive; *Xlim*-1 mRNA accumulates rather quickly after the addition of activin to animal explants and can be easily observed after 2 hr. Expression in induced explants is transient as it is in the whole embryo. In addition to activin, retinoic acid also induces the expression of *Xlim*-1, and there is a definite synergism between activin and retinoic acid in this induction. By contrast, *Mix*.1, which is rapidly induced by activin, is totally unaffected by retinoic acid. Retinoic acid is not a mesoderm inducer and does not cause the differentiation of an animal explant. Nevertheless, this compound has important biological effects on early embryogenesis. When frog embryos are cultured in retinoic acid they do not develop anterior structures and in the extreme case become entirely acephalic (Durston et al., 1989). A variety of experiments suggest retinoic acid posteriorizes mesoderm and neural tissue, and enhances the expression of posterior marker genes (Durston et al., 1989; Ruiz i Altaba and Jessel, 1991; Cho and De Robertis, 1990). This conclusion is somewhat confusing in the present context, because retinoic acid induces the *Xlim*-1 gene which, at the gastrula stage, is a dorsoanterior determinant. So rather than enhancing the expression of a gene that is normally expressed in the posterior and ventral region, as it does in several other examples, in the present case retinoic acid induces a gene that is expressed anteriorly and dorsally.

We speculate that the distribution of *Mix*.1 and *Xlim*-1 in the embryo may reflect the distribution of the activating substances. For example, activin may be distributed throughout the entire marginal zone and retinoic acid may have a source at the dorsal side. If the distributions of activin and retinoic acid did overlap, then the observed distribution of expression of the two homeobox genes could result. In this model, the homeobox genes are seen as reporter genes for the expression of the growth factors. We believe that one major way in which growth factors express their developmental message, is through the control of homeobox genes.

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QUESTIONS AND ANSWERS

Q: *Have you looked to see whether retinoic acid might be inducing TGF-β2 in the frog embryos?*

A: I haven't looked at that. I doubt that the effect on *Xlim*-1 goes through TGF-β2 because at least up to 100 ng/ml TGF-β2 does not induce *Xlim*-1.

Q: *What's known about the* Xlim-*1 protein's function?*

A: The LIM type homeobox genes have been recently discovered so there is not a great deal known about them. The two *C. elegans* genes have been picked up as cell lineage determinants. In mutants for the mec-3 gene, a cell that should make one sort of mechanosensory neuron doesn't die but makes some other cell. In lin-11, the cell that should make a vulva cell instead makes a body wall cell. So it's clearly a switch gene in development. Of course, since it is a homeobox gene we feel it is a transcription factor. What the LIM domains themselves are doing is not at all clear. Just from the structure you would think that they are zinc-binding or at least metal-binding proteins. In fact there is some unpublished evidence to support this from a laboratory other than ours (note added in proof: see Li et al., 1991). But the structure of the cysteine repeat is not a classical zinc finger although it can be fit to a zinc finger-like domain. The question is whether it is involved in DNA binding or in protein–protein interactions. There is also a parallel between LIM-type homeobox genes and POU-type homeobox genes. There are no sequence similarities between the POU and LIM-type domains, but these genes are similar in that they have a homeobox and an associated second motif. Of course, the POU domain has been shown to be required for DNA binding and for dimerization.

Q: *Have you tested the effect of antibodies to TGF-*β *and FGF on the ability of the embryos to differntiate?*

A: There is no data on that. We have obtained some antibodies to activin B that we plan to use. The problem is to get the antibodies into the embryo. In fact, you probably can't. But you can disperse the cells of the embryo and under certain conditions they will still interact with each other if they are close to each other. Antibodies against FGF have also not been used in this way, but Kirschner and his colleagues have done a very nice experiment with the FGF receptor where they made a dominant negative mutant by removing the intracellular domain. When overexpressed in the embryo the mutant inhibits some aspects of mesoderm formation (Musci et al., 1991). So it's clear that the FGF receptor is required for some aspects of mesoderm development.

Q: *What happens in differentiation to an animal cap explant that consists of ectodermal tissue removed asymmetrically from one side of the animal cap rather than from the middle?*

A: This is exactly the experiment done by Sokol and Melton (1991).: They divided the animal caps into dorsal- and ventral sides. They found that the dorsal sides can form complete embryoids, whereas the ventral sides do not form embryoids. Their conclusion was that some region of the animal hemisphere already has some inclination to differentiate although it will not do so without receiving a signal from activin. This fits very nicely with what we've done. There is therefore some agreement that by midblastula stage which is only 6 hr after fertilization, the tissue has already formed some differences that give it a virtual dorsal–ventral polarity that can be brought out by adding activin.