

## Induction of dorsal mesoderm by soluble, mature Vg1 protein

Daniel S. Kessler and Douglas A. Melton

Department of Molecular and Cellular Biology, Howard Hughes Medical Institute, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138, USA

### SUMMARY

Mesoderm induction during *Xenopus* development has been extensively studied, and two members of the transforming growth factor- $\beta$  family, activin  $\beta$ B and Vg1, have emerged as candidates for a natural inducer of dorsal mesoderm. Heretofore, analysis of Vg1 activity has relied on injection of hybrid Vg1 mRNAs, which have not been shown to direct efficient secretion of ligand and, therefore, the mechanism of mesoderm induction by processed Vg1 protein is unclear. This report describes injection of *Xenopus* oocytes with a chimeric activin-Vg1 mRNA, encoding the pro-region of activin  $\beta$ B fused to the mature region of Vg1, resulting in the processing and secretion of mature Vg1. Treatment of animal pole explants with mature Vg1 protein resulted in differentiation of dorsal, but not ventral, mesodermal tissues and dose-dependent activation of both dorsal and ventrolateral mesodermal

markers. At high doses, mature Vg1 induced formation of 'embryoids' with a rudimentary axial pattern, head structures including eyes and a functional neuromuscular system. Furthermore, truncated forms of the activin and FGF receptors, which block mesoderm induction in the intact embryo, fully inhibited mature Vg1 activity. To examine the mechanism of inhibition, we have performed receptor-binding assays with radiolabeled Vg1. Finally, follistatin, a specific inhibitor of activin  $\beta$ B which is shown not to block endogenous dorsal mesoderm induction, failed to inhibit Vg1. The results support a role for endogenous Vg1 in dorsal mesoderm induction during *Xenopus* development.

Key words: *Xenopus*, mesoderm induction, Vg1, activin receptor, FGF receptor, BMP receptor, follistatin

### INTRODUCTION

At the blastula stage of *Xenopus* development, vegetal blastomeres produce signals that induce mesoderm formation in overlying equatorial cells (Nieuwkoop, 1969a; Sudarwati and Nieuwkoop, 1971). Much effort has been directed at the molecular analysis of mesoderm induction and, consequently, a number of molecules with mesoderm-inducing or patterning activities have been identified. Among the described mesoderm-inducing factors, three endogenous secreted proteins, activin and bone morphogenetic protein (BMP) (members of the TGF- $\beta$  family), and fibroblast growth factor (FGF), are implicated in the formation of dorsal, ventral and ventrolateral mesoderm, respectively (reviewed by Kessler and Melton, 1994). Expression of dominant inhibitory activin, FGF or BMP receptors, lacking the intracellular kinase domains, supports the involvement of these factors in endogenous mesoderm induction and patterning (Hemmati-Brivianlou and Melton, 1992; Amaya et al., 1991; Suzuki et al., 1994; Graff et al., 1994). Additional molecules, noggin, chordin, and members of the wnt family, can alter the dorsoventral pattern of mesoderm while lacking intrinsic mesoderm-inducing activity (reviewed by Kessler and Melton, 1994; Sasai et al., 1994). Despite the potent activities observed for these molecules, each fails to fulfill a strong expectation for an endogenous mesoderm inducer: localization to the vegetal pole blastomeres that are responsible for mesoderm induction and

patterning during normal development. Localization of maternal activin, BMP or FGF protein has yet to be observed (Asashima et al., 1991; Fukui et al., 1993; Nishimatsu et al., 1993; Ueno et al., 1992).

Vg1 is a maternal mRNA localized to the vegetal pole of *Xenopus* eggs and early embryos (Rebagliati et al., 1985; Weeks and Melton, 1987; reviewed by Vize and Thomsen, 1994). Vg1 mRNA is synthesized early in oogenesis and becomes tightly localized to the vegetal cortex by the end of oogenesis (Melton, 1987; Mowry and Melton, 1992; Yisraeli and Melton, 1988) and, therefore, Vg1 mRNA and protein become partitioned within vegetal pole blastomeres (inducing tissue) in the early embryo (Dale et al., 1989; Tannahill and Melton, 1989). TGF- $\beta$ -like proteins are synthesized as inactive precursors, which form disulfide-linked dimers and are proteolytically cleaved, releasing a mature C-terminal bioactive dimer (Massagué et al., 1994). However, endogenous Vg1 protein accumulates as an unprocessed precursor ( $46 \times 10^3 M_r$ ) and little or no mature Vg1 ( $18 \times 10^3 M_r$ ) has been detected (Dale et al., 1989; Tannahill and Melton, 1989; Thomsen and Melton, 1993). Consistent with this observation, injection of embryos with Vg1 mRNA produces high levels of Vg1 precursor, but no processed protein and, consequently, neither mesoderm induction nor developmental effects are observed (Dale et al., 1989, 1993; Tannahill and Melton, 1989; Thomsen and Melton, 1993). In contrast, injection of activin mRNA directs efficient production of mature protein and animal pole

explants are induced to form mesoderm (Thomsen et al., 1990). Therefore, although Vg1 localization suggests that it is an *in vivo* mesoderm-inducing signal, an inability to produce processed protein has hindered analysis of mature Vg1 activity.

Recent studies using chimeric BMP-Vg1 molecules have demonstrated mesoderm-inducing activity for mature Vg1 (Thomsen and Melton, 1993; Dale et al., 1993). A fusion protein, consisting of the N-terminal pro-region of BMP and the C-terminal mature region of Vg1, was shown to direct precursor processing, and permitted an analysis of mature Vg1 function. Injection of BMP2-Vg1 mRNA, incorporating the BMP2 tetrabasic cleavage site, resulted in induction of dorsal mesoderm (muscle and notochord) in animal pole explants and complete dorsal axis formation in ultraviolet-ventralized embryos, demonstrating the inductive activity of the Vg1 hybrid (Thomsen and Melton, 1993). In contrast, a BMP4-Vg1 hybrid, maintaining the Vg1 tetrabasic cleavage site, weakly induced mesoderm and axis formation (Dale et al., 1993).

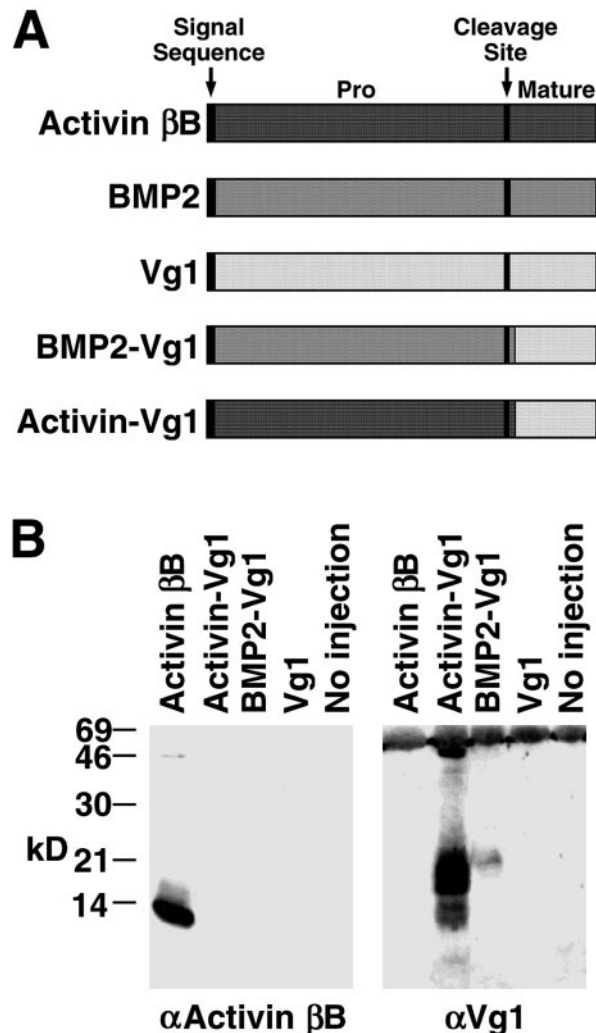
While processing is enhanced by the hybrid constructs, the secretion of soluble, mature Vg1 by BMP4-Vg1-injected oocytes is inefficient (Dale et al., 1993), bringing into question the mechanism of processed Vg1 action in injected embryos. Specifically, it is possible that hybrid Vg1 molecules act intracellularly, or that they inhibit translation or processing of other endogenous TGF- $\beta$ s, such as BMPs. Furthermore, injection of hybrid mRNAs does not allow the analysis of competence, mesoderm induction versus dorsalization, specific activity and receptor-binding specificity. In an attempt to elucidate the activity of mature Vg1 and its role during normal development, we have undertaken the production of soluble, mature Vg1.

Utilizing a hybrid activin  $\beta$ B-Vg1 molecule, expressed in *Xenopus* oocytes, we have obtained efficient secretion of soluble, biologically active, processed Vg1. Soluble mature Vg1 strongly induces dorsal mesoderm in animal pole explants, resulting in the formation of 'embryoids' displaying a rudimentary axial pattern. In addition, both a truncated activin type II receptor and a truncated FGF receptor fully inhibited the inducing activity of mature Vg1, while a truncated BMP receptor did not. Receptor-binding studies indicated that, although the truncated activin receptor can block Vg1 function, it does not bind Vg1 ligand. Moreover, follistatin, an inhibitor of activin, which we show is unable to block endogenous dorsal mesoderm formation, failed to inhibit mature Vg1. Our results establish the dorsal mesoderm-inducing activity of secreted Vg1 ligand and suggest that inhibition of endogenous mesoderm induction by truncated receptors (Amaya et al., 1991; Hemmati-Brivanlou and Melton, 1992) may be due, in part, to an inhibition of signaling by endogenous Vg1.

## MATERIALS AND METHODS

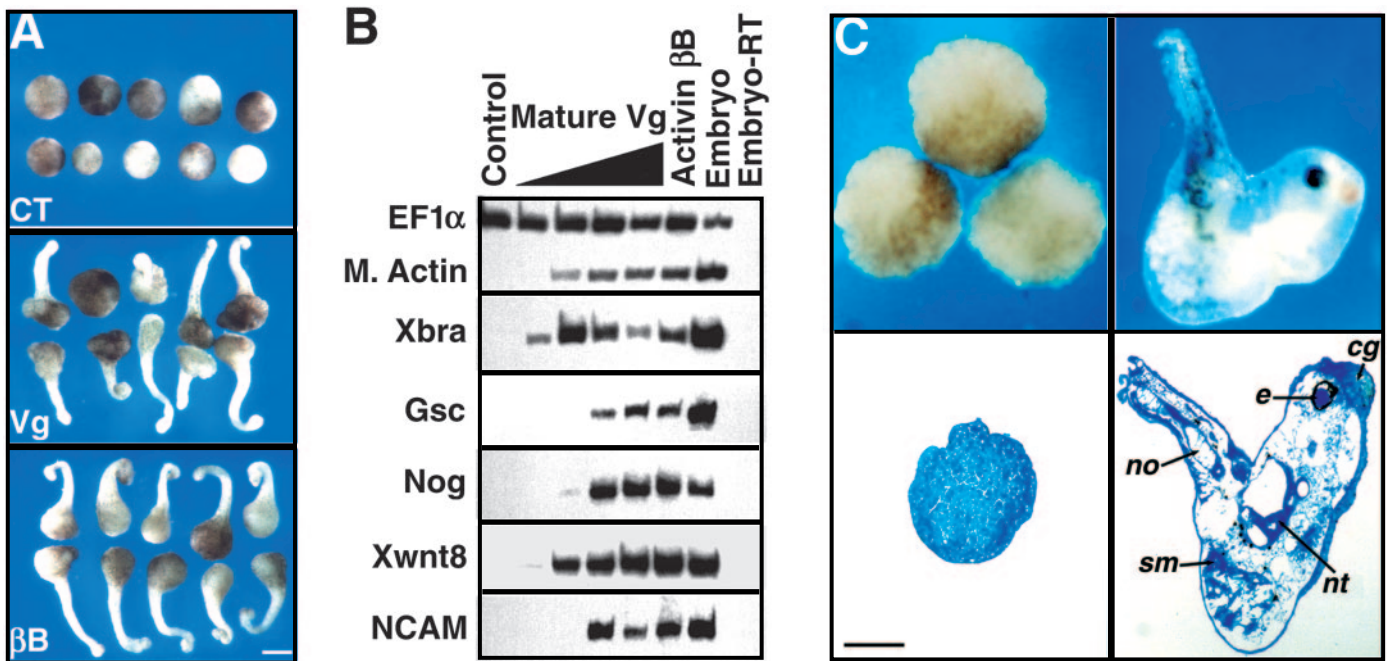
### Embryological procedures and histology

*Xenopus* eggs were fertilized, microinjected and cultured using standard procedures (Thomsen and Melton, 1993). For animal cap experiments, embryos were injected at the 2-cell stage, animal cap explants prepared at stage 8 and cultured in 0.5 $\times$  MMR supplemented with 50 ng/ml recombinant basic FGF (Gibco/BRL) or oocyte-conditioned media. For marginal zone experiments, dorsal or ventral explants were prepared from stage 10.25 embryos (Nieuwkoop and



**Fig. 1.** (A) Chimeric Vg1 constructs. Schematic representation of activin  $\beta$ B, BMP2, Vg1, and chimeric BMP2-Vg1 and activin-Vg1. Members of the TGF- $\beta$  superfamily, these genes contain a signal sequence, pro-region, tetrabasic cleavage site and mature region. Activin  $\beta$ B and BMP2, but not Vg1, form disulfide-linked dimers that are subsequently cleaved, releasing the mature C-terminal peptide as a secreted bioactive dimer. The chimeric constructs, fused four amino acids downstream of the cleavage site, are designed to facilitate processing and secretion of mature Vg1. (B) Western blot analysis of oocyte supernatants. Oocytes were injected with the indicated mRNA and conditioned supernatants (10  $\mu$ l) analyzed by western blotting of reducing SDS-PAGE using activin-specific (left) or Vg1-specific (right) antisera. Activin  $\beta$ B mRNA directs secretion of mature protein. While Vg1 mRNA directs no secretion and BMP2-Vg1 directs low levels of secretion, activin-Vg1 mRNA results in secretion of abundant mature Vg1 protein.

Faber, 1967) and cultured in 0.5 $\times$  MMR supplemented with oocyte-conditioned media. Oocytes were surgically removed from anesthetized females, follicle cells removed by digestion with 2 mg/ml collagenase (Sigma, type IA) in OR2 (5 mM Tris-HCl (7.8), 82.5 mM NaCl, 2.5 mM KCl, 1 mM Na<sub>2</sub>HPO<sub>4</sub>) (Peng, 1991), injected with 50 ng of capped, *in vitro* transcribed RNA and cultured at 19°C in OR2 supplemented with 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub> and 0.5 mg/ml BSA. Oocyte-conditioned media was prepared by incubating oocytes in OR2 with MgCl<sub>2</sub>, CaCl<sub>2</sub> and BSA (10  $\mu$ l/oocyte) in 96-well microtiter



**Fig. 2.** (A) Mature Vg1 induces morphogenetic movements in ectodermal explants. Blastula-stage animal pole explants were treated with 10% supernatant (1/10 dilution) until the gastrula stage and cultured to the neurula stage (stage 15). Mature Vg1 (Vg) results in a dramatic elongation indicative of mesoderm induction (Symes and Smith, 1987). Activin  $\beta$ B ( $\beta$ B) results in similar elongation, while supernatant of uninjected oocytes (CT) have no effect. Scale bar, 300  $\mu$ m. (B) Dose-dependent induction of mesodermal markers by mature Vg1. Blastula-stage animal pole explants were treated with increasing doses of mature Vg1 supernatant (1%, 3%, 10%, 30%), or control (30%) or activin  $\beta$ B supernatant (10%), cultured to the neurula stage and analyzed by RT-PCR. At low doses (1%) the general marker Xbra is induced; at intermediate doses (3%) the ventrolateral marker Xwnt8 and the dorsolateral marker cardiac actin (M. Actin) are induced; and at high doses (10–30%) the dorsoanterior markers goosecoid (Gsc) and noggin (Nog), and the neural marker NCAM are induced. Activin  $\beta$ B treatment results in a similar response and control supernatants have no effect. EF1 $\alpha$  is a loading and reverse transcription control and the embryo and embryo-RT are additional positive and negative controls. (C) Induction of embryoids by mature Vg1. Blastula animal pole explants treated with a high dose of mature Vg1 and cultured to the late tadpole stage (stage 40), differentiated into embryoids displaying a rudimentary axial organization, with anterior-posterior pattern and head structures. The embryoid shown (top right) has a clear head-to-tail pattern and pigmented eye and cement gland. A histological section (bottom right) reveals differentiated notochord (no), somitic muscle (sm), neural tube (nt), eye (e) and cement gland (cg). Treatment with supernatant of uninjected oocytes has no effect and explants form atypical epidermis (left). Scale bar, 150  $\mu$ m.

plates. Media was conditioned for 3–4 days and stored at 4°C. For histology, samples were fixed in Bouin's fixative and processed for Paraplast sectioning and Giemsa staining.

#### Construction of activin $\beta$ B-Vg1 hybrid and mRNA synthesis

The activin  $\beta$ B-Vg1 hybrid was constructed by PCR amplification of a fragment of a *Xenopus* activin  $\beta$ B gene (Thomsen et al., 1990) encoding the N-terminal pro-region, the tetrabasic cleavage site and the first four amino acids of the C-terminal mature region. This fragment was ligated, in frame, to a PCR amplified fragment of the Vg1 gene (Weeks and Melton, 1987) encoding the C-terminal mature region lacking the first four amino acids. The ligation product was reamplified, digested at terminal restriction sites and cloned into a derivative of pSP64T (Krieg and Melton, 1987). The activin-Vg1 construct and nucleotide sequence are available upon request. This hybrid is analogous to the BMP2-Vg1 construct previously described (Thomsen and Melton, 1993; Fig. 1A).

For *in vitro* synthesis of capped mRNA, linearized templates for activin  $\beta$ B-Vg1, BMP2-Vg1 (Thomsen and Melton, 1993), activin  $\beta$ B (Sokol et al., 1991), Vg1 (Tannahill and Melton, 1989), noggin (Smith and Harland, 1992), follistatin (Hemmati-Brivanlou et al., 1994), truncated FGF receptor (Amaya et al., 1991), truncated activin receptor (Hemmati-Brivanlou and Melton, 1992), truncated BMP2/4 receptor (Graff et al., 1994) or  $\beta$ -galactosidase (Smith and Harland,

1991) were used to program the Megascript SP6 transcription kit (Ambion).

#### Western blotting and quantitation of secreted protein

Supernatants of injected oocytes were resolved by 15% reducing SDS-PAGE, electroblotted to nylon membranes and probed using a mature region-specific Vg1 monoclonal antiserum (Tannahill and Melton, 1989) or an affinity-purified activin  $\beta$ B polyclonal antiserum specific for the mature region (provided by P. Klein). Cross-reacting peptides were detected using alkaline phosphatase-conjugated secondary antibodies, visualized by chemiluminescence (Bio-Rad). The concentration of secreted proteins in oocyte supernatants was determined by western blotting and quantitation of serial dilutions of supernatant and a standard curve of *in vitro* translated Vg1 and activin. The concentration of *in vitro* translated proteins was calculated by measuring incorporation of [<sup>35</sup>S]methionine.

#### Reverse transcription-polymerase chain reaction

RNA isolation, reverse transcription, PCR conditions and primer sequences have been previously described (Wilson and Melton, 1994). PCR products were resolved on 5% non-denaturing acrylamide gels. The ubiquitous EF1 $\alpha$  mRNA (Krieg et al., 1989) was used as a RNA extraction and reverse transcription control. Xbra is a general mesodermal marker (Smith et al., 1991) and Xwnt8 is a marker of ventrolateral mesoderm (Christian et al., 1991). Cardiac actin is a

marker of somitic muscle (Stutz and Sphor, 1986) and gooseoid and noggin are markers of dorsal mesoderm (Blumberg et al., 1991; Smith and Harland, 1992). NCAM is a general marker of neural tissue (Kintner and Melton, 1987).

### Activin receptor ligand-binding analysis

An epitope-tagged version of the activin type II receptor (XARmyc), containing a COOH-terminal c-Myc peptide (Evan et al., 1985), was used for receptor crosslinking studies (A. Hemmati-Brivanlou, unpublished). Defolliculated oocytes were injected with 10 ng of XARmyc mRNA and cultured for 2 days prior to binding analysis. Metabolically labeled activin and mature Vg1 was prepared as described above with the addition of [<sup>35</sup>S]methionine (50  $\mu$ Ci/ml) to the culture medium. In a total volume of 200  $\mu$ l, ten oocytes expressing XARmyc were incubated with 0.4 nanomolar labeled ligand ( $\sim 10^4$  cts/minute/ $\mu$ g), in the presence or absence of 4 nanomolar unlabeled competitor, for 30 minutes at 4°C. Oocytes were washed extensively with cold OR2 supplemented with 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub> and ligand-receptor complexes chemically crosslinked with the reducible crosslinker DTSSP (2 mM; Pierce) for 30 minutes at 4°C. Following lysis in RIPA (50 mM Tris-HCl (8.0), 150 mM NaCl, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS), ligand-receptor complexes were immunoprecipitated from cleared lysates (5 minutes at 5000 $\times$ g) using the Myc-epitope specific monoclonal antibody 9E10 (5  $\mu$ g/ml) (Evan et al., 1985). Ligand-receptor complexes were reduced, co-precipitating ligand resolved by 15% reducing SDS-PAGE. Co-precipitating activin is released from complexes by reducing the crosslinker, and is therefore resolved as an  $\sim 14 \times 10^3$   $M_r$  band, distinguishing the binding of mature ligand from unprocessed protein, which was present at low levels in the preparations used.

## RESULTS

### Secretion of processed Vg1 by *Xenopus* oocytes

Expression of hybrid BMP-Vg1 molecules in embryos directs precursor processing, but little evidence for efficient secretion of processed Vg1 is available. BMP2-Vg1 expression in COS cells (DSK, G. Thomsen and DAM, unpublished) or *Xenopus* oocytes resulted in low or undetectable levels of secreted mature Vg1 (Fig. 1A,B), although intracellular processing of precursor was apparent. BMP4-Vg1 expression in oocytes failed to direct efficient secretion of mature protein, although polyanion addition did release low levels of processed protein. The biological activity of this material is unknown since no activity assessment was reported (Dale et al., 1993). Consistent with these observations, secretion of BMP2 or BMP4 is not detected in these systems (D. S. K. and D. A. M., unpublished), suggesting that the pro-region may be a target for the regulation of secretion (Gray and Mason, 1990). In addition, these observations raise the possibility that the effects of BMP-Vg1 in embryos may not be due to secretion of mature protein, but rather to an intracellular activity of the hybrid. Alternatively, oocytes and embryos may differ in their capacity to process and secrete various members of the TGF- $\beta$  family.

In contrast to the BMP-Vg1 hybrids, functional activin  $\beta$ B ligand is efficiently secreted in both COS cells and oocytes (Fig. 1B; Thomsen et al., 1990). Therefore, to obtain soluble mature Vg1, an additional hybrid molecule was prepared containing the activin  $\beta$ B signal sequence, pro-region, tetrabasic cleavage site and four amino acids of the activin  $\beta$ B mature region fused to the mature region of Vg1 (Fig. 1A). To test the function of this activin-Vg1 hybrid, animal pole explants of

injected embryos were prepared at the blastula stage, cultured and scored for mesoderm formation. Injection of activin-Vg1 mRNA resulted in tissue elongation and expression of muscle-specific cardiac actin, similar to that observed with BMP2-Vg1 injection (data not shown). Induction by the activin-Vg1 hybrid was not due to expression of the activin pro-region since an activin-BMP2 hybrid induced expression of ventral, not dorsal mesodermal markers, consistent with the activity of BMP2 (data not shown).

Defolliculated oocytes were injected with 50 ng of in vitro transcribed RNA and cultured for 3 days. Conditioned supernatants were analyzed by western blotting using antisera specific for the mature region of activin  $\beta$ B or Vg1 (Fig. 1B). Injection of activin  $\beta$ B mRNA resulted in secretion of processed protein, detected as a reduced monomer of  $\sim 14 \times 10^3$   $M_r$ . No secreted mature Vg1 was detected following injection of native Vg1 mRNA, and a small amount of mature Vg1 secretion was directed by BMP2-Vg1. Injection of activin-Vg1 mRNA resulted in secretion of abundant mature Vg1, detected as a series of bands of  $\sim 18 \times 10^3$   $M_r$ . The different mature species presumably reflect varying glycosylation states, as described for the endogenous precursor (Tannahill and Melton, 1989; Dale et al., 1989, 1993). The ability of activin-Vg1 to direct secretion of mature protein was comparable in efficiency to activin  $\beta$ B and, in both cases, a lesser amount of secreted precursor ( $\sim 46 \times 10^3$   $M_r$ ) is also detected. The concentration of mature Vg1 and activin in oocyte-conditioned medium was determined by western blotting and quantitation against a standard curve of Vg1 or activin protein (see Materials and Methods). In a typical experiment, 200 oocytes were injected, producing approximately 2 ml of supernatant containing soluble, processed Vg1 and activin  $\beta$ B at concentrations of 100-500 ng/ml.

### Mature Vg1 induces dorsal mesoderm in animal pole explants

Oocyte supernatants were tested for mesoderm-inducing activity on animal pole explants. Blastula-stage animal poles (prospective ectoderm) were explanted, incubated with supernatant, cultured to the neurula stage and scored for the formation of mesodermal tissues and expression of mesodermal markers. Supernatants of uninjected, Vg1- or BMP2-Vg1-injected oocytes failed to induce morphogenetic movements or expression of mesodermal markers (Fig. 2A,B; data not shown). Supernatants of activin-Vg1- or activin  $\beta$ B-injected oocytes strongly induced morphogenetic movements indicative of mesoderm induction, expression of mesodermal markers and differentiation of the mesodermal tissues, muscle and notochord (Fig. 2A-C).

Low doses of mature Vg1 induced expression of the general mesodermal marker Xbra (Smith et al., 1991). Intermediate doses induced the ventrolateral marker Xwnt-8 (Christian et al., 1991) and the dorsolateral marker cardiac actin (Stutz and Sphor, 1986). High doses of mature Vg1 induced expression of the dorsal mesodermal markers gooseoid (Blumberg et al., 1991) and noggin (Smith and Harland, 1992), and, by secondary induction, the neural marker NCAM (Kintner and Melton, 1987). These markers were also induced by activin  $\beta$ B, which was a positive control, but not by supernatant of uninjected oocytes (Fig. 2B). Globin, a definitive marker of ventral mesoderm was not induced by Vg1 or activin (data not shown).

Induction of morphogenetic movements in Vg1-treated explants was consistent with the response profile of mesodermal markers. At low doses (Xbra positive), no elongation was apparent and, at higher doses (cardiac actin positive), marked elongation is observed (data not shown). Using this preparation of mature Vg1, the lowest dose that induces mesoderm is approximately 1 ng/ml or 40 pM. The specific activity of mature Vg1 is similar to that found for activin  $\beta$ B and other members of the TGF- $\beta$  family (Vale et al., 1990).

Examination of mature Vg1-treated explants cultured to the late neurula stage by immunohistochemistry and histology revealed the differentiation of mesodermal tissues, notochord and somitic muscle, as well as neural tissue (data not shown). Explants cultured to the late tadpole stage, following treatment with mature Vg1, often developed a high degree of axial organization. These 'embryoids' displayed a rudimentary anterior-posterior pattern with organized head structures, including eyes, and a functional neuromuscular system, similar to results with activin protein (Sokol et al., 1990). Histological sections showed the presence of muscle, notochord, neural tissue and differentiated eye (Fig. 2C).

### Inhibition of mature Vg1 activity by the truncated activin and FGF receptors

To address the relation between mature Vg1 and endogenous mesoderm-inducing signals, we tested the ability of dominant inhibitory receptors to block the activity of mature Vg1. A truncated form of an activin type II receptor (tAR), lacking the intracellular kinase domain, blocked formation of detectable mesoderm and expression of mesodermal markers in embryos (Hemmati-Brivanlou and Melton, 1992). Animal pole explants of embryos injected with 4 ng of tAR mRNA were treated with mature Vg1 or activin  $\beta$ B. tAR expression fully inhibited induction of morphogenetic movements and expression of brachyury and cardiac actin by mature Vg1. As expected, tAR also blocked activin  $\beta$ B activity and resulted in NCAM expression regardless of treatment with supernatants (Table 1). This confirms similar results reported by Schulte-Merker et al. (1994). A dominant inhibitory FGF receptor (tFGFR), lacking the kinase domain, blocked formation of trunk and posterior mesoderm, resulting in loss of axial mesoderm and tail structures (Amaya et al., 1991). In addition to blocking FGF activity, tFGFR also blocks mesoderm induction by activin  $\beta$ B

**Table 1. Mesoderm induction by mature Vg1 is blocked by truncated activin and FGF receptors**

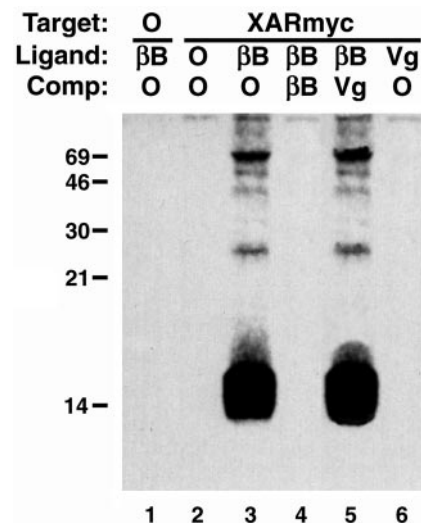
	$\beta$ Gal	tAR	tFGFR	tBR
Control	-	-	-	-
Mature Vg1	+	-	-	+
Activin $\beta$ B	+	-	-	+

At the 2-cell-stage embryos were injected with 4 ng of a truncated activin type II receptor (tAR), a truncated FGF receptor (tFGFR), a truncated BMP2/4 receptor (tBR) or  $\beta$ -galactosidase ( $\beta$ Gal) mRNA. Blastula-stage animal pole explants ( $n \geq 10$ ) were prepared and treated with mature Vg1, activin  $\beta$ B or control supernatants (10%). Following culture to the neurula stage, mesoderm induction was assessed by explant elongation and expression of brachyury and cardiac actin, as was done for the experiments shown in Fig. 5. Induction by mature Vg1 was fully inhibited by the truncated activin and FGF receptors, similar to their effects on activin. No inhibition was detected with the truncated BMP receptor, or the control  $\beta$ -galactosidase injection. These results were obtained in three independent experiments.

in animal pole explants (LaBonne and Whitman, 1994; Cornell and Kimelman, 1994). Injection of 4 ng of tFGFR mRNA resulted in a complete block of mesoderm induction by both mature Vg1 and activin  $\beta$ B (Table 1; Schulte-Merker et al., 1994). These observations suggest that the ability of the truncated activin and FGF receptors to perturb mesoderm induction in the embryo may, in fact, be due to an inhibition of endogenous Vg1 signaling. In contrast, animal cap expression of a truncated BMP2/4 receptor (tBR), capable of converting ventral mesoderm to dorsal mesoderm in embryos (Suzuki et al., 1994; Graff et al., 1994), failed to inhibit the mesoderm-inducing activity of mature Vg1 (Table 1). In these experiments tFGFR and tBR blocked induction by FGF and BMP2, respectively (data not shown).

### The activin type II receptor is not a high affinity receptor for mature Vg1

The inhibition of mature Vg1 activity by the truncated activin receptor suggested that the activin type II receptor (XAR1) may also function as a Vg1 receptor. This possibility was examined by binding analysis of radiolabeled ligands (see Methods) to an epitope-tagged version of XAR1 (XARmyc) expressed in oocytes. Ligand binding was assessed by competition studies using chemical crosslinking and immunoprecipitation



**Fig. 3.** Mature Vg1 is not bound by the activin type II receptor. Uninjected oocytes, or oocytes expressing a myc-tagged activin type II receptor (XARmyc) were bound with radiolabeled activin  $\beta$ B or mature Vg1, complexes chemically crosslinked, immunoprecipitated with a myc-specific antiserum and visualized by 15% reducing SDS-PAGE and fluorography. Co-precipitating activin is released from complexes by reducing the crosslinker, and is therefore resolved as an  $\sim 14 \times 10^3 M_r$  band. Uninjected oocytes did not bind radiolabeled activin  $\beta$ B (lane 1) and XARmyc-expressing oocytes displayed no binding in the absence of radiolabeled ligand (lane 2). Receptor-expressing oocytes strongly bound activin (lane 3) and this binding was competed by a 10-fold excess of unlabeled activin (lane 4). In contrast, a 10-fold excess of unlabeled mature Vg1 was unable to compete activin binding (lane 5). Furthermore, a 10-fold higher dose of radiolabeled Vg1 than that required for activin binding did not result in detectable binding (lane 6). The higher molecular weight bands (lanes 3, 5) may be due to non-reduced ligand-receptor complexes and co-precipitating unprocessed activin.

itation. While radiolabeled activin  $\beta$ B was not bound by control oocytes, expression of XARmyc resulted in strong binding of activin, which was competed by a 10-fold excess of unlabeled activin (Fig. 3). Although a complete competition of activin binding is apparent in this experiment, quantitation of additional experiments shows an approximately 7-fold reduction of activin binding by a 10-fold excess of unlabeled activin. In contrast, radiolabeled Vg1 showed no detectable binding to XARmyc, even at a 10-fold higher dose than that required for activin binding. Furthermore, an excess of unlabeled mature Vg1 was unable to compete for activin binding. These observations suggest that the activin type II receptor is not a Vg1 receptor, and that inhibition of Vg1 signaling by the truncated activin receptor is due to an interaction with an endogenous Vg1-specific receptor.

#### Follistatin does not block mature Vg1 activity or endogenous dorsal mesoderm induction

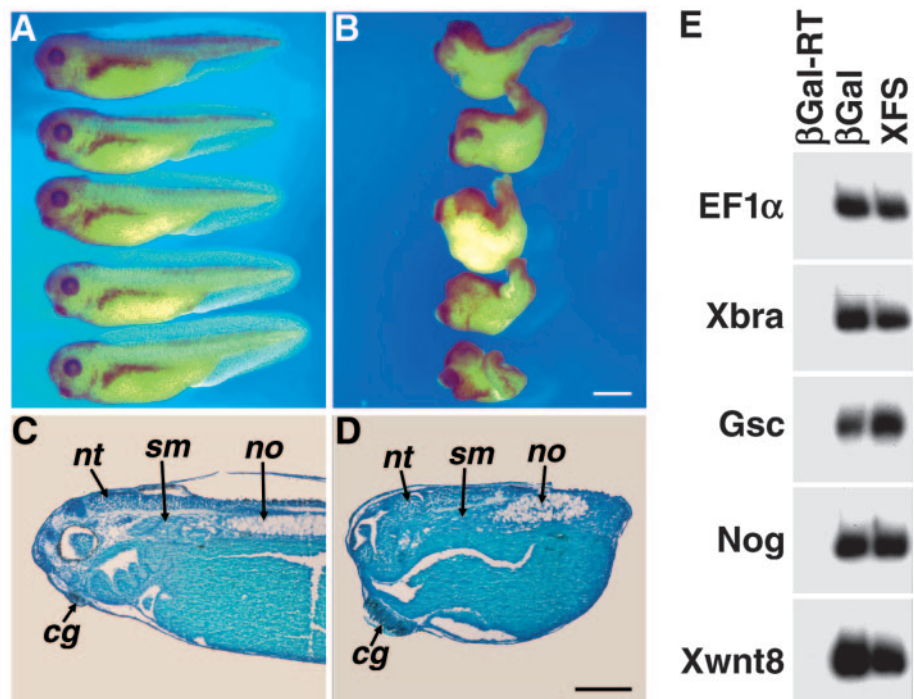
A natural inhibitor of activin function is the activin-binding protein follistatin (Kogawa et al., 1991; Nakamura et al., 1990). *Xenopus* follistatin is maternally expressed and can block activin induction of animal pole explants (Tashiro et al., 1991; Fukui et al., 1993, 1994; Hemmati-Brivanlou et al., 1994). It has been reported that, unlike the truncated activin receptor, rat follistatin has no effect on the early embryo (Slack, 1991; Schulte-Merker et al., 1994). To examine more closely the effect of follistatin expression on normal development, *Xenopus* follistatin mRNA (XFS-319; Hemmati-Brivanlou et al., 1994) was injected into both blastomeres at the 2-cell stage. In contrast to previous reports (Schulte-Merker et al., 1994), we do observe an effect of follistatin on early development. At the tadpole stage, XFS overexpression results in a consistent loss of posterior structures and an apparent enhancement of dorsoanterior structures (Fig. 4A,B). As assessed by both histology and molecular markers, no suppression of dorsal mesoderm is detected in response to XFS. Even at high doses of XFS mRNA, differentiated dorsal mesodermal tissues (notochord and muscle) are present in injected embryos (Fig. 4C,D), consistent with the maintenance of dorsal mesodermal marker expression at the gastrula stage (Fig. 4E). These observations indicate that XFS does not inhibit endogenous induction of dorsal mesoderm and do not support a role for activin in the formation of dorsal mesoderm in vivo.

To examine further the relation of mature Vg1 to endogenous mesoderm induction, the ability of follistatin to inhibit mature Vg1 activity was examined. Mature Vg1 or activin supernatants were combined with an equal

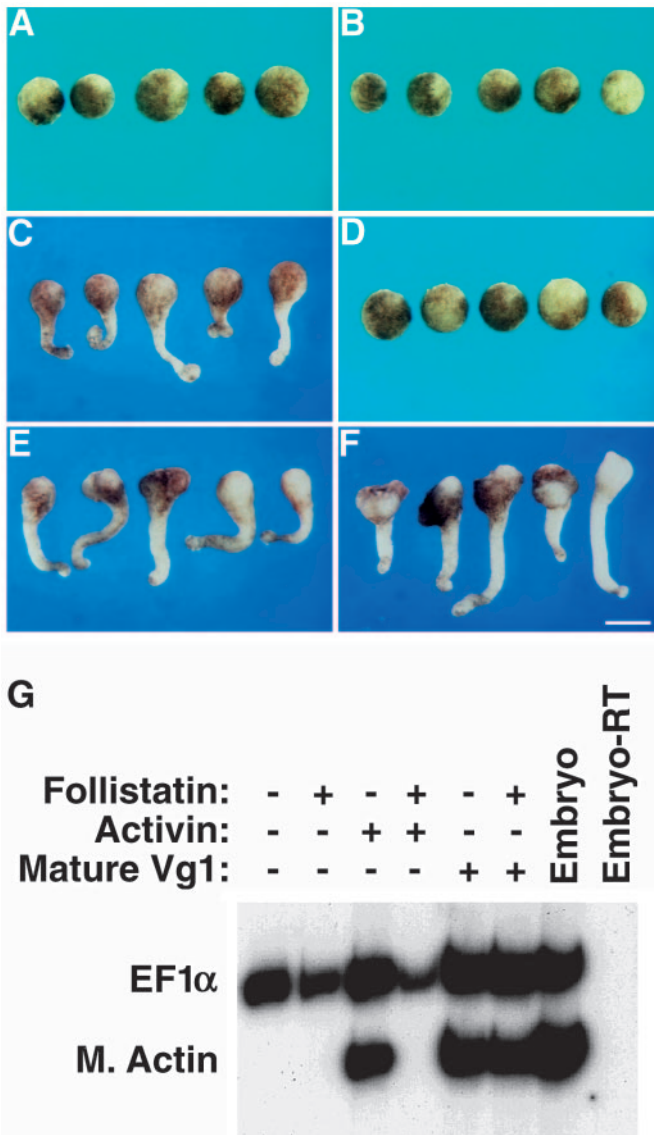
volume of XFS supernatant prior to addition to blastula animal pole explants. At these doses, XFS fully blocked activin-induced morphogenetic movements and expression of cardiac actin, whereas mature Vg1 activity was unaffected (Fig. 5). Even at a dose of follistatin 100-fold higher than that required to inhibit activin, no inhibition of mature Vg1 was observed (data not shown).

#### Mature Vg1 does not dorsalize gastrula ventral mesoderm

At the gastrula stage the organizer produces a signal that can alter the fate of lateral mesodermal tissues, resulting in formation of mesodermal tissues with more dorsal character (Dale and Slack, 1987; Slack and Foreman, 1980; Yamada, 1950). While neither activin nor FGF display this dorsalizing activity (Slack et al., 1992), soluble noggin protein, expressed in the organizer (Smith and Harland, 1992), can induce muscle in gastrula ventral marginal zone explants (Smith et al., 1993). We tested the dorsalizing activity of mature Vg1 by incubating blastula or gastrula ventral marginal zone explants in supernatants containing mature Vg1, activin  $\beta$ B, or noggin and examined the expression of cardiac actin. Mature Vg1, activin and noggin treatment resulted in expression of muscle-specific cardiac actin in blastula ventral marginal zone explants.

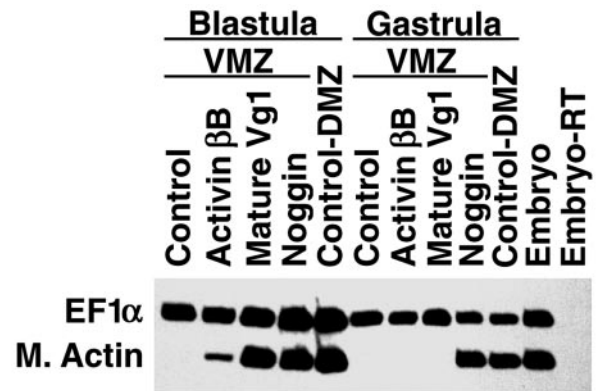


**Fig. 4.** Follistatin does not inhibit endogenous dorsal mesoderm induction. Both blastomeres of 2-cell-stage embryos were injected with  $\beta$ -galactosidase mRNA (A) or follistatin mRNA (B) (1 ng total mRNA) and cultured to the tadpole stage. Follistatin resulted in a loss of posterior structures with maintenance or enhancement of dorsoanterior structures. Histological analysis reveals the presence of dorsal mesodermal tissues in both  $\beta$ -galactosidase (C)- and follistatin (D)-injected embryos (no, notochord; sm, somitic muscle; nt, neural tube; cg, cement gland). (E) Follistatin does not inhibit expression of mesodermal markers at the gastrula stage. 2-cell-stage embryos were injected with a total of 2 ng of  $\beta$ -galactosidase ( $\beta$ Gal) or follistatin (XFS) mRNA, harvested at the mid-gastrula stage (stage 11) and analyzed by RT-PCR. The expression of a general mesodermal marker, brachyury (Xbra), the dorsal markers, goosecoid (Gsc) and noggin (Nog), and a ventrolateral marker, Xwnt8, were unaffected by follistatin. EF1 $\alpha$  is a loading and reverse transcription control. Scale bars, 1 mm (A,B) and 0.5 mm (C,D).



**Fig. 5.** Follistatin does not inhibit mature Vg1 activity. Blastula-stage animal pole explants were treated with control (A,B), activin  $\beta$ B (C,D), or mature Vg1 (E,F) supernatants following mixture and preincubation with control (A,C,E) or follistatin (B,D,F) supernatants. While follistatin fully blocks activin-induced morphogenetic movements, induction by mature Vg1 was unaffected. (G) Follistatin fails to inhibit cardiac actin (M. Actin) induction by mature Vg1. Blastula animal pole explants were treated with mature Vg1 or activin only, or with these supernatants preincubated with follistatin. Upon reaching the neurula stage, samples were analyzed by RT-PCR. While follistatin fully inhibits cardiac actin induction by activin, no effect on mature Vg1 activity is detected. EF1 $\alpha$  is a loading and reverse transcription control. Scale bar, 300  $\mu$ m.

However, neither mature Vg1 nor activin induced muscle actin expression in gastrula stage ventral marginal zone explants, while noggin-treated explants and control dorsal marginal zone explants expressed substantial levels of cardiac actin (Fig. 6). The inability of mature Vg1 to dorsalize the gastrula ventral marginal zone is consistent with the loss of competence to respond to mesoderm induction at the gastrula stage (Jones and



**Fig. 6.** Mature Vg1 does not dorsalize gastrula ventral marginal zone explants. Dorsal or ventral marginal zone explants were prepared from blastulae (stage 8) or early gastrulae (stage 10.25) and incubated in supernatants (30%) containing activin, mature Vg1 or noggin, or in a supernatant of uninjected oocytes (control). At the neurula stage, samples were analyzed by RT-PCR. While all three factors induced cardiac actin (M. Actin) in blastula ventral marginal zone explants, only noggin did so in gastrula-stage explants. EF1 $\alpha$  is a loading and reverse transcription control.

Woodland, 1987), and is an expected characteristic of a natural mesoderm inducer.

## DISCUSSION

We report the preparation of soluble, biologically active mature Vg1 by expression of an activin  $\beta$ B-Vg1 chimera in *Xenopus* oocytes. The results demonstrate that the mesoderm-inducing activity of Vg1 does reside in the secreted mature region, establishing the mechanism of action of injected BMP-Vg1 hybrids (Dale et al., 1993; Thomsen and Melton, 1993). Soluble, mature Vg1 is a potent inducer of dorsal mesoderm when applied to animal pole explants, resulting in expression of dorsal markers (goosecoid, noggin and cardiac actin), a ventrolateral marker (Xwnt8) and a general mesodermal marker (Xbra), but not the ventral mesodermal marker globin. Differentiation of mesodermal tissues (muscle and notochord) and secondary induction of neural tissue is observed and, in many cases, explants form a well-organized axial pattern, including head structures. Based on dosage and degree of response, it can be concluded that mature Vg1 and activin  $\beta$ B have markedly similar activities on animal pole explants. Furthermore, we find a precise correspondence between the inhibition profile of mature Vg1 and that of endogenous dorsal mesoderm induction. Truncated forms of the activin and FGF receptors, which interfere with endogenous mesoderm induction, fully inhibit mesoderm induction by mature Vg1. In contrast, a truncated BMP receptor, which stimulates formation of dorsal mesoderm, does not inhibit mature Vg1. Additional experiments demonstrate that follistatin, an inhibitor of activin function, does not block endogenous dorsal mesoderm induction, and fails to block mature Vg1 activity. In addition to supporting a role for Vg1 in dorsal mesoderm induction, these results argue against the involvement of endogenous activin in dorsal mesoderm induction. In sum, our observations support a role for processed Vg1 in natural dorsal mesoderm

induction and indicate that the inhibitory effects of truncated receptors may reflect an inhibition of Vg1 signaling in the developing embryo.

Our experiments differ from previous studies of follistatin in several ways (Slack, 1991; Schulte-Merker et al., 1994). In these previous studies, an alternative splice product of the rat follistatin gene was used and this has reduced activity compared to the *Xenopus* gene (Fukui et al., 1993). Rat follistatin failed to display any biological activity at low doses, such as the neuralizing activity previously described for XFS (Hemmati-Brivanlou et al., 1994), and high doses resulted in embryo death. In addition, it is not clear if a sufficient excess of follistatin was used in the analysis of Vg1 inhibition (Schulte-Merker et al., 1994). The studies presented here utilized *Xenopus* follistatin that is active and non-toxic, even at high doses. In addition, the described preparations of concentrated, soluble mature Vg1 and XFS allowed the use of a large excess of XFS in inhibition studies, conclusively demonstrating the inability of follistatin to inhibit mature Vg1.

Overexpression of follistatin in embryos and animal pole explants can stimulate neural markers, but inhibition of endogenous mesoderm formation has not been reported (Hemmati-Brivanlou et al., 1994; Schulte-Merker et al., 1994). In contrast, our observations indicate that follistatin can block development of posterior structures, suggesting a potential role in the patterning of mesoderm. An examination of the ability of follistatin to stimulate dorsal mesoderm formation is now underway. In addition, a dose of follistatin 100-fold higher than that required to inhibit activin does not inhibit the mesoderm-inducing activity of mature Vg1. The failure of follistatin to block either mature Vg1 or endogenous induction of dorsal mesoderm is consistent with the function of endogenous Vg1 in dorsal mesoderm induction.

The mechanism of mature Vg1 inhibition by the truncated activin type II receptor is not clear. Results of receptor-binding analysis indicate that mature Vg1 does not bind to the activin type II receptor with high affinity. The TGF- $\beta$ -related receptor family is a diverse group of related molecules that form heterodimeric complexes (Massagué et al., 1994), and inhibition of signaling by overexpression of a truncated receptor could result from inactivation of an associated subunit within an existing or novel receptor complex. Therefore, the activin type II receptor could be a subunit of a Vg1 receptor complex or have no involvement in an endogenous Vg1 receptor complex.

### A model for the role of Vg1 in dorsal mesoderm formation

An oriented cortical rotation during the first cell cycle establishes initial dorsoventral polarity in *Xenopus* (Elinson and Rowing, 1988; Vincent and Gerhart, 1987). The dorsoventral pattern is reflected in the capacity of dorsal vegetal cells to induce notochord and muscle, while ventral vegetal cells induce mesenchyme, blood and small amounts of muscle (Nieuwkoop, 1969b; Boterenbrood and Nieuwkoop, 1973; Dale and Slack, 1987). In addition, as early as the 32-cell stage, dorsal vegetal blastomeres can organize a complete body axis following transplantation to the ventral vegetal region (Gimlich and Gerhart, 1984; Kageura, 1990). The dorsal vegetal cells that constitute this organizing center (Nieuwkoop center) have an endodermal fate and induce dorsal mesoderm,

resulting in formation of Spemann's organizer and axial organization (Elinson and Kao, 1989; Gerhart et al., 1989).

It is proposed that cortical rotation directs the localized processing of Vg1 precursor, resulting in formation of the Nieuwkoop center and, subsequently, the Spemann organizer. The proteolytic activation of Vg1 could be dependent on a locally activated protease or restricted release of a cleavage site inhibitor. It has been shown that ventral vegetal injection of ultraviolet-ventralized embryos with BMP2-Vg1 results in complete axial rescue and injected cells exclusively populate the endoderm, consistent with the formation of a Nieuwkoop center (Thomsen and Melton, 1993). While both noggin and Xwnt-8 injection result in axial rescue and Nieuwkoop center formation (Smith and Harland, 1991, 1992; Sokol et al., 1991), these factors, unlike Vg1, are not localized to vegetal pole blastomeres during normal development and do not induce dorsal mesoderm. Vg1 is a promising candidate for an endogenous factor required for Nieuwkoop center formation. The induction of dorsal mesoderm in explants by mature Vg1 may depend on the prior formation of an endodermal organizing center. Consistent with this suggestion would be the induction of endodermal markers by mature Vg1 and the detection of endodermal tissues in apposition to mesodermal tissues in induced explants. These predictions are currently being tested. It should be noted that the induction of an endoderm-specific antigen by mesoderm-inducing factors has been reported (Jones et al., 1993), consistent with the proposed expectations.

We are grateful to Peter Klein for critical reading of the manuscript and for the activin antiserum; Cord Dohrmann for assistance in constructing the activin  $\beta$ B-Vg1 hybrid; Olivia Kelly for assistance in the production and analysis of follistatin protein; Ali Hemmati-Brivanlou for the myc-tagged activin receptor; and Richard Harland for noggin protein and expression constructs. D. S. K. was supported by a post-doctoral fellowship from the Jane Coffin Childs Memorial Fund for Medical Research. D. A. M. received support from the Howard Hughes Medical Institute and the National Institutes of Health.

### 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.
- Asashima, M., Nakano, H., Uchiyama, H., Sugino, H., Nakamura, T., Eto, Y., Ejima, D., Nishimatsu, S. I., 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., De Robertis, E. M. and Cho, K. W. (1991). Organizer-specific homeobox genes in *Xenopus laevis* embryos. *Science* **253**, 194-196.
- Boterenbrood, E. C. and Nieuwkoop, P. D. (1973). The formation of the mesoderm in urodelean amphibians. V. Its regional induction by the endoderm. *Roux Arch. EntwMech. Org.* **173**, 319-332.
- Christian, J. L., McMahon, J. A., McMahon, A. P. and Moon, R. T. (1991). Xwnt-8, a *Xenopus* Wnt-1/int-1-related gene responsive to mesoderm inducing factors may play a role in ventral mesodermal patterning during embryogenesis. *Development* **111**, 1045-1056.
- Cornell, R. and Kimelman, D. (1994). Activin-mediated mesoderm induction requires FGF. *Development* **120**, 453-462.
- Dale, L. and Slack, J. M. W. (1987). Regional specification within the mesoderm of early embryos of *Xenopus laevis*. *Development* **100**, 279-295.
- Dale, L., Matthews, G., Tabe, L. and Colman, A. (1989). Developmental expression of the protein product of Vg1, a localized maternal mRNA in the frog *Xenopus laevis*. *EMBO J.* **8**, 1057-1065.
- Dale, L., Matthews, G. and Colman, A. (1993). Secretion and mesoderm-



- inducing activity of the TGF- $\beta$ -related domain of *Xenopus* Vg1. *EMBO J.* **12**, 4471-4480.
- Elinson, R. P. and Rowning, B.** (1988). A transient array of parallel microtubules in frog eggs: potential tracks for a cytoplasmic rotation that specifies the dorso-ventral axis. *Dev. Biol.* **128**, 185-97.
- Elinson, R. P. and Kao, K. R.** (1989). The location of dorsal information in frog early development. *Dev. Growth Diff.* **31**, 423-430.
- Evan, G. I., Lewis, G. K., Ramsay, G. and Bishop, J. M.** (1985). Isolation of monoclonal antibodies specific for human *c-myc* protooncogene product. *Mol. Cell Biol.* **5**, 3610-3616.
- Fukui, A., Nakamura, T., Sugino, K., Takio, K., Uchiyama, H., Asashima, M. and Sugino, H.** (1993). Isolation and characterization of *Xenopus* follistatin and activin. *Dev. Biol.* **159**, 131-139.
- Fukui, A., Nakamura, T., Uchiyama, H., Sugino, K., Sugino, H. and Asashima, M.** (1994). Identification of activins A, AB, and B and follistatin proteins in *Xenopus* embryos. *Dev. Biol.* **163**, 279-281.
- Gerhart, J., Danilchik, M., Doniach, T., Roberts, S., Rowning, B. and Stewart, R.** (1989). Cortical rotation of the *Xenopus* egg: consequences for the anteroposterior pattern of embryonic dorsal development. *Development* **107** (Suppl.), 37-51.
- Gimlich, R. L. and Gerhart, J. C.** (1984). Early cellular interactions promote embryonic axis formation in *Xenopus laevis*. *Dev. Biol.* **104**, 117-130.
- Graff, J. M., Thies, R. S., Song, J. J., Celeste, A. J. and Melton, D. A.** (1994). Studies with a *Xenopus* BMP receptor suggest that ventral mesoderm-inducing signals override dorsal signals in vivo. *Cell* **79**, 169-179.
- Gray, A. M. and Mason, A. J.** (1990). Requirement for activin A and transforming growth factor- $\beta$ 1 pro-regions in homodimer assembly. *Science* **247**, 1328-1330.
- Hemmati-Brivanlou, A., Kelly, O. G. and Melton, D. A.** (1994). Follistatin, an antagonist of activin, is present in the Spemann organizer and displays direct neuralizing activity. *Cell* **77**, 283-295.
- Hemmati-Brivanlou, A. and Melton, D. A.** (1992). A truncated activin receptor dominantly inhibits mesoderm induction and formation of axial structures in *Xenopus* embryos. *Nature* **359**, 609-614.
- Jones, E. A. and Woodland, H. R.** (1987). The development of animal cap cells in *Xenopus*: a measure of the start of animal cap competence to form mesoderm. *Development* **101**, 557-563.
- Jones, E. A., Abel, M. H. and Woodland, H. R.** (1993). The possible role of mesodermal growth factors in the formation of endoderm in *Xenopus laevis*. *Roux's Arch. Dev. Biol.* **202**, 233-239.
- Kageura, H.** (1990). Spatial distribution of the capacity to initiate a secondary embryo in the 32-cell embryo of *Xenopus laevis*. *Dev. Biol.* **142**, 432-8.
- Kessler, D. S. and Melton, D. A.** (1994). Vertebrate embryonic induction: mesodermal and neural patterning. *Science* **266**, 596-604.
- Kintner, C. R. and Melton, D. A.** (1987). Expression of *Xenopus* N-CAM RNA in ectoderm is an early response to neural induction. *Development* **99**, 311-325.
- Kogawa, K., Nakamura, T., Sugino, K., Takio, K., Titani, K. and Sugino, H.** (1991). Activin-binding protein is present in pituitary. *Endocrinology* **128**, 1434-1440.
- Krieg, P., Varnum, S., Wormington, M. and Melton, D. A.** (1989). The mRNA encoding elongation factor 1 $\alpha$  (EF1 $\alpha$ ) is a major transcript at the mid-blastula transition in *Xenopus*. *Dev. Biol.* **133**, 93-100.
- Krieg, P. A. and Melton, D. A.** (1987). In vitro RNA synthesis with SP6 RNA polymerase. *Meth. Enzymol.* **155**, 397-415.
- LaBonne, C. and Whitman, M.** (1994). Mesoderm induction by activin requires FGF-mediated intracellular signals. *Development* **120**, 463-472.
- Massagué, J., Attisano, L. and Wrana, J. L.** (1994). The TGF- $\beta$  family and its composite receptors. *Trends Cell Biol.* **4**, 172-178.
- Melton, D. A.** (1987). Translocation of a localized maternal mRNA to the vegetal pole of *Xenopus* oocytes. *Nature* **328**, 80-82.
- Mowry, K. and Melton, D.** (1992). Vegetal messenger RNA localization directed by a 340-nt sequence element in *Xenopus* oocytes. *Science* **255**, 991-994.
- Nakamura, T., Takio, K., Eto, Y., Shibai, H., Titani, K. and Sugino, H.** (1990). Activin-binding protein from rat ovary is follistatin. *Science* **247**, 836-838.
- Nieuwkoop, P. D. and Faber J.** (1967). Normal Table of *Xenopus laevis* (Daudin). Amsterdam: North Holland.
- Nieuwkoop, P. D.** (1969a). The formation of mesoderm in urodelean amphibians. I. Induction by the endoderm. *Roux Arch. EntwMech. Org.* **162**, 341-373.
- Nieuwkoop, P. D.** (1969b). The formation of the mesoderm in urodelean amphibians II. The origin of the dorso-ventral polarity of the mesoderm. *Roux Arch. EntwMech. Org.* **163**, 298-315.
- Nishimatsu, S., Takebayashi, K., Suzuki, A., Murakami, K. and Ueno, N.** (1993). Immunodetection of *Xenopus* bone morphogenetic protein-4 in early embryos. *Growth Factors* **8**, 173-6.
- Peng, H. B.** (1991). Solutions and protocols. In *Xenopus laevis: Practical Uses in Cell and Molecular Biology* (eds. B. K. Kay, H. B. Peng), pp. 657-662. New York: Academic Press.
- Rebagliati, M. R., Weeks, D. L., Harvey, R. P. and Melton, D. A.** (1985). Identification and cloning of localized maternal mRNAs from *Xenopus* eggs. *Cell* **42**, 769-777.
- Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L. K. and De Robertis, E. M.** (1994). *Xenopus* chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* **79**, 779-790.
- Schulte-Merker, S., Smith, J. C. and Dale, L.** (1994). Effects of truncated activin and FGF receptors and of follistatin on the inducing activities of BVg1 and activin: does activin play a role in mesoderm induction? *EMBO J.* **13**, 3533-3541.
- Slack, J. M. W.** (1991). The nature of the mesoderm-inducing signal in *Xenopus*: a transfilter induction study. *Development* **113**, 661-669.
- Slack, J. M., Isaacs, H. V., Johnson, G. E., Lettice, L. A., Tannahill, D. and Thompson, J.** (1992). Specification of the body plan during *Xenopus* gastrulation: dorsoventral and anteroposterior patterning of the mesoderm. *Development Suppl.*, 143-149.
- Slack, J. M. W. and Foreman, D.** (1980). An interaction between dorsal and ventral regions of the marginal zone in early amphibian embryos. *J. Embryol. Exp. Morph.* **56**, 283-299.
- 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*) in 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.
- Smith, W. C., Knecht, A. K., Wu, M. and Harland, R. M.** (1993). Secreted noggin protein mimics the Spemann organizer in dorsalizing *Xenopus* mesoderm. *Nature* **361**, 547-549.
- Sokol, S., Wong, G. 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**, 741-752.
- Stutz, F. and Sphor, G.** (1986). Isolation and characterization of sarcomeric actin genes expressed in *Xenopus laevis* embryos. *J. Mol. Biol.* **187**, 349-361.
- Sudarwati, S. and Nieuwkoop, P. D.** (1971). Mesoderm formation in the anuran *Xenopus laevis* (Daudin). *Roux Arch. EntwMech. Org.* **166**, 189-204.
- Suzuki, A., Thies, R. S., Yamaji, N., Song, J. J., Wozney, J. M., Murakami, K. and Ueno, N.** (1994). A truncated BMP receptor affects dorsal-ventral patterning in the early *Xenopus* embryo. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 10255-10259.
- Symes, K. and Smith, J. C.** (1987). Gastrulation movements provide an early marker of mesoderm induction in *Xenopus laevis*. *Development* **101**, 339-349.
- Tannahill, D. and Melton, D. A.** (1989). Localized synthesis of the Vg1 protein during early *Xenopus* development. *Development* **106**, 775-785.
- Tashiro, K., Yamada, R., Asano, M., Hasimoto, M., Muramatsu, M. and Shiokawa, K.** (1991). Expression of mRNA for activin-binding protein (follistatin) during early embryonic development of *Xenopus laevis*. *Biochem. Biophys. Res. Comm.* **174**, 1022-1027.
- 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.
- Thomsen, G. H. and Melton, D. A.** (1993). Processed Vg1 protein is an axial mesoderm inducer in *Xenopus*. *Cell* **74**, 433-41.
- Ueno, N., Shoda, A., Takebayashi, K., Suzuki, A., Nishimatsu, S.-I., Kikuchi, T., Wakimasu, M., Fujino, M. and Murakami, K.** (1992). Identification of bone morphogenetic protein-2 in early *Xenopus laevis* embryos. *Growth Factors* **7**, 233-240.
- Vale, W., Hsueh, A., Rivier, C. and Yu, J.** (1990). Inhibin/activin family of

- hormones and growth factors. In *Peptide growth factors and their receptors* vol. 2 (eds. M. B. Sporn and A. B. Roberts), pp. 211-236. New York: Springer-Verlag.
- Vincent, J.-P. and Gerhart, J. C.** (1987). Subcortical rotation in *Xenopus* eggs: an early step in embryonic axis specification. *Dev. Biol.* **123**, 526-539.
- Vize, P. D. and Thomsen, G. H.** (1994). Vg1 and regional specification in vertebrates: a new role for an old molecule. *Trends Genet.* **10**, 371-376.
- Weeks, D. L. and Melton, D. A.** (1987). A maternal mRNA localized to the vegetal hemisphere in *Xenopus* eggs codes for a growth factor related to TGF- $\beta$ . *Cell* **51**, 861-867.
- Wilson, P. A. and Melton, D. A.** (1994). Mesodermal patterning by an inducer gradient depends on secondary cell-cell communication. *Curr. Biol.* **4**, 676-686.
- Yamada, T.** (1950). Regional differentiation of the isolated ectoderm of the *Triturus* gastrula induced through a protein extract. *Embryologia* **1**, 1-20.
- Yisraeli, J. and Melton, D. A.** (1988). The maternal mRNA Vg1 is correctly localized following injection into *Xenopus* oocytes. *Nature* **336**, 592-595.

(Accepted 30 March 1995)