Cell, Vol. 71, 731–739, November 27, 1992, Copyright © 1992 by Cell Press

Responses of Embryonic Xenopus Cells to Activin and FGF Are Separated by Multiple Dose Thresholds and Correspond to Distinct Axes of the Mesoderm

Jeremy B. A. Green, Helen V. New, and J. C. Smith
Laboratory of Developmental Biology
National Institute for Medical Research
The Ridgeway, Mill Hill
London NW7 1AA
England

Summary

The potent mesoderm-inducing factors activin and FGF are present as maternally synthesized proteins in embryos of X. laevis. We show that activin can act on explanted blastomeres to induce at least five different cell states ranging from posterolateral mesoderm to dorsoanterior organizer mesoderm. Each state is induced in a narrow dose range bounded by sharp thresholds. By contrast, FGF induces only posterolateral markers and does so over relatively broad dose ranges. FGF can modulate the actions of activin, potentiating them and broadening the threshold-bounded dose windows. Our results indicate that orthogonal gradients of activin and FGF would be sufficient to specify the main elements of the body plan.

Introduction

The mesoderm of the amphibian embryo is formed from the equatorial region of the blastula-staged embryo as the result of an inductive signal derived from the vegetal hemisphere (Nieuwkoop, 1969; see reviews by Smith, 1989; Whitman and Melton, 1989; Dawid and Sargent, 1990). Of the several classes of molecule suggested to be involved in mesoderm induction (see above reviews and papers by Smith and Harland, 1991; Sokol et al., 1991; Chakrabarti et al., 1992; Köster et al., 1991; Dale et al., 1992; Jones et al., 1992), two are particularly strong candidates for natural mesoderm-inducing factors. These are members of the fibroblast growth factor (FGF) family and some members of the transforming growth factor beta (TGFβ) superfamily, particularly the activins. Members of both these peptide growth factor families induce mesoderm from isolated animal caps of the Xenopus blastula at picomolar concentrations (see references above). Evidence that these molecules play a role in normal development comes from the observations that they are present in the embryo at appropriate stages (Kimelman and Kirschner, 1987; Kimelman et al., 1988; Slack and Isaacs, 1989; Shiurba et al., 1991; Thomsen et al., 1990; Asashima et al., 1991) and that expression of a dominant negative mutation of the FGF receptor causes defects in posterior mesoderm formation (Amaya et al., 1991). The posterior defects observed in the latter experiments are consistent with the mesoderm-inducing activities of the two classes of factor: activins tend to induce genes and cell types characteristic of anterior and dorsal differentiation while FGF elicits mesoderm of posterior and ventral character (Ruiz i Altaba and Melton, 1989a; Green et al., 1990; Cho and De Robertis, 1990).

Mesodermal patterns could arise from these mesoderm-inducing factors in a number of ways. Because the effects of the two are different, a simple pattern would arise if activin were confined to one region of the embryo and FGF to another. The effects of uniform distributions of either or both factors could then be modulated by localized expression of other molecules such as members of the Wnt family (see Christian et al., 1992). In addition, however, each factor could generate a complex pattern directly via concentration-dependent effects (Green and Smith, 1991); we have previously shown that dispersed animal pole cells can distinguish between small differences in activin dose to produce at least three different cell types (Green and Smith, 1990).

In this paper, we go on to demonstrate that activin can elicit a complete spectrum of mesodermal responses, from posterolateral mesoderm to prospective organizer, with each component of the spectrum having a tightly restricted dose window bounded by concentration thresholds. We also show that FGF can elicit multiple responses, that these responses are different from the activin responses, and that FGF can modulate the thresholds revealed by activin induction. We discuss the relationship between the effects we observe with the factors and the axes of the mesodermal fate map.

Results

Activin Induces a Complete Organizer-to-Tail Spectrum of Mesodermal Markers in Threshold-Bounded Dose Windows

Our previous work (Green and Smith, 1990) showed that when cells from animal caps of Xenopus blastulae are dispersed and exposed to different concentrations of activin before being washed, reaggregated, and cultured, muscle differentiation only occurs within a narrow range of activin doses. Figure 1 shows the results of a single-cell aggregate experiment in which the dose of activin was varied and expression of a spectrum of mesoderm-specific genes was examined by RNAase protection. Analysis was performed at stage 17/18 (tailbud stage), when the fundamental body pattern is complete but many region-specific genes, such as homeobox genes, are still expressed. Five distinct cell responses or fates can be observed. At low or zero activin, cells express epidermis-specific keratin (XK81A-type epidermis; Jonas et al., 1989). At slightly higher doses, expression of the genes *Xhox3*, *XlHbox6*, and *Xbra* is induced. These genes are expressed predominantly in posterior and lateral mesoderm at these stages (Ruiz i Altaba and Melton, 1989b; Wright et al., 1990; Smith et al., 1991), and although *Xbra* is also expressed elsewhere (see below), its combination with *Xhox3* and *XlHbox6* is characteristic of this region. It should be added that *Xhox3* and *XlHbox6* are also expressed in neural tissue (Ruiz i Altaba and Melton, 1989b; Wright et al., 1990),

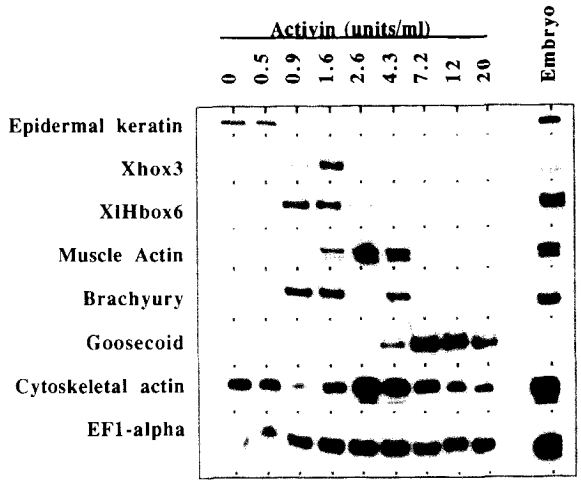


Figure 1. Induction by Activin of an Organizer-to-Posterior Spectrum of Genes Showing Multiple Dose Thresholds

RNAase protection assays of RNA extracted from cell aggregates at stage 17.5. Single cells had been exposed at blastula stages to a 1.7-fold dilution series of activin. This experiment was performed four times with the same results. For each experiment, two separate assays were performed on RNA from the same aggregates, one with keratin and actin probes (loading control: cytoskeletal actin), the other with all other probes together (loading control: EF1-α). Embryo track corresponds to RNA from three whole stage 17.5 embryos. Genes probed were:

Epidermal keratin XK81A (Jonas et al., 1989); *Xhox3* posterior-enriched homeobox gene (Ruiz i Altaba and Melton, 1989a, 1989b; Saha and Grainger, 1992); *XlHbox6* antennapaedia family posterolateral specific homeobox (Fritz and De Robertis, 1988; Wright et al., 1990); Actin, muscle and cytoskeletal (Mohun et al., 1984); *Xbra*, Xenopus brachyury gene homolog (Smith et al.,1991); *goosecoid* organizer-specific homeobox gene (type A, Blumberg et al., 1991; Cho et al., 1991).

but our previous work showed that activin-induced aggregates do not express neural markers (Green and Smith, 1990). *Xhox3*, *XlHbox6*, and *Xbra* are switched off with increasing dose and muscle-specific actin is switched on. The muscle window, observed previously (Green and Smith, 1990), overlaps with a second *Xbra*-expressing window. Thus, as activin dose is increased, *Xbra* expression is first turned on, then off, then on again and finally, at high doses, off. These two windows of *Xbra* induction may relate to the two domains of *Xbra* expression in vivo; at neurula stages, *Xbra* is expressed in the notochord and around the blastopore (Smith et al., 1991). Whole-mount in situ hybridization (Figure 2) shows that expression occurs all around the blastopore, thus including prospective posterolateral mesoderm as well as prospective notochord. High doses of activin induce expression of *goosecoid*, which is expressed in the dorsal lip, or "organizer," of the Xenopus gastrula (Cho et al., 1991). In vivo expression of this gene falls during gastrulation (Blumberg et al., 1991) but is still detectable by RNAase protection until neurula stages. Separate experiments show that similarly high concentrations of activin are required for expression of *gsc* during gastrula stages. The combination of *goosecoid* expression and the neural-inducing ability of

Figure 2. Whole-Mount In Situ Hybridization Showing Expression of *Xbra* in Notochord and Posterior Domains

Anterior is to the left. In situ hybridization with a Xenopus *Brachyury* probe was carried out according to Harland (1991).

cells in this high-dose range (Green and Smith, 1990) is consistent with their being of organizer character.

Muscle and Notochord Are Induced by Activin Doses That Overlap but Are Not Identical

The correlation of two *Xbra*-inducing activin dose ranges with two *Xbra* embryonic expression domains suggests that the higher dose window should induce notochord. Table 1 shows the results of experiments in which single-cell aggregates were allowed to differentiate and then analyzed for notochord and muscle formation. The results show that notochord induction overlaps with, but occurs at higher activin doses than, muscle, in agreement with the prediction from the gene expression data above. Our previous work (Green and Smith, 1990) also showed that the activin concentrations required for muscle and notochord differentiation overlap, and our initial impression was that notochord respected the same dose thresholds as muscle. However, improved technique, including faster dissection and gentler cell dissociation, has allowed us to resolve the muscle and notochord dose ranges, and we are now able to generate notochord in the absence of muscle. Figure 3 shows a section from an aggregate that consisted essentially entirely of notochord cells, as shown by ubiquitous staining with MZ15 (Smith and Watt, 1985), vacuolation, and large nuclei, features characteristic of and unique to this tissue. In both parts of the first experiment recorded in Table 1, entire aggregates differentiated as notochord. In the same experiments (and in others not shown) quantitation of muscle tissue revealed that this tissue never constitutes more than about 35% of the volume of muscle-expressing aggregates (see Experimental Procedures). This is consistent with our previous observation that aggregates contain only patches of muscle (Green and Smith, 1990).

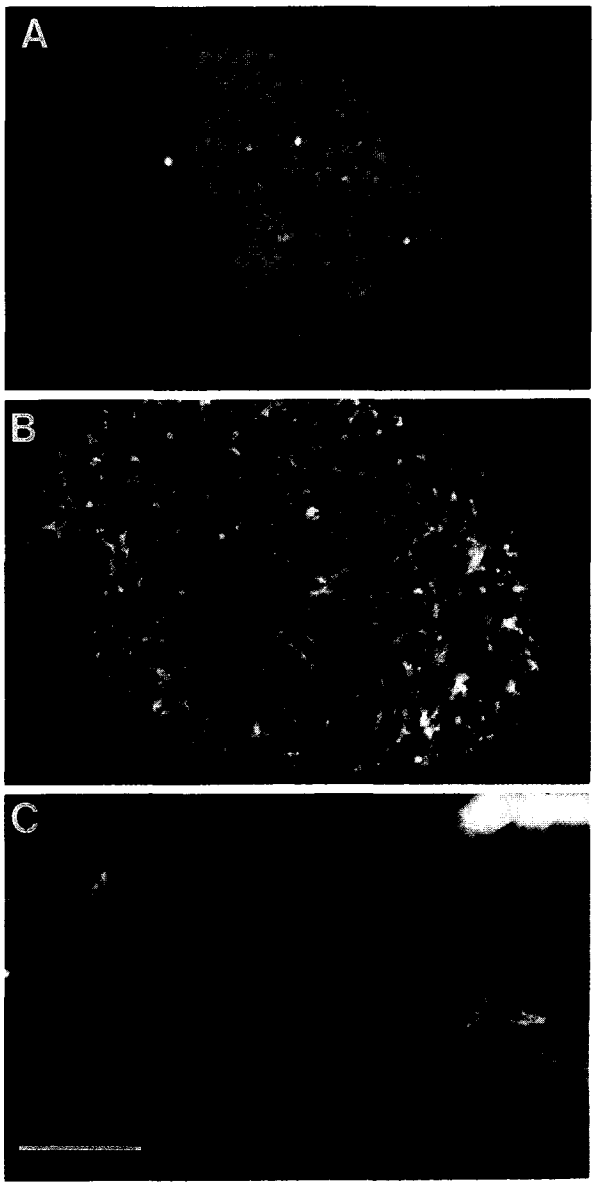
FGF Responses Plateau at High Doses

The morphological responses of animal cap cells to FGF are different from those to activin (Slack et al., 1987; Green et al., 1990), and FGF is unable to activate certain activin-inducible genes such as *Mix.1* and *goosecoid* (Rosa, 1989; Cho et al., 1991). To examine dose-dependent effects of FGF we used it in the same single-cell aggregate protocol as we used for activin. A typical result is shown in Figure

Table 1. Separate Activin Induction Dose Windows for Muscle and Notochord and Their Modulation by Addition of FGF

	Activin (U/ml)							
	0.0	0.5	1.0	2.0	4.0	8.0	16.0	
Experiment 1								
Muscle	–	–	(+)	++	–	–	–	
Notochord	–	–	–	+	++	–	–	
Muscle	–	++	++	+	–	–	–	FGF
Notochord	–	–	++	++	++	+	+	(8 U/ml)
Experiment 2								
Muscle	–	–	–	++	++	–	–	
Notochord	–	–	–	–	++	–	–	
Muscle	–	+	++	++	+	–	–	FGF
Notochord	–	–	–	+	+	+	+	(8 U/ml)

Dispersed blastula stage animal cap cells were treated for 1 hr with the factors shown, reaggregated, and incubated to control stage 42. After fixation and sectioning, they were stained with anti-muscle antibody 12/101 and anti-notochord sheath antibody MZ15. Every second section was scored. ++ indicates at least 20% (by area) of the majority of sections were positive; + indicates at least two cells in at least two sections in a given aggregate were clearly positive. Parentheses indicate weak staining.



4, where, to aid comparison between the two factors, FGF concentration is increased at the same intervals used for activin in Figure 1. A number of differences from the activin response are obvious. First, epidermal keratin is suppressed relatively gradually. Then, instead of a sharp transition to induced genes, there is an apparent gap, or at least an attenuation, in which neither keratin nor any of the induced markers is strongly expressed. As the FGF dose is further increased, *XlHbox6* is switched on sharply and plateaus. *Xbra* is also switched on sharply and at the same doses as *XlHbox6* , but is induced more strongly after a further increase in dose of approximately 3-fold, after which its expression also plateaus. The lower limit of *Xhox3* induction is somewhat indistinct, partly because the amount of *Xhox3* message is so low that (in these experiments where the amount of material obtainable from one aggregate is limiting) it is hard to distinguish signal from background. It should be added that the spatial expression of *Xhox3* in vivo also lacks sharp boundaries (Ruiz i Altaba et al., 1991). Nonetheless, the FGF dose required for full induction of *Xhox3* is certainly higher than that for *XlHbox6*. In contrast with activin induction of muscle, FGF induces muscle over a broad range of doses that are bounded not by sharp thresholds but by more gradual expression changes. The muscle appears to decline somewhat at higher doses, although when the signal is normalized to cytoskeletal actin, this decline is by less than a factor of two over the range tested. This combination of

Figure 3. Induction of Notochord without Coinduction of Muscle

All panels are sections of Stage 42 specimens stained with the anti-notochord sheath antibody MZ15 (Smith and Watt, 1985). (A) shows a negative control section through an aggregate of untreated cells. (B) shows a section through an aggregate of cells that were treated as dispersed cells for 1 hr at blastula stages with 2.5 U/ml activin. A fine tracery of positive staining is seen throughout the section (though somewhat less intensely at the center of the section where strucutral preservation is less good), as is vacuolation characteristic of notochord differentiation. (C) shows a section through a whole embryo, a glancing longitudinal section revealing notochord. All panels same scale; scale bar = 200 μm.

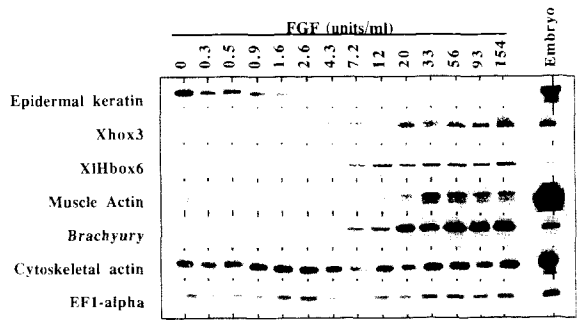


Figure 4. Induction by FGF of Posteriorly-Expressed Genes Showing Response Plateaus

RNAase protection assays of RNA extracted from cell aggregates at stage 17.5. Single cells had been exposed at blastula stages to a 1.7-fold dilution series of basic FGF. This experiment was performed four times with the same results. For each experiment, two separate assays were performed on RNA from the same aggregates, one with keratin and actin probes (loading control: cytoskeletal actin), the other with all other probes together (loading control: EF1-α). Embryo track corresponds to RNA from three whole embryos. Genes probed were as in Figure 2. *goosecoid* expression was not induced at any FGF dose (data not shown).

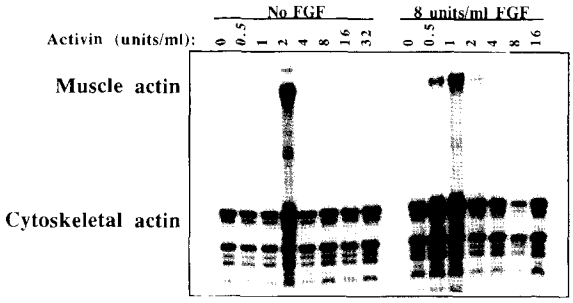


Figure 5. FGF Modulates Activin Induction Thresholds

Constant concentration of FGF was added to varying activin concentration. RNAase protection assays of RNA extracted from cell aggregates at stage 17.5. Single cells had been exposed at blastula stages to a 2-fold dilution series of XTC-MIF (activin) in the presence or absence of a constant concentration of FGF. This experiment was performed four times with the same results.

markers and their dose profiles is consistent with previous induction experiments using intact animal caps (Ruiz i Altaba and Melton, 1989a; Cho and De Robertis, 1990) and with the posterior character of FGF inductions. For all the markers, the dose range over which they are induced is at least twice as broad as that for activin.

Histological examination of FGF-induced aggregates shows that muscle is induced in proportions similar to those observed with activin; that is, it comprises less than 35% of the aggregate by volume. Induction of notochord has not been observed (data not shown). This is consistent with previous observations that FGF does not induce notochord in the dose ranges tested (Green et al., 1990) and suggests that FGF-induced *Xbra* expression represents that seen in posterolateral mesoderm.

FGF Modulates Activin Dose Thresholds

Both FGF and activin are present in the Xenopus embryo at early stages (see Introduction for references) and this suggests that mesoderm induction in vivo will be most accurately reflected by experiments in which the factors are used in combination rather than just singly. Figure 5 and Table 1 show the results of such experiments. Activin dose was varied in the presence or absence of a fixed concentration of FGF. We used a concentration of FGF that was slightly less than that needed to maximally induce muscle on its own so that positive effects of activin would be readily observable as enhancement of muscle induction. The addition of FGF affects the activin dose-response profile in two ways. It broadens the dose ranges for both muscle and notochord and shifts their optimum inducing doses downwards. Thus, the concentration of activin required for muscle induction is lower in the presence of FGF than in its absence. This is consistent with the results of Kimelman and Kirschner (1987) who found that FGF and TGFβ2 were "synergistic" for induction of muscle in animal caps. However, there is a further subtlety: FGF is not purely synergistic for muscle induction nor is it purely additive. First, the total amount of muscle in a given aggregate is not greatly increased by the presence of FGF, either as assayed by actin gene expression (Figure 5) or histologically (data not shown). Secondly, there are some activin concentrations at which FGF actually reduces muscle gene expression (e.g., 2 and 4 U/ml activin in Figure 5), apparently in compensation for increased notochord differentiation (Table 1). Thirdly, a simple synergy would be expected to shift dose thresholds to lower concentrations but not to broaden the muscle- and notochord-inducing ranges. Finally, although FGF does not induce notochord by itself (Green et al., 1990), it does potentiate induction of notochord by activin (Table 1). The implications of these observations are discussed below.

Discussion

In this paper we describe the responses of Xenopus blastula cells to different doses of activin and FGF, applied separately and in combination. To characterize differentiation and position in the body plan, we use immunological and histological markers for muscle and notochord and transcriptional probes for *Xhox3*, *XlHbox6*, *Xbra*, muscle actins, *goosecoid*, and epidermal keratin. The results tell us about the instructiveness of mesoderm-inducing factors, the differences between the factor responses, and their possible relationships to the patterning of the mesoderm as a whole.

Activin Dose Is Instructive and Specifies at Least Five Different Cell Types

Our results (Figure 1) suggest that different concentrations of activin can specify at least five different cell states. In order of increasing activin concentration these may be described as:

- Epidermis (expresses epidermal keratin)
- Posterolateral mesoderm (expresses *XlHbox6*, *Xhox3*, *Xbra*)

—Muscle (expresses muscle-specific actin, 12/101 antigen)
—Notochord (expresses *Xbra* and low level *gsc*, MZ15 antigen, has vacuolated cells)
—Organizer (expresses *gsc*; has neural-inducing activity [Green and Smith, 1990]).

This ability of activin to specify different cell types according to its concentration defines it as a "morphogen," and suggests that cells respond to the factor according to predetermined thresholds, essentially as predicted by Wolpert (1969). These five different responses are elicited over a dose range of approximately 20-fold. The cellular mechanism by which this might occur is completely unknown (see Green and Smith, 1991), but, as we discuss below, different concentrations of activin may be involved in establishing the body plan in vivo.

Is activin specifying cell fate or is it merely activating or selecting different predisposed subpopulations of cells? The fact that all cells in our aggregates can differentiate as notochord is most simply explained as specification, the only other explanation being selection by drastic cell death. The fact that we do not see reproducible differences in cell survival with different doses argues against this possibility. Furthermore, were there to be predispositions among cells one might expect them to favor muscle differentiation, which is the most abundant mesodermal cell type in vivo and is 10 times more abundant than notochord (Cooke, 1981). In fact, our results show that induction of single cells by activin yields much less muscle than it does notochord. Thus, we favor the view that activin is sufficient to specify cell fate. It remains unclear why only a proportion of cells in an aggregate can be induced to form muscle, although it is known that cell–cell contacts are particularly important for muscle differentiation (Gurdon et al., 1984), and that a "community effect" can restrict differentiation in cells previously specified to make muscle (Gurdon, 1988). Such effects may operate in cell aggregates.

We attribute the previous and persistent overlap of muscle and notochord induction (Green and Smith, 1990) to an irreducible heterogeneity among animal cap cells. At least part of this heterogeneity is likely to be experimental, because embryos within a batch are never absolutely synchronous and, in addition, it is necessary to dissect animal caps over a period of 1–1.5 hr, during which time further variation in the environments experienced by cells must arise. There is also evidence for systematic dorsoventral differences within the animal hemisphere (Sokol and Melton, 1991; Ruiz i Altaba and Jessell, 1991). These might arise through cell–cell contacts, the extracellular matrix, or other extracellular components. Such differences are minimized in our cell disaggregation experiments but may have a role in modulating responses to mesoderm-inducing factors in vivo (see below).

The Response to FGF Differs from that to Activin

Like activin, FGF induces the expression of different genes at different concentrations (see Figure 4). Activin, however, induces gene expression only over narrow dose ranges; FGF, by contrast, activates genes over wide dose ranges that tend to plateau at high concentrations. The fact that posterior and lateral genes are not extinguished at high FGF concentrations and that *goosecoid* expression and notochord differentiation are not observed in response to this factor is consistent with the view that FGF induces posterior mesoderm (Ruiz i Altaba and Melton, 1989a; Green et al., 1990; Cho and De Robertis, 1990; Amaya et al., 1991). We discuss below the potential role of FGF in patterning the mesoderm.

Dose-Dependent Effects of Activin and FGF Match Two Axes of the Mesodermal Fate Map

Our data may be summarized by plotting them on a graph, with activin concentration as the abscissa and FGF concentration as the ordinate (Figure 6A). We interpolate threshold values assuming only that the effects of FGF on activin thresholds vary smoothly with dose. If the activin axis is taken to point to the dorsal side of the blastula and the FGF axis to the animal pole, the resulting plot resembles in form the fate map of the Xenopus blastula marginal zone (Figures 6A and 6B). This resemblance may not be trivial and may point to specific gradients of activin- and FGF-like activities at blastula stages, with activin concentration high near the organizer, and FGF concentration high in the upper marginal zone but with radial symmetry (Figure 6C). There are a number of lines of evidence from diverse sources that reinforce this view.

Most is known about the distribution of FGF in the embryo. Shiurba et al. (1991) show that FGF epitopes are rare in the animal hemisphere and more abundant in the rest of the embryo. At morula and blastula stages, staining is stronger in the marginal zone than in more vegetal cells, but the distribution appears to be uniform in the dorsoventral axis, that is, radially symmetrical. The functional receptor depletion experiments of Amaya et al. (1991) are consistent with this distribution being that of a functional FGF activity.

Nothing is known about the localization of activin protein in the Xenopus embryo, but the results from experimental embryology and the spatial distribution of activin-induced immediate-early genes suggest that the highest concentration of activin would be near the dorsal marginal zone of the blastula, the prospective organizer. For example, it is only dorso-vegetal blastomeres that are capable, like activin, of inducing notochord from animal pole tissue (Boterenbrood and Nieuwkoop, 1973; Dale et al., 1985) and genes such as *goosecoid*, *Xlim-1*, and *Xfkh1*, which are induced by activin but not by FGF (Cho et al., 1991; Dirksen and Jamrich, 1992; Taira et al., 1992), are expressed exclusively at the dorsal lip of the blastopore. It may be that activin activity is not restricted exclusively to this region because another activin-inducible gene, *Mix.1*, is expressed over the entire vegetal hemisphere as well as in the marginal zone (Rosa, 1989).

The two gradients we propose are similar to those proposed by Dalcq and Pasteels (1937), though with slightly different distributions. Dalcq and Pasteels postulated two physicochemical gradients: a cortical gradient high on the dorsal side and a vitelline gradient high toward the vegetal

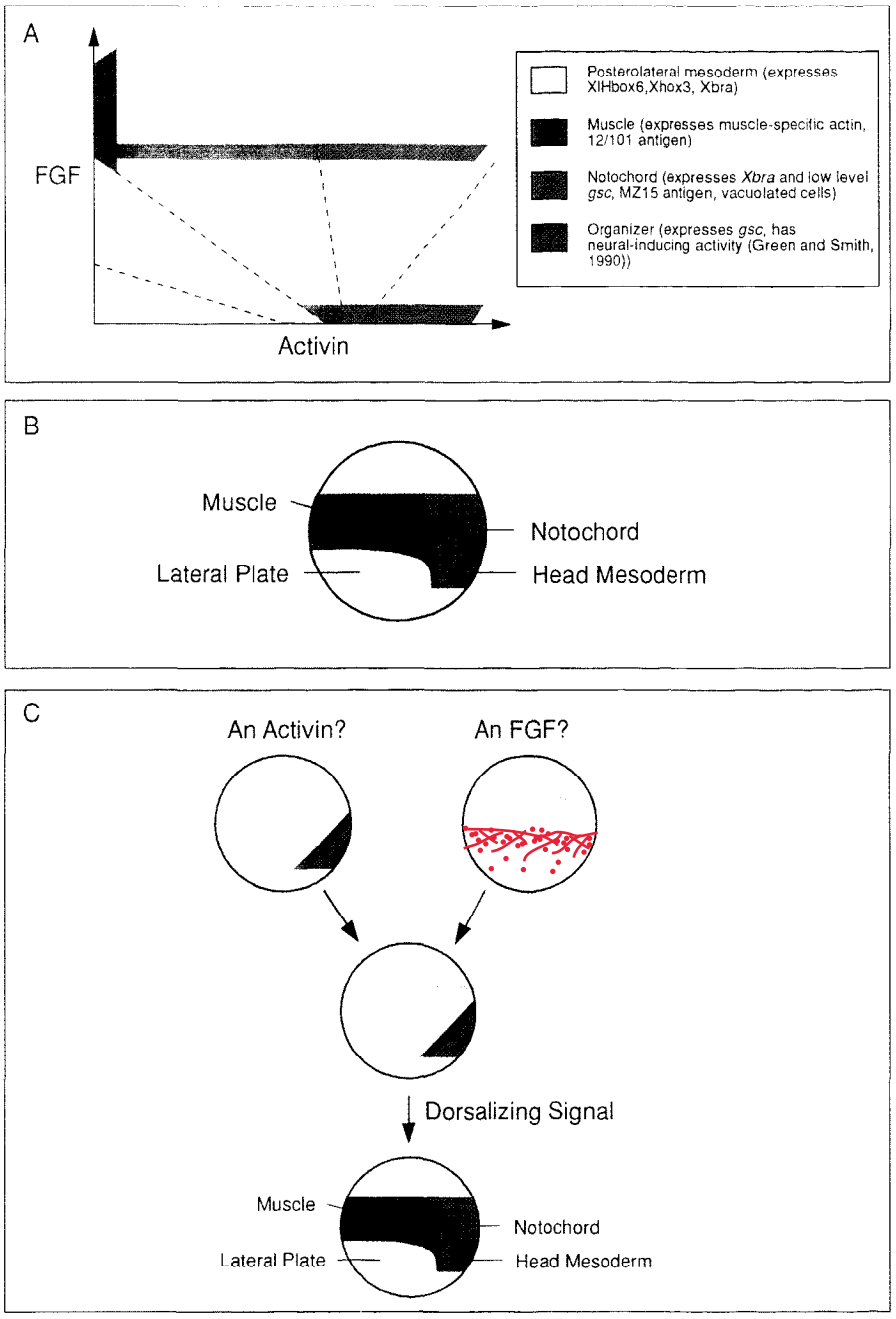


Figure 6. Dose-Dependent Effects of Activin and FGF and Their Correspondence to Two Axes of the Mesodermal Blastula Fate Map

(A) Summary of the data shown in Figures 1–4 and Table 1. Dashed lines interpolate thresholds at different FGF concentrations assuming only that they vary smoothly. (B) Schematic diagram of the Xenopus blastula fate map based on the fate maps of Cooke and Webber (1985) and Keller (1976). (C) Two-gradient hypothesis for how gradients of activin- and FGF-like activities might specify the fate map. A putative activin-like gradient has a high point near the prospective dorsal lip, and a putative FGF-like gradient is radially symmetrical about the animal–vegetal axis. It is high in the upper marginal zone and trails off gradually towards the vegetal pole. Both gradients are in the form of maternal proteins. Simple superimposition of the separate effects of the two gradients would give rise to the blastula specification map (Dale and Slack, 1987). Interaction between the gradients takes place during gastrula stages and may require the action of an activin-induced third signal ("dorsalization"; Dale and Slack, 1987). See text for further explanation.

pole; combinations of the gradient values would constitute a coordinate system for morphogenesis. The form of their model differs from ours only in that their vitelline gradient has the opposite polarity to the FGF gradient we suggest.

We, however, are able to go further than Dalcq and Pasteels by directly demonstrating graded cellular responses to the factors, by suggesting specific identities for the gradient molecules, and by showing what their morphoge-

netic effects are at different concentrations and in different combinations.

The model also resembles the "three signal" model of Smith and Slack (1983). This proposes that separate signals are required for induction of ventral mesoderm and of the organizer, and that regionalization within the ventral mesoderm is then achieved through a third "dorsalizing" signal produced by the organizer. Activin and FGF would, respectively, represent the signals inducing the organizer and the ventral mesoderm; activin can indeed induce organizer activity in animal pole tissue (Ruiz i Altaba and Melton, 1989a; Cooke, 1989). The third signal could be activin itself, spreading from the dorsal lip, or a different signal whose production is induced by activin. We prefer the latter possibility because dorsalization occurs at stages when cells are losing their competence to respond to activin (Green et al., 1990). One promising candidate for a dorsalizing molecule is the protein encoded by the gene *noggin* (Smith and Harland, 1992). Thus, a possible refinement of our model is that the dorsal part of the fate map is induced by activin directly while the ventral part is generated by spread of the activin-induced dorsalization signal at later stages.

Clearly, the model in Figure 6 is not complete. In particular, it does not account for formation of the most anterior head structures, which are not induced by activin (Thomsen et al., 1990; Bolce et al., 1992). Induction of these tissues may require the activity of members of the wnt family; injection of Xwnt-8 RNA into ventral blastomeres of a Xenopus embryo causes formation of a new and complete dorsal axis, and the presence of Xwnt-8 RNA dorsalizes the response of animal caps to both FGF and activin (Christian et al., 1992; R. T Moon, personal communication). These results have led to the suggestion that pattern may arise through localized (perhaps graded) expression of wnts modifying the effects of uniform distributions of inducing factors (Christian et al., 1992). It is not possible to address this question using the quantitative techniques described in this paper because the wnts are not available as soluble proteins, and injection of RNA can lead to uncontrolled variation between embryos in amounts of translated protein (Vize et al., 1991).

Even if activin and FGF do not have exactly the roles we propose for them, it seems very likely that they stimulate two systems that do act in the two axes of the blastula fate map, namely dorsoanterior-posterolateral and posteroventral, respectively. Two gradient systems are known to specify pattern in different axes in Drosophila (Nüsslein-Volhard and Roth, 1989), and it is intriguing that one of the morphogens operating in the dorso-ventral axis, the decapentaplegic gene product, is, like activin, a member of the TGFβ superfamily (Ferguson and Anderson, 1992).

Experimental Procedures

Embryos and Dissections

Xenopus embryos were obtained by artificial fertilization as described by Smith and Slack (1983). They were dejellied with cysteine hydrochloride (pH 8.1) and staged according to Nieuwkoop and Faber (1967). Other procedures were as in Green and Smith (1990) with minor modifications. Briefly, animal caps were excised, after a minimum of 10 min preincubation, in calcium- and magnesium-free medium (CMFM) at blastula stage 8–8.5. Incubation in two or three changes of CMFM with very gentle pipetting allowed thorough disaggregation of inner layer cells and removal of intact outer layers. Pipettes treated briefly with a 0.4% poly-HEMA (poly-hydroxyethylmethacrylate: Aldrich) solution in ethanol–acetone (1:1) were used for this and all subsequent cell handling. Aliquots of the disaggregated cells, typically derived from inner layers of 15–20 caps, were transferred to 35 mm agarose-lined dishes containing CMFM with 0.1% bovine serum albumin fraction V (Sigma) and appropriate concentrations of factor(s). Cells were kept dispersed by occasional gentle pipetting. After 1 hr incubation cells plus some medium were transferred to poly-HEMA treated Eppendorf tubes and centrifuged for 3 min, 165 × g at room temperature. All but about 15 μl of supernatant was removed and 1.5 ml CMFM added (i.e., 100-fold dilution). Cells were resuspended by gentle pipetting, recentrifuged, and resuspended in 1.5 ml 75% NAM (a further 100-fold dilution) before a final centrifugation. This procedure was always completed before stage 10. Pelleted cells were allowed to reaggregate at 18°C overnight and incubated at 18–23°C until harvested for RNA or fixed for immunohistology. For incubations of more than 24 hr, aggregates were transferred after their first overnight incubation to 40% NAM.

Mesoderm-Inducing Factors

Either Xenopus activin A or human recombinant activin A was used with no apparent difference in effects. Xenopus activin A was purified from XTC cell–conditioned medium as in Smith et al. (1990). Recombinant human activin A was purified from conditioned medium from CHO cells transfected with the human β-A gene (gift of Gordon Wong, Genetics Institute, Cambridge, MA). The purification protocol was a modification of that used by Albano et al. (1990). Briefly, activin was purified on a phenyl–Sepharose CL-4B column (Pharmacia) followed by two reverse-phase high-pressure liquid chromatography steps. This resulted in an almost complete purification, as evaluated by silver-stained polyacrylamide gel electrophoresis. FGF was recombinant Xenopus basic FGF prepared using the bacterial expression plasmid XF140 as described by Kimelman et al. (1988). It was purified on a conventional heparin Sepharose column with gradient elution (see Slack and Isaacs, 1989). Activity of each batch of each factor was assayed by the animal cap assay (Cooke et al., 1987), one unit per milliliter being defined as the minimum concentration necessary to obtain morphologically observable mesoderm induction of animal cap explants. For activin, 1 U/ml was approximately 0.13 ng/ml or 5 pM. For FGF, specific activity varied significantly from batch to batch but remained stable within each batch. The lowest specific activity observed was 1 U/ml = 20 ng/ml or 1 nM; the highest specific activity was 1 U/ml = 0.2 ng/ml or 10 pM.

Histological Procedures and In Situ Hybridization

Embryos and aggregates were fixed in 2% trichloroacetic acid overnight at 4°C, embedded in polyacrylamide (Hausen and Dreyer, 1981), and sectioned. Antibodies were 12/101 anti-muscle (Kintner and Brockes, 1984) and MZ15 anti-notochord sheath (Smith and Watt, 1985). Areas of muscle and notochord tissue were measured using a BioRad confocal microscope and its associated software. Other histological procedures were as in Green et al. (1990). In situ hybridization was carried out as described by Harland (1991).

RNA Isolation and RNAase Protections

Embryos or single-cell aggregates were frozen on dry ice in a minimum volume of 75% NAM and stored at −80°C. RNA was extracted as described by Smith et al. (1991). RNAase protections were carried out essentially as described by Green et al. (1990). Probes used were as follows:

Epidermal keratin XK81A1 type 1 epidermal keratin probe exactly as in Jonas et al. (1989), probe length 550 nt, protected fragment 236 nt. *XlHbox6* homeobox gene probe consisting of the 5′ EcoRI fragment of clone P1 (Figure 1 of Fritz and De Robertis, 1988) cloned into the SmaI site of pGEM2 (Promega), linearized with EcoRI, probe 515nt, protected length 475nt. *Xhox3* homeobox gene probe exactly as in Saha and Grainger (1992), 300nt, protected length 230nt. *Xbra*, Xenopus *brachyury* gene homolog exactly as in Smith et al. (1991), probe

293nt, protected length 214nt. Actin probe pSP21 (pSPa1) exactly as in Mohun et al. (1988) probe 380nt, protected lengths 285nt (muscle-specific cardiac actin) and 135nt (cytoskeletal actin). *goosecoid* organizer-specific homeobox gene probe consisting of a PstI–ApaI fragment from the 5′ end of the *gscA* cDNA clone (Cho et al., 1991) cloned into BluescriptII KS (Stratagene), probe 440nt, protected length 367nt. Autoradiography used preflashed Kodak X-Omat film except for Figure 4 for which a PhosphorImager (Molecular Dynamics) was used to reduce exposure time. For all genes except *Xhox3*, a background subtraction of 5 counts per pixel was applied; for *Xhox3*, 50 counts per pixel were subtracted to filter out an underlying smear that was also observed in the tRNA-only control track.

Acknowledgments

This work is supported by the Medical Research Council. We are grateful to all those who kindly sent us probes, antibodies and growth factors. These include Doug Melton, Ariel Ruiz i Altaba, Margaret Saha and Rob Grainger (*Xhox3*), Eddy De Robertis, Ken Cho and Nancy Papalopulu (*XlHbox6* and *goosecoid*), Igor Dawid and Alison Snape (epidermal keratin), Jeremy Brockes (12/101), David Kimelman and Marc Kirschner (FGF) and Gordon Wong (activin-secreting CHO cells). We thank Alison Snape and Jonathan Cooke for comments on the manuscript and Brenda Price for help with whole-mount in situs.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 USC Section 1734 solely to indicate this fact.

Received August 11, 1992; revised September 22, 1992.

References

Albano, R. M., Godsave, S. F., Huylebroeck, D., van Nimmen, K., Isaacs, H. V., Slack, J. M. W., and Smith, J. C. (1990). A mesoderm-inducing factor produced by WEHI-3 murine myelomonocytic leukemia cells is activin A. Development *110*, 435–443.

Amaya, E., Musci, T. J., and Kirschner, M. W. (1991). Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in Xenopus embryos. Cell *66*, 257–270.

Asashima, M., Nakano, H., Uciyama, H., Sugino, H., Nakamura, T., Eto, Y., Ejima, D., Nishimatsu, S., Ueno, N., and Kinoshita, K. (1991). Presence of activin (erythroid differentiation factor) in unfertilized eggs and blastulae of *Xenopus laevis*. Proc. Natl. Acad. Sci. USA *88*, 6511–6514.

Blumberg, B., Wright, C. V. E., De Robertis, E. M., and Cho, K. W. Y. (1991). Organizer-specific homeobox genes in *Xenopus laevis* embryos. Science *253*, 194–196.

Bolce, M. E., Hemmati-Brivanlou, A., Kushner, P. D., and Harland, R. M. (1992). Ventral ectoderm of *Xenopus* forms neural tissue, including hindbrain, in response to activin. Development *115*, 681–688.

Boterenbrood, E. C., and Nieuwkoop, P. D. (1973). The formation of the mesoderm in urodelean amphibians. V. Its regional induction by the endoderm. Wilhelm Roux's Arch. Entw. Mech. Org. *173*, 319–332.

Chakrabarti, A., Matthews, G., Colman, A., and Dale, L. (1992). Secretory and inductive properties of *Drosophila* wingless (Dwnt-1) protein in *Xenopus* oocytes and embryos. Development *115*, 355–369.

Cho, K. W. Y., and De Robertis, E. M. (1990). Differential activation of *Xenopus* homeobox genes by mesoderm-inducing growth factors and retinoic acid. Genes Dev. *4*, 1910–1916.

Cho, K. W. Y., Blumberg, B., Steinbeisser, H., and De Robertis, E. M. (1991). Molecular nature of Spemann's organizer: the role of the Xenopus homeobox gene *goosecoid*. Cell *67*, 1111–1120.

Christian, J. L., Olson, D. J., and Moon, R. T. (1992). Xwnt-8 modifies the character of mesoderm induced by FGF in isolated *Xenopus* ectoderm. EMBO J. *11*, 33–41.

Cooke, J. (1981). Scale of body pattern adjusts to available cell number in amphibian embryos. Nature *290*, 775–778.

Cooke, J. (1989). Mesoderm-inducing factors and Spemann's organizer phenomenon in amphibian development. Development *107*, 229–241.

Cooke, J., and Webber, J. (1985). Dynamics of the control of body pattern in the development of *Xenopus laevis* I. Timing and pattern in the development of dorsoanterior and posterior blastomere pairs, isolated at the four-cell stage. J. Embryol. Exp. Morph. *88*, 85–112.

Cooke, J., Smith, J. C., Smith, E. J., and Yaqoob, M. (1987). The organization of mesodermal pattern in *Xenopus laevis*: experiments using a *Xenopus* mesoderm-inducing factor. Development *101*, 893–908.

Dalcq, A., and Pasteels, J. (1937). Une conception nouvelle des bases physiologiques de la morphogénèse. Arch. Biol. *48*, 669–710.

Dale, L., and Slack, J. M. W. (1987). Regional specification within the mesoderm of early embryos of *Xenopus leavis*. Development *100*, 279–295.

Dale, L., Smith, J. C., and Slack, J. M. W. (1985). Mesoderm induction in *Xenopus laevis*: a quantitative study using a cell lineage label and tissue-specific antibodies. J. Embryol. Exp. Morph. *89*, 289–312.

Dale, L., Howes, G., Price, B. M. J., and Smith, J. C. (1992). Bone morphogenetic protein 4: a ventralizing factor in early *Xenopus* development. Development *115*, 573–585.

Dawid, I. B., and Sargent, T. D. (1990). The role of growth factors in embryonic induction in amphibians. Curr. Top. Dev. Biol. *24*, 31–55.

Dirksen, M. L., and Jamrich, M. (1992). A novel, activin-inducible, blastopore lip-specific gene of *Xenopus laevis* contains a fork head DNA-binding domain. Genes Dev. *6*, 599–608.

Ferguson, E. L., and Anderson, K. V. (1992). Localized enhancement and repression of the activity of the TGF-β family member, decapentaplegic, is necessary for dorsal-ventral pattern formation in the *Drosophila* embryo. Development *114*, 583–597.

Fritz A. F., and De Robertis, E. M. (1988). *Xenopus* homeobox-containing cDNAs expressed in early development. Nucl. Acids Res. *16*, 1453–1469.

Green, J. B. A., and Smith, J. C. (1990). Graded changes in dose of a *Xenopus* activin A homologue elicit stepwise transitions in embryonic cell fate. Nature *347*, 391–394.

Green, J. B. A., and Smith, J. C. (1991). Growth factors as morphogens: do gradients and thresholds establish body plan? Trends Genet. *7*, 245–250.

Green, J. B. A., Howes, G., Symes, K., Cooke, J., and Smith, J. C. (1990). The biological effects of XTC-MIF: quantitative comparison with *Xenopus* bFGF. Development *108*, 173–183.

Gurdon, J. B. (1988). A community effect in animal development. Nature *336*, 772–774.

Gurdon, J. B., Brennan, S., Fairman, S., and Mohun, T. J. (1984). Transcription of muscle-specific actin genes in early Xenopus development: nuclear transplantation and cell dissociation. Cell *38*, 691–700.

Harland, R. M. (1991). In situ hybridization: an improved whole-mount method for *Xenopus* embryos. Meth. Cell Biol. *36*, 685–695.

Hausen, P., and Dreyer, C. (1981). The use of polyacrylamide as an embedding medium for immunohistochemical studies of embryonic tissues. Stain Tech. *56*, 287–293.

Jonas, E. A., Snape, A. M., and Sargent, T. D. (1989) Transcriptional regulation of a *Xenopus* embryonic epidermal keratin gene. Development *106*, 399–405.

Jones, C. M., Lyons, K. M., Lapan, P. M., Wright, C. V. E., and Hogan, B. M L. (1992). DVR-4 (bone morphogenetic protein-4) as a posterior ventralizing factor in *Xenopus* mesoderm induction. Development *115*, 639–647.

Keller, R. (1976). Vital dye mapping of the gastrula and neurula of *Xenopus laevis* II. Prospective areas and morphogenetic movements of the deep layer. Dev. Biol. *51*, 118–137.

Kimelman, D., and Kirschner, M. (1987). Synergistic induction of mesoderm by FGF and TGF-β and the identification of an mRNA coding for FGF in the early Xenopus embryo. Cell *51*, 869–877.

Kimelman, D., Abraham, J. A., Haaparanta, T., Palisi, T. M., and Kirschner, M. W. (1988). The presence of fibroblast growth factor in the frog egg: its role as a natural mesoderm inducer. Science *242*, 1053–1056.

Kintner, C. R., and Brockes, J. P. (1984). Monoclonal antibodies iden-

tify blastemal cells derived from differentiating muscle in newt limb regeneration. Nature *308*, 67–69.

Köster, M., Plessow, S., Clement, J. H., Lorenz, A., Tiedemann, H., and Knöchel, W. (1991). Bone morphogenetic protein 4 (BMP-4), a member of the TGF-β family, in early embryos of *Xenopus laevis*: analysis of mesoderm-inducing activity. Mech. Dev. *33*, 191–199.

Mohun, T. J., Garrett, N., Stutz, F., and Spohr, G. (1988). A third striated muscle actin gene is expressed during early development in the amphibian *Xenopus laevis*. J. Mol. Biol. *202*, 67–76.

Nieuwkoop, P. D. (1969). The formation of mesoderm in Urodelean amphibians. I. Induction by the endoderm. Wilhelm Roux's Arch. Entw. Mech. Org. *162*, 341–373.

Nieuwkoop, P. D., and Faber, J., eds. (1967). Normal Table of *Xenopus laevis* (Daudin), 2nd edition, (Amsterdam: North-Holland).

Nüsslein-Volhard, C., and Roth, S. (1989). Axis determination in insect embryos. In CIBA Foundation Symposium 144: Cellular Basis of Morphogenesis, D. Evered and J. Marsh, eds., pp. 37–55.

Rosa, F. M. (1989). *Mix.1*, a homeobox mRNA inducible by mesoderm inducers, is expressed mostly in the presumptive endodermal cells of Xenopus embryos. Cell *57*, 965–974.

Ruiz i Altaba, A., and Melton, D. A. (1989a). Interaction between peptide growth factors and homeobox genes in the establishment of antero-posterior polarity in frog embryos. Nature *341*, 33–38.

Ruiz i Altaba, A., and Melton, D. A. (1989b). Bimodal and graded expression of the *Xenopus* homeobox gene *Xhox3* during embryonic development. Development *106*, 173–183.

Ruiz i Altaba, A., and Jessell, T. (1991). Retinoic acid modifies mesodermal patterning in early *Xenopus* embryos. Genes Dev. *5*, 175–187.

Ruiz i Altaba, A., Choi, T., and Melton, D. A. (1991). Expression of the Xhox3 homeobox protein in *Xenopus* embryos: blocking its early function suggests the requirement of Xhox3 for normal posterior development. Dev. Growth Differ. *33*, 651–669.

Saha, M. S., and Grainger, R. M. (1992). A labile period in the determination of the anterior-posterior axis during early neural development in Xenopus. Neuron *8*, 1003–1014.

Shiurba, R. A., Jing, N., Sakakura, T., and Godsave, S. F. (1991). Nuclear translocation of fibroblast growth factor during *Xenopus* mesoderm induction. Development *113*, 487–493.

Slack, J. M. W., and Isaacs, H. V. (1989). Presence of basic fibroblast growth factor in the early *Xenopus* embryo. Development *105*, 147–153.

Slack, J. M. W., Darlington, B. G., Heath, J. K., and Godsave, S. F. (1987). Mesoderm induction in early *Xenopus* embryos by heparin-binding growth factors. Nature *326*, 197–200.

Smith, J. C. (1989). Mesoderm induction and mesoderm-inducing factors in early amphibian development. Development *105*, 665–677.

Smith, J. C., and Slack, J. M. W. (1983). Dorsalization and neural induction: properties of the organizer in *Xenopus laevis*. J. Embryol. Exp. Morph. *78*, 299–317.

Smith, J. C., and Watt, F. M. (1985). Biochemical specificity of *Xenopus* notochord. Differentiation *29*, 109–115.

Smith, J. C., Price, B. M. J., Van Nimmen, K., and Huylebroeck, D. (1990). Identification of a potent *Xenopus* mesoderm inducing factor as activin A. Nature *345*, 729–731.

Smith, J. C., Price, B. M. J., Green, J. B. A., Weigel, D., and Herrmann, B. G. (1991). Expression of a Xenopus homolog of *Brachyury (T)* is an immediate-early response to mesoderm induction. Cell *67*, 79–87.

Smith, W. C., and Harland, R. M. (1991). Injected Xwnt-8 RNA acts early in Xenopus embryos to promote formation of a vegetal dorsalizing center. Cell *67*, 753–765.

Smith, W. C., and Harland, R. M. (1992). Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. Cell *70*, 829–840.

Sokol, S., and Melton, D. A. (1991). Pre-existent pattern in *Xenopus* animal pole cells revealed by induction with activin. Nature *351*, 409–411.

Sokol, S., Christian, J. L., Moon, R. T., and Melton, D. A. (1991). Injected Wnt RNA induces a complete body axis in Xenopus embryos. Cell *67*, 741–752.

Taira, M., Jamrich, M., Good, P. J., and Dawid, I. B. (1992). The LIM domain-containing homeobox gene *Xlim-1* is expressed specifically in the organizer region of *Xenopus* gastrula embryos. Genes Dev. *6*, 356–366.

Thomsen, G., Woolf, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W., and Melton, D. A. (1990). Activins are expressed early in Xenopus embryogenesis and can induce axial mesoderm and anterior structures. Cell *63*, 485–493.

Vize, P. D., Melton, D. A., Hemmati-Brivanlou, A. and Harland, R. M. (1991). Assays for gene function in developing *Xenopus* embryos. Meth. Cell Biol. *36*, 367–387.

Whitman, M., and Melton, D. A. (1989). Growth factors in early embryogenesis. Annu. Rev. Cell Biol. *5*, 93–117.

Wolpert, L. (1969). Positional information and the spatial pattern of cellular differentiation. J. Theor. Biol. *25*, 1–47.

Wright, C. V. E., Morita, E. A., Wilkin, D. J., and De Robertis, E. M. (1990). The *Xenopus* XlHbox 6 homeo protein, a marker of posterior neural induction, is expressed in proliferating neurons. Development *109*, 225–234.