Development 118, 1335-1342 (1993)
Printed in Great Britain © The Company of Biologists Limited 1993

Mesoderm formation in *Xenopus* ectodermal explants overexpressing Xwnt8: evidence for a cooperating signal reaching the animal pole by gastrulation

S. Y. Sokol

Department of Microbiology and Molecular Genetics, Harvard Medical School and Molecular Medicine Unit, Beth Israel Hospital, 330 Brookline Avenue, Boston MA 02215, USA

SUMMARY

It is demonstrated here that the ability of injected Xwnt8 RNA to trigger mesoderm formation in *Xenopus* presumptive ectoderm (animal caps) depends on the time of explantation. Animal caps isolated from Xwnt8 injected embryos at the late blastula/early gastrula stages differentiate mesodermal tissues whereas caps isolated from early blastula do not. This finding suggests that an endogenous signal reaches the animal cap by the late blastula stage and cooperates with Xwnt8 to induce mesoderm. Similarly, late animal caps isolated at st. 10 from lithium-treated embryos, but not those from control embryos, elongate and express muscle-specific actin transcripts. In addition, the data presented suggests that the cooperating signal is distributed homogeneously with respect to the future dorsoventral axis and may require FGF- and activin-dependent signal transduction pathways. These observations support a model in which mesoderm is induced in vivo by a combined action of several different signals.

Key words: mesoderm induction, *Wnt*, *Xenopus*, growth factors

INTRODUCTION

Induction of mesoderm is thought to be the first step in a cascade of inductive interactions that occur during early vertebrate development. At least two different types of factors may participate in dorsal mesoderm formation during *Xenopus* embryogenesis. One factor, a general mesoderm inducer (Sokol and Melton, 1992; Moon and Christian, 1992), is present in vegetal cells and triggers mesoderm formation in animal/vegetal recombinants (Niewkoop, 1969). Activin and FGF-related growth factors have been shown to mimic the endogenous inducing activity by triggering mesodermal differentiation in ectodermal explants *in vitro* (see Smith, 1989; Melton, 1991, for reviews). The presence of another factor in the embryo has been suggested by the observations that dorsal and ventral animal caps respond to mesoderm inducers differently (Sokol and Melton, 1991; Ruiz i Altaba and Jessell, 1991). This factor (the dorsal modifier) is normally active in dorsal blastomeres (Gimlich and Gerhart, 1984; Gallager et al., 1991; see Elinson and Kao, 1989, for review) and can be mimicked by Wnts, LiCl and noggin, which alter the cell response to general mesoderm inducers (Slack et al., 1988; Christian et al., 1992; Sokol and Melton, 1992; Smith and Harland, 1992).

Injection of Wnt1 and Xwnt8 mRNAs into fertilized eggs has been shown to trigger muscle-specific actin RNA expression in isolated animal caps (Sokol et al., 1991). Other reports showed that Wnt-containing animal caps differentiated only 'ventral' mesodermal tissues including mesothelium and mesenchyme, but not muscle (Chakrabarti et al., 1992) or did not form any mesodermal structures at all (Christian et al., 1991; Christian et al., 1992).

In the present study, conditions required for the inductive response of animal cap cells to the injected Xwnt8 mRNA are investigated. I find that the time at which animal caps are isolated from the injected embryo is a critical parameter in determining the inductive response, such that mesoderm forms in animal caps isolated at stage 9 or 10 but not in those isolated at stage 8. This observation suggests that mesoderm is induced in such explants as a result of a combined action of the overexpressed Xwnt8 and an endogenous cooperating signal which emanates from the vegetal hemisphere or marginal zone and reaches animal cap cells by the late blastula stage.

Injections of Xwnt8 mRNA are used to study further the properties of endogenous cooperating signal. Additional data presented here indicate that the endogenous signal may be distributed homogeneously along the future dorsoventral axis but not along the animal-vegetal axis. The cooperating signal seems to require both FGF- and activin-dependent signal transduction systems. These observations are discussed with respect to the available molecular and embryological data on the formation of dorsal mesoderm in *Xenopus*.

MATERIALS AND METHODS

Eggs and embryos

Eggs were obtained from female *Xenopus laevis* by injecting them with 800 units of human chorionic gonadotropin. Fertilization and embryo culture was done as described (Newport and Kirschner, 1982). Staging was according to Nieuwkoop and Faber (1967). More specifically, stage 8 starts 5 hours after fertilization and corresponds to 11 cell divisions (at 22-24°C). Stage 10 is characterized by the appearance of a dorsal lip of a blastopore (9 hours after fertilization; Nieuwkoop and Faber, 1967).

In vitro transcription and microinjection of embryos with mRNAs

Capped synthetic RNAs were generated as described (Krieg and Melton, 1984) by in vitro transcription of plasmids, containing full coding sequence of Xwnt8 (Christian et al., 1991) or modified forms of FGF receptor cDNA (Amaya et al., 1991). XFD/Xss, dominant negative mutant form of FGF receptor, and d50/Xss inactive control containing a short deletion were kindly provided by E. Amaya (UCSF) (Amaya et al., 1991). Full-length (XAR1) and truncated (Δ1XAR1) activin receptor constructs were obtained from A. Hemmati-Brivanlou (Hemmati-Brivanlou and Melton, 1992). Embryos, incubated in 3% Ficoll, 1× MMR (100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM Hepes, (pH 7.6), 0.1 mM EDTA; Newport and Kirschner, 1982) were injected with 5 nl of mRNA solution in distilled water at the 16-32 cell stage into a ventrovegetal blastomere or with 10 nl into an animal pole of a fertilized egg before the first cleavage. After 2 hours of incubation the medium was changed to 0.1× MMR with 50 μg/ml of gentamicin for long-term culture. Death rate for the injected embryos was usually below 5%.

Histology

After 2 days of culturing in 0.1× MMR, embryos were fixed in MEMFA (0.1 M Mops, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$ and 3.7% formaldehyde) for 1-2 hours (Hemmati-Brivanlou and Harland, 1989), dehydrated, embedded in Paraplast, and 7 μm sections were cut on a rotary microtome.

Explant culture, RNA isolation and northern blot analysis

Animal caps were cut out from animal hemispheres of the injected embryos and cultured in 0.5× MMR solution as described (Fig. 2C; Sokol et al., 1990). When inductive responses of large and small caps were compared (Table 2), mean radial size of 'large' animal caps was 212 μm (s.e.m.=26 μm, n=14) if measured after explants heal and acquire a spherical shape. 'Small' animal caps were 96 μm in radius (s.e.m.=12 μm, n=13). Explants isolated at different stages of development were of approximately the same size. Though cells in a late blastula explant may represent a subpopulation of cells present in the earlier explanted animal caps due to epiboly, this does not affect my conclusions. Explants were scored positive in the elongation assay if their length:width ratio exceeded 1.5 after 8 hour culture in 0.5× MMR solution. This assay is also based on external morphology; the induced explants acquire a characteristic pear-like shape.

The prospective dorsal and ventral sides were determined by pigmentation differences in the early embryo (Nieuwkoop and Faber, 1967). Prospective ventral blastomeres are more heavily pigmented than their dorsal counterparts. The accuracy of this determination was tested in each experiment by leaving a group of control embryos to develop, making sure that the pigmentation differences correctly predicted the position of dorsal blastopore lip. The usual error of such determination was 5-7%.

After explant culturing at room temperature, RNA was extracted with proteinase K/SDS buffer and analyzed by northern blot as previously described (Sokol et al., 1990). Radioactively labeled antisense RNA probes specific to *Xenopus* cardiac actin, fibronectin and EF1α were prepared by transcribing the corresponding templates (Dworkin-Rastl et al., 1986; Krieg et al., 1989; Yisraeli et al., 1990) with SP6 RNA polymerase. The anti-Xbra probe (Smith et al., 1991) was prepared from a plasmid kindly provided by P. Wilson.

RESULTS

Response of animal caps to Xwnt8 strictly depends on the time of explantation

The conditions required for the response of animal cap cells to Wnts were further studied by injecting 5-10 pg of Xwnt8 mRNA into the animal pole of a fertilized egg. Animal caps were isolated from injected or from uninjected embryos and cultured in 0.5× MMR solution.

When animal caps are explanted from different stages a marked difference in the ability to form mesoderm is revealed by the morphogenetic movements (elongation) of explants (Symes and Smith, 1987) and by the production of muscle-specific actin mRNA (Fig. 1; Mohun et al., 1984). Elongation of animal caps is a reliable early response marker that correlates tightly with mesoderm induction (Symes and Smith, 1987). No elongation or muscle-specific actin mRNA expression is observed in animal caps from injected embryos isolated at the early or mid blastula stages (stages 7 and 8). Morphologically these explants are not distinguishable from the control explants isolated from uninjected embryos. Even after prolonged culture for an additional 48 hours, vesicular structures characteristic of FGF-like 'ventral type' inductions do not form in these explants. In contrast, Xwnt8-containing animal caps isolated at the late blastula (st. 9) and early gastrula (st. 10) stages elongate and differentiate mesodermal structures including muscle, notochord, mesothelium and mesenchyme (Table 1). Note that control uninjected animal caps explanted at either early or late stages do not form mesodermal tissues.

Stage 8 animal caps do not elongate in response to any dose of the injected Xwnt8 mRNA ranging from 0.4 to 3000 pg per embryo (only two out of 80 explants tested elongated slightly, even after 48 hours in culture). In contrast, stage 10 animal caps respond to Xwnt8 mRNA injections specifically and in a dose dependent manner. While doses of 0.4-4 pg of the mRNA caused slight elongation of the late animal caps, 40-400 pg of the RNA led to a pronounced morphogenetic response (Fig. 2B). Similar doses of mouse Wnt4 mRNA (a gift from A. McMahon) failed to cause mesoderm induction in stage 10 animal caps (data not shown). The response of st. 10 animal caps does not depend on the time at which Xwnt8 mRNA was injected, varying from 30 minutes to 3 hours after fertilization (data not shown) or on the size of the explants: both small and large animal caps show vigorous elongation (Table 2). All experiments described below were performed on embryos that had been injected with about 10 pg of the mRNA each.

These experiments indicate that the response of animal cap explants to the injected Xwnt8 mRNA depends on the time of explantation. This result suggests that Xwnt8 injections reveal an endogenous inducing signal that spreads in

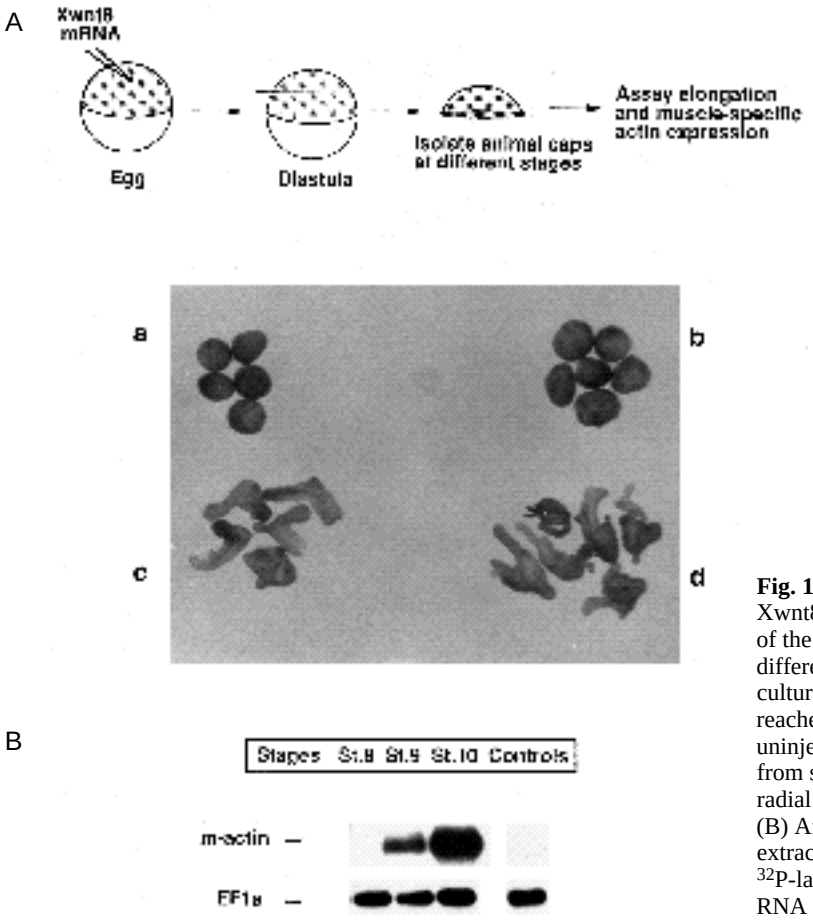

Fig. 1. (A) Fertilized eggs were injected with 5 pg of Xwnt8 RNA. Animal caps of equivalent size (about 1/5th of the embryo) were isolated from the injected embryos at different times (stage 8 (b), stage 9 (c) and stage10 (d)) and cultured in 0.5× MMR solution untill the control embryos reached stage 14. Control animal caps were obtained from uninjected st. 10 embryos (a). Control animal caps isolated from stages 9 and 8 look identical to those in 'a'. Average radial size of control animal caps is about 200 μm.
(B) After 2 days, RNA from the cultured explants was extracted and probed on northern blots with a mixture of ^{32}P-labeled EF-1α and muscle actin anti-sense RNAs. RNA from 5 animal cap equivalents is in each lane.

Table 1. Injected Xwnt8 mRNA causes mesoderm formation in ectoderm explants isolated at stage 10, but not at stage 8

Stage of explantation	Number of explants sectioned	Muscle	Notochord	Neural tissue	Eyes	Mesenchyme or Mesothelium
8	14	1 (7%)	0	0	0	2 (15%)
10	22	20 (90%)	5 (23%)	7 (32%)	0	9 (41%)

Fertilized eggs were injected in the animal pole with 10 pg of Xwnt8 mRNA. Animal caps were explanted from injected embryos at stages 8 and 10 and cultured in isolation. After 2 days explants were fixed in MEMFA, dehydrated, embedded in Paraplast and sectioned at 7 μm. Control explants (n = 20 for each stage) isolated from uninjected embryos did not differentiate any mesodermal structures. Less than 10% of them contained a small number of loose mesenchymal cells (not shown).

the late blastula animal caps, which is well beyond the normal area for presumptive mesoderm (Fig. 2A).

Xwnt8 is required in animal cap cells for the most efficient response

Xwnt8 mRNA was reported to be most effective in inducing a dorsal axis when injected in the vegetal or marginal cells (Sokol et al., 1991; Smith and Harland, 1991). To test if Xwnt8 has to be present in the vegetal cells for the efficient inductive response of animal caps, different blastomeres of the 32-cell embryo were injected with 10 pg of Xwnt8 mRNA and the response of animal caps isolated from these embryos at stage 10 was measured by elongation. The effect of Xwnt8 mRNA is most pronounced when it is injected in the animal blastomeres of the 32-cell embryo (Table 2a). Injection into the tier 3 or tier 4 vegetal cells results in only slight elongation of explants and formation of vesicular structures characteristic of 'ventral' type of inductive response after culturing for an additional 48 hours. The conclusion from this experiment is that Xwnt8 gene product is required in the animal cap cells for the efficient inductive response. Recent findings that Xwnt8 mRNA must be present in animal cap cells in order to cooperate efficiently with FGF in animal cap induction (Christian et al., 1992) suggest that mesoderm induction in Xwnt8-containing late animal caps may also be a result of cooperation of Xwnt8 with an endogenous inducing agent in a manner similar to cooperation of Xwnt8 with FGF.

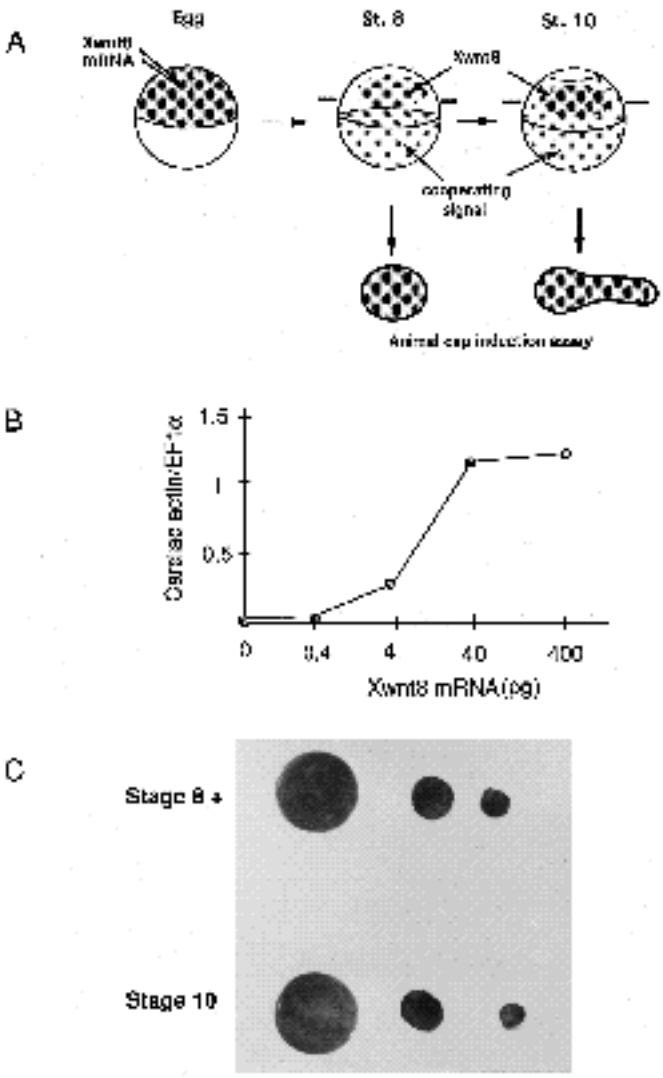

Fig. 2. (A) A model for the cooperation of injected Xwnt8 and an endogenous inducing signal in the formation of mesoderm in animal caps. A vegetally derived mesoderm inducing signal gradually spreads in the animal pole cells of the late blastulae. Overexpressed Xwnt8 product cooperates with the endogeneous inducer to induce mesoderm in animal caps isolated at stage 10 but not at stage 8. (B) Response of animal caps to the injected Xwnt8 is dose-dependent. Animal caps from the embryos injected with different doses of Xwnt8 mRNA were explanted at st. 10, cultured for 30 hours, and their mRNA was isolated for northern analysis. The response is plotted as a ratio of cardiac actin signal to EF1α signal (control for loading). (C) Relative size of large and small animal caps isolated at stages 8 and 10 and used in these experiments as compared to the size of the embryo. This picture has been taken before the explants healed into tight spheres which are usually of a smaller size.

A signal cooperating with Xwnt8 in mesoderm formation gradually reaches the animal pole by early gastrula stage

To test whether all animal pole cells or a certain group of cells respond to Xwnt8 mRNA, 5 pg of Xwnt mRNA were injected into the animal pole of fertilized eggs, and animal caps of different size (Fig. 2C) were isolated from three different stages (st. 8, 9 and 10). Elongation was scored as an early marker for inductive response.

Neither large nor small animal caps elongate in response to the injected Xwnt8 mRNA when isolated at st. 8 (Table 2b). In contrast, only large animal caps isolated from the stage 9 embryos injected with Xwnt8 mRNA show a pronounced elongation. Explantation at stage 10 results in the elongation of both large and small animal caps (Table 2b). The elongated explants express high levels of muscle-specific cardiac actin mRNA (not shown). Control animal caps isolated at each stage from uninjected embryos do not show any evidence for mesoderm induction. This experiment suggests that there is a cooperating inducing signal (produced by the vegetal hemisphere or by the marginal zone) and that this signal reaches the animal cap by st. 9 and further spreads towards the animal pole by the beginning of gastrulation (Fig. 2A).

The endogenous inducing signal is present in both the presumptive ventral and dorsal ectoderm

It was previously reported that dorsal and ventral ectoderm respond differently to activin and FGF (Sokol and Melton, 1991; Ruiz i Altaba and Jessell, 1991; Kimelman and Maas, 1992). To determine if there is also a dorsoventral difference in response to Xwnt8, Xwnt8 mRNA was injected into the animal pole of a fertilized egg, the animal caps were isolated at stage 10 and separated into presumptive dorsal and ventral halves. The inductive responses of the half-caps are compared in Table 3.

Both dorsal and ventral ectoderm respond to Xwnt8 in an identical manner. The frequencies and the degree of elongation and the spectra of induced tissues are indistinguishable (Table 3). The amounts of induced cardiac actin mRNA are also very similar (not shown). The two-fold difference in the dose of the endogenous signal would have been readily detected because the frequencies of notochord (Table 3) and the amount of the induced muscle are quite low and can be readily increased in the explants by addition of activin (Sokol and Melton, 1992). This result implies that there is no dorsoventral polarity in the response of animal cap cells to Xwnt8 and that the endogenous cooperating signal may be distributed homogeneously in the embryo.

Truncated forms of FGF receptor and activin receptor inhibit the response of animal caps to Xwnt8 mRNA

Experiments using truncated FGF and activin receptors suggest that FGF and activin may be necessary for different aspects of mesoderm induction in *Xenopus* (Amaya et al., 1991; Hemmati-Brivanlou and Melton, 1992). These modified receptors may act as dominant negative mutants and can be used to study the molecular nature of the spreading signal revealed by Xwnt8 mRNA injections. For example, if the cooperating activity is related to FGF, then dominant negative mutant FGF receptor, but not the truncated activin receptor, should block mesoderm induction in animal caps injected with Xwnt8 mRNA and isolated at stage 10.

Table 2. Animal cap elongation in response to Xwnt8 depends on the place of injection, size of the explant and time of explantation

Type of explant	(a) Site of injection (explanted at stage 10)				(b) Time of explantation		
	Animal	Marginal	Vegetal	Uninjected	Stage 8	Stage 9	Stage 10
Large	18/18	3/19	2/20	2/34	2/37	31/36	38/39
Small	–	–	–	0/15	0/18	3/20	16/18

Fertilized eggs were allowed to develop to the 16-32 cell stage at which time they were injected in an animal, marginal or vegetal blastomere with 5 pg of Xwnt8 mRNA (a). Alternatively, 5 pg of Xwnt8 mRNA was injected in the animal pole of a fertilized egg (b). Large and small animal caps (about 1/5 and 1/10th of the embryonic size, correspondingly) were isolated from injected embryos and from uninjected controls and cultured in 0.5× MMR for 6-8 hrs. Results are expressed as number of elongated explants/total number of explants. Combined data from three different experiments are presented. Note that the majority of Xwnt8 mRNA injected explants in (a) cultured for an additional 48 hours formed vesicular structures characteristic of weak inductive response. Vesicular structures formed in less than 5% of uninjected explants (a) and in explants isolated at stage 8 (b) (not shown).

Table 3. Presumptive dorsal and ventral ectoderm respond to Xwnt8 similarly

Type of explant	Xwnt8 RNA injected	Number of explants	Explants elongated	Notochord	Muscle	Neural tissue	Mesenchyme or Mesothelium
D	–	18	0	0	0	0	0
V	–	19	0	0	0	0	2 (10%)
D	+	21	18 (86%)	5 (24%)	21 (100%)	10 (47%)	5 (24%)
V	+	18	17 (94%)	6 (33%)	16 (89%)	8 (44%)	4 (22%)

Fertilized eggs were injected with 10 pg of Xwnt8 mRNA in a volume of 10 nl. At stage 10 dorsal (D) and ventral (V) animal cap explants were isolated from injected embryos and from uninjected controls and cultured in 0.5× MMR solution. After 2 days explants were fixed in MEMFA, dehydrated, embedded in Paraplast and sectioned at 7 μm. Note that frequencies of notochord are well below those observed for animal caps treated with activin (Sokol et al. 1990).

Embryos were coinjected with 10 pg of Xwnt8 mRNA and with 2 ng of mRNA coding for either a dominant negative mutant of the FGF receptor (XFD mRNA; Amaya et al., 1991) or a truncated activin receptor (Δ1XAR1 RNA; Hemmati-Brivanlou and Melton, 1992). Inactive FGF receptor RNA and mRNA encoding full-length activin receptor served as controls (Table 4, Fig. 3). Animal caps were isolated at stage 10 and cultured for 7 hours at room temperature in 0.5× MMR solution. Results were assessed morphologically and with molecular markers (Table 4, Fig. 3). The majority of the explants that received Xwnt8 mRNA alone or together with d50 mRNA or XAR1 mRNA elongated dramatically, while the morphogenetic response to Xwnt8 was strongly inhibited by both the dominant negative FGF receptor and by the truncated activin receptor mRNAs (Fig. 3A, Table 4). The inhibition was not complete, because the explants cultured for an additional 48 hours often developed vesicular structures characteristic of weak mesoderm inductive response. This incomplete effect may be due to the heterogeneous distribution of microinjected mRNAs (Sokol et al., 1991), or to the possible degradation of the dominant negative receptor mRNA and protein products later during gastrulation. No inductive response has been observed in animal explants that received truncated activin or FGF receptor mRNAs or control inactive FGF receptor mRNA without Xwnt8 mRNA.

In accordance with these observations, the expression of an early mesodermal marker, Xbra (Smith et al., 1991), and of muscle-specific cardiac actin mRNA in the stage 10 animal caps injected with Xwnt8 is blocked by either the truncated FGF receptor mRNA or by the truncated activin receptor mRNA (Fig. 3B). These observations suggest that both FGF and activin functions may be required for cells to respond to Xwnt8.

Lithium causes mesoderm to form in animal caps isolated from stage 10 embryos

The ability of both Wnts and lithium chloride to dorsalize mesoderm inductive response (Cooke et al., 1989; Christian et al., 1992; Kao and Elinson, 1989; Slack et al., 1988) suggests that LiCl may also cooperate with the endogenous signal in the animal caps. Since lithium chloride has not been previously reported to induce mesoderm in *Xenopus* animal caps on its own, this result would further support the idea that both Xwnt8 and lithium reveal the presence of endogenous signaling factors in animal cap by cooperating with them in mesoderm induction.

To test the possibility that late (stage 10-10.5) animal caps isolated from Li-treated embryos may be able to form mesoderm they were explanted and cultured in isolation. An inductive response (assayed by both elongation and muscle-specific actin mRNA expression) is observed for animal caps isolated from Li-treated embryos but not from untreated control embryos (Fig. 4). Among animal caps isolated from the Li-treated embryos at stages (7, 8, 9 and 10+), only explants isolated after stage 10 formed mesoderm (Fig. 4B and unpublished observations).

DISCUSSION

Here it is shown that Xwnt8 mRNA injected in an animal pole of an egg causes formation of mesoderm in animal caps explanted from the late blastula/early gastrula embryos, but not from the early or midblastula embryos. I propose that

Table 4. Truncated FGF and activin receptors inhibit cell response to Xwnt8 mRNA

	No coinjection		d50 mRNA		XFD mRNA		XAR1 mRNA		Δ1XAR1 mRNA	
Xwnt6 RNA injected	−	+	−	+	−	+	−	+	−	+
Total number of explants	74	82	38	87	34	85	31	38	42	63
Explants elongated (%)	2 (3%)	78 (95%)	1 (2%)	76 (87%)	0	11 (13%)	8 (26%)	37 (97%)	1 (2%)	5 (8%)

Fertilized eggs were coinjected in the animal pole with 10 pg of Xwnt8 mRNA and 2 ng of the following mRNAs: XFD or d50 mRNAs coding for dominant negative mutant and inactive control forms of FGF receptor, respectively; and XAR1 or Δ1XAR1 mRNAs coding for the full-length and truncated activin receptors. At stage 10, animal cap explants were isolated from injected embryos and cultured. After 7 hours, explant morphology was examined and explant elongation scored. Results from four independent experiments are presented. Note that XAR1 mRNA injection led to animal cap elongation even in the absence of Xwnt8. The majority of explants injected with Xwnt8 formed vesicular structures characteristic of the weak inductive response after 48 hrs in culture (not shown).

the induction of mesoderm with Xwnt8 depends on the presence of an endogenous inducing signal, which gradually spreads in the animal cap during blastula stages. This signal may be too weak to induce mesoderm in the animal cap region on its own, but it is revealed by Xwnt8 mRNA injection or by treatment with lithium chloride. This explanation is supported by the observation that treatment of Xwnt8-containing animal caps with a dose of activin, which is too low to affect cell fates on its own, results in their mesodermal differentiation (Sokol and Melton, 1992). Thus, modifiers may act by lowering a threshold of cell responsiveness to a general mesoderm inducer (activin or FGF), so that the same effect is achieved with a lower dose of the inducer when the inducer acts in combination with Wnt or lithium chloride (Christian et al., 1992; Sokol and Melton, 1992).

These data demonstrate that the stage at which animal caps are isolated is the critical variable for mesoderm induction with Xwnt8. It was suggested earlier, that differences in the explant size may explain variability in mesoderm induction assays (Christian et al., 1992). In this study neither large nor small animal caps isolated at stage 8 from the Xwnt8 mRNA injected embryos form mesoderm (Table 2). The explant size, however, clearly is important for the response of animal caps isolated at stage 9 (Table 2), in agreement with the existence of a spreading cooperating signal. It is likely that inconsistencies in the previous reports with regard to the ability of Wnts to cause mesoderm induction in ectoderm explants (Sokol et al., 1991; Christian et al., 1991; Chakrabarti et al., 1992) may be connected with differences in the time of explantation. Thus, animal cap assays should be interpreted with caution considering that the animal cap size may vary between different laboratories and that the explants (both small and large) do receive low levels of inducing factors before gastrulation starts.

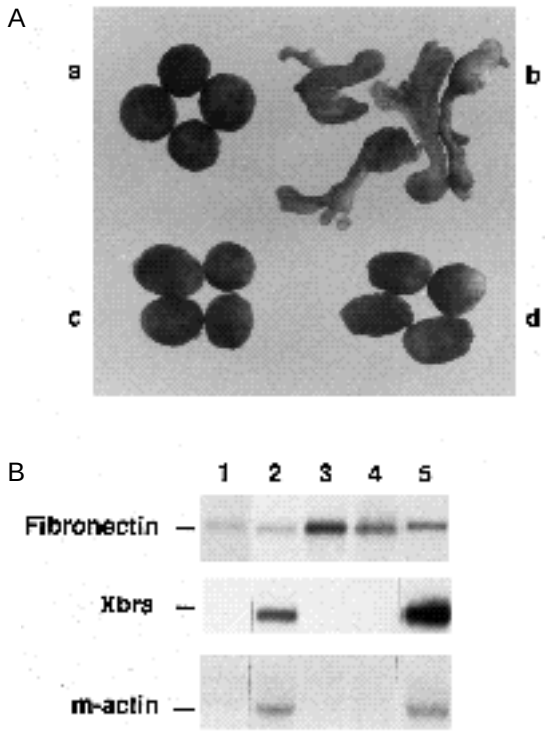

Fig. 3. Cooperating activity requires FGF and activin signalling pathways. (A) Animal caps were explanted from uninjected embryos (a), and from embryos coinjected with Xwnt8 mRNA and either d50 control mRNA (b), XFD mRNA (c) or Δ1XAR1 mRNA (d). Morphology of the explants cultured for 6 hours is shown. (B) Brachyury and muscle-specific actin gene expression is blocked in the animal caps coinjected with Xwnt8 and truncated FGF and activin receptors. Total RNA was extracted from uninjected control caps (1), animal caps injected with Xwnt8 mRNA and either d50 mRNA (2), or XFD mRNA (3) or Δ1XAR1 mRNA (4) and hybridized on northern blots with anti-sense Xbra (early marker for mesoderm), fibronectin (control for loading) and cardiac (muscle-specific) actin probes. Ten animal cap equivalents of total RNA were loaded in each lane. Lane 5 contains total RNA from a normal embryo at the corresponding stage. Xbra and fibronectin gene expression was analyzed when the control embryos reached stage 12. Cardiac actin was assayed when the controls were at stage 22. Faint cross-hybridization of the m-actin probe to cytoplasmic actin reflects equal RNA loading. Note that injections of Xwnt8 mRNA alone gave a response indistinguishable morphologically or molecularly from the response of animal caps to Xwnt8 coinjected with the control d50 mRNA (not shown, but see Fig.1, Table 4).

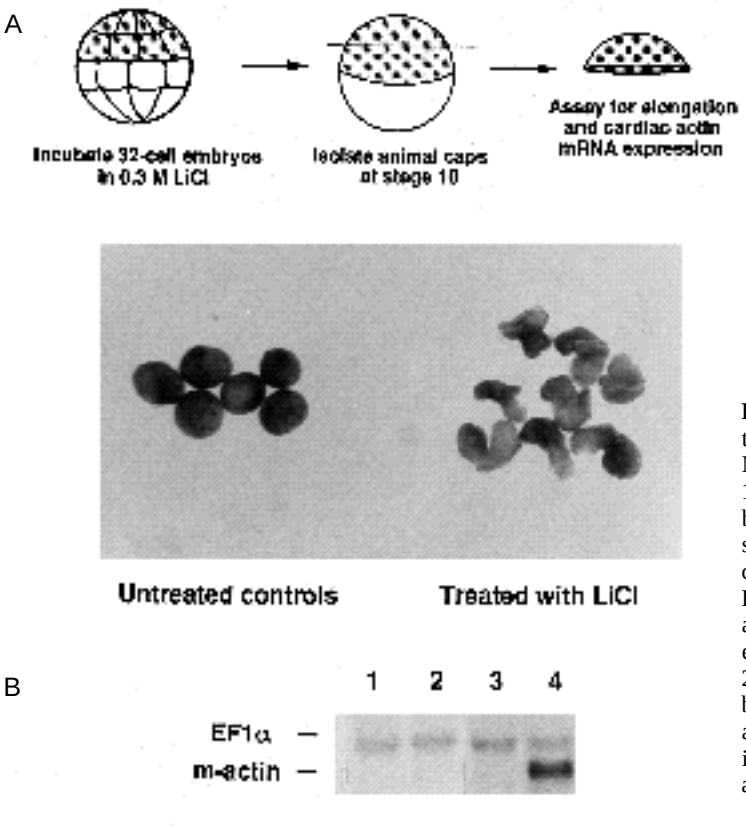

Fig. 4. LiCl treatment may lead to mesoderm formation in the animal caps. Developing embryos were treated with 0.3 M LiCl at the 32 cell stage for 5-10 minutes (Kao et al., 1986), washed with 0.1× MMR and left to develop until the beginning of gastrulation. Animal caps were explanted at stage 10 from treated and from untreated embryos and cultured in isolation. (A) Morphology of the explants. Radial size of healed control explants is 200 μm on average. (B) After 48 hours in culture, total RNA was extracted from animal caps isolated at stage 8 (lanes 1 and 2) or at stage 10+ (lanes 3 and 4) and probed on northern blots with a mixture of ^{32}P-labeled EF1α, and cardiac actin anti-sense RNAs. RNA from four animal caps was loaded in each lane. 1 and 3, animal caps from normal embryos; 2 and 4, animal caps from LiCl-treated embryos.

The ability of lithium chloride to trigger mesoderm formation in late animal caps supports the classification of the lithium ion as a dorsal modifier (Sokol and Melton, 1992) and may be relevant to the previously reported mesoderm-inducing effect of lithium on *Triturus* gastrula ectoderm (Masui, 1961). This result further points at the similarity of lithium chloride and several Wnt mRNA effects (see Christian et al., 1992; Sokol and Melton, 1992). Both agents are reported to induce a second dorsal axis (Kao et al., 1986; Sokol et al., 1991), to rescue UV-treated dorsal axis-deficient embryos (Kao et al., 1986; Sokol et al., 1991; Smith and Harland, 1991) and to cooperate with activin and FGF in mesoderm induction in animal caps (Slack et al., 1988; Kao and Elinson, 1989; Cooke et al., 1989; Christian et al., 1992; Sokol and Melton, 1992). Lithium ions are known to interfere with phosphoinositol-mediated signal transduction pathway (Maslanski et al., 1992), but the events underlying Wnt signaling remain obscure. It is likely that both Xwnt8 and lithium may affect signal transduction pathway utilized by the endogenous dorsal modifier during normal development (Christian et al., 1992).

The endogenous cooperating signal seems to be distributed homogeneously with respect to the future dorsoventral axis as indicated by the equal capacity of presumptive dorsal and ventral ectoderm to respond to Xwnt8 mRNA injections. This is additional evidence that the endogenous signal revealed in these experiments is different from the dorsally localized modifier responsible for the establishment of the dorsoventral polarity in mesoderm (Sokol and Melton, 1991). The endogenous cooperating signal may be the same as the vegetal inducing signal (Nieuwkoop, 1969). While Nieuwkoop has shown that vegetal blastomeres produce a signal that can restore mesoderm formation in vitro (in the animal/vegetal recombinates), these experiments demonstrate that the cooperating signal does spread throughout the embryo in vivo and that it can be revealed later in the animal cap induction assay. My finding that this signal spreads from vegetal hemisphere towards animal cap may be related to the vegetal-marginal succession of dorsal organizing centers observed throughout blastula stages (Gerhart et al., 1991).

The observation that both truncated FGF and activin receptors inhibit the response of animal caps to Xwnt8 means that both growth factors may be involved in the formation of mesoderm in Xwnt8-containing animal caps. Mesoderm induction studies with a transfilter experimental system suggest that neither activin nor bFGF directly represent the vegetal inducing signal (Slack, 1991). Additional experiments, however, are needed to determine whether activin or FGF correspond to the spreading signal revealed by Xwnt8 or whether these growth factors may be secreted after cells receive the initial inductive stimulus.

In conclusion, mesoderm induction may consist of two steps: establishment of predisposition in the responding tissue (due to a localized modifier action) and induction of mesodermal fates by a general mesoderm inducing agent (Sokol and Melton, 1991, 1992; Christian et al., 1992). This view includes a single mesoderm inducing signal and a modifying signal, which does not induce mesoderm on its own, and is different from the alternative model for mesoderm induction postulating the existence of two different vegetal inducers (dorsal and ventral mesoderm inducers; Smith et al., 1985).

I am grateful to P. Klein, C. Jennings, M. Ku, P. Vize, L. Bally-

Cuif, R. Elinson, G. Thomsen and P. Wilson for helpful discussions of the manuscript. I thank P. Wilson for the Xbra probe, E. Amaya for cDNA plasmids encoding mutant FGF receptors and A. Hemmati-Brivanlou for activin receptor cDNA clones. I especially acknowledge experimental help and critical advice of D. Melton during early stages of this work. This work was supported by a NIH grant to D. Melton and by the Beth Israel Hospital.

REFERENCES

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.

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

Christian, J. L., McMahon, J. A., McMahon, A. P. and Moon, R. T. (1991). Xwnt8, a *Xenopus* Wnt1/int-1-related gene responsive to mesoderm inducing growth factors, may play a role in ventral mesodermal patterning during embryogenesis. *Development* **111**, 1045-1055.

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

Cooke, J., Symes, K. and Smith, E. J. (1989). Potentiation by the lithium ion of morphogenetic responses to a Xenopus inducing factor. *Development* **105**, 549-558.

Dworkin-Rastl, E., Kelly, D. B. and Dworkin, M. B. (1986). Localisation of specific mRNA sequences in Xenopus laevis by in situ hybridisation. *J. Embryol exp. Morph.* **91**, 153-168.

Elinson, R. P. and Kao, K. R. (1989). The location of dorsal information in frog early development. *Dev. Growth Differ.* **31**, 423-430.

Gallagher, B. C., Hainski, A. M. and Moody, S. A. (1991). Autonomous differentiation of dorsal axial structures from an animal cap cleavage stage blastomere in *Xenopus*. *Development* **112**, 1103-1114.

Gerhart, J., Doniah, T. and Stewart, R. (1991). Organizing the *Xenopus* organizer. In *Gastrulation. Movements, Patterns and Molecules* (ed. by R. Keller, W. H. Clark, Jr. and F. Griffin) pp. 57-79. New York: Plenum Press.

Gimlich, R. L. and Gerhart, J. C (1984). Early cellular interactions promote embryonic axis formation in Xenopus laevis. *Dev. Biol*. **104**, 117-130.

Hemmati-Brivanlou, A. and Harland, R. M. (1989). Expression of an engrailed-related protein is induced in the anterior neural ectoderm of early *Xenopus* embryos. *Development* **106**, 611-617.

Hemmati-Brivanlou, A. and Melton, D. A. (1992). A truncated activin receptor inhibits mesoderm induction and formation of axial structures in *Xenopus* embryos. *Nature* **359**, 609-614.

Kao, K. R., Masui, Y. and Elinson, R. P. (1986). Lithium-induced respecification of pattern in Xenopus laevis embryos. *Nature* **322**, 371-373.

Kao, K. R. and Elinson, R. P. (1989). Dorsalization of mesoderm induction by lithium. *Dev. Biol.* **132**, 81-90.

Kimelman, D. and Maas, A. (1992). Induction of dorsal and ventral mesoderm by ectopically expressed *Xenopus* basic fibroblast growth factor. *Development* **114**, 261-269.

Krieg, P. A., Varnum, S. M., Wormington, W. M. and Melton, D. (1989). The mRNA encoding elongation factor 1α (EF-1α) is a major transcript at the midblastula transition in Xenopus. *Dev. Biol.* **133**, 93-100.

Krieg, P. A. and Melton, D. A. (1984). Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. *Nucl. Acids Res.* **12**, 7057-7070.

Maslanski, J. A., Leshko, L. and Busa, W. B. (1992). Lithium-sensitive production of inositol phosphates during amphibian embryonic mesoderm induction. *Science* **256**, 243-245.

Masui, Y. (1961). Mesodermal and endodermal differentiation of the presumptive ectooderm of Triturus gastrula through influence of lithium ion. *Experientia* **17**, 458-459.

Melton, D. A. (1991). Pattern formation during animal development. *Science* **252**, 234-241.

Mohun, T. J., Brennan, S., Dathan, N., Fairman, S. and Gurdon, J. B. (1984). Cell type specific activation of actin genes in the early amphibian embryo. *Nature* **311**, 715-721.

Moon, R. T. and Christian, J. L. (1992). Competence modifiers synergize with growth factors during mesoderm induction and patterning in *Xenopus. Cell* **71**, 709-712.

Newport, J. and Kirschner, M. (1982). A major developmental transition in early Xenopus embryos. *Cell* **30**, 687-703.

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

Nieuwkoop, P. D. and Faber, J. (1967). Normal table of Xenopus laevis (Daudin). Amsterdam: North-Holland.

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

Slack, J. M. W., Isaacs, H. V. and Darlington, B. G. (1988). Inductive effects of fibroblast growth factor and lithium ion on *Xenopus* ectoderm. *Development* **103**, 581-590.

Slack, J. M. W. (1991). The nature of the mesoderm inducing signal in *Xenopus*: a transfilter study. *Development* **113**, 661-669.

Smith, J. C., Dale, L. and Slack, J. M. W. (1985). Cell lineage of labels and region-specific markers in the analysis of inductive interactions. *Development* **89Supplement,** 317-331.

Smith, J. C. (1989). Mesoderm induction and mesoderm-inducing factors in early amphibian development. *Development* **105**, 665-678.

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 Xwnt8 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., Wong, G. and Melton, D. A. (1990). A mouse macrophage factor induces head structures and organizes a body axis in *Xenopus. Science* **249**, 561-564.

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**, 745-752.

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

Sokol, S. and Melton, D. A. (1992). Interaction between Wnt and activin in dorsal mesoderm formation in *Xenopus*. *Dev. Biol.* **154**, 348-355.

Symes, K. and Smith, J. C. (1987). Gastrulation movements provide an early marker of mesoderm induction in *Xenopus laevis. Development* **101**, 339-349.

Yisraeli, J. K., Sokol, S. and Melton, D. A. (1990). A two step model for the localization of maternal mRNA in *Xenopus* oocytes: involvement of microtubules and microfilaments in the translocation and anchoring of Vg1 mRNA. *Development* **108**, 289-298.

(Accepted 26 April 1993)