The EMBO Journal vol.13 no.15 pp.3533–3541, 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?

S.Schulte-Merker, J.C.Smith[1] and L.Dale[2]

Laboratory of Developmental Biology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA and [2]Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK

[1]Corresponding author

Communicated by J.C.Smith

Activin and Vg1, two members of the TGF-β family, are believed to play roles in mesoderm induction and axis formation in the amphibian embryo. Both molecules are provided maternally, either as protein (activin) or as RNA and protein (Vg1), and experiments with a truncated form of a type IIB activin receptor have led to the conclusion that activin is required for induction of mesoderm *in vivo*. In this paper we first show that truncated versions of two different *Xenopus* activin receptors also have severe effects on the activity of the mature region of Vg1, suggesting that such receptors may block the function of several members of the TGF-β family. We go on to demonstrate that follistatin, a secreted protein which binds activin and blocks its activity, does not interfere with Vg1 signalling. Furthermore, overexpression of follistatin mRNA in *Xenopus* embryos does not perturb mesoderm formation. Taken together, our data show that the effects of truncated activin receptors on *Xenopus* development can be explained by the inhibition of Vg1 activity, while the lack of effect of follistatin argues against a function for activin in mesoderm induction.

Key words: activin receptor/follistatin/mesoderm induction/TGF-β/Vg1

Introduction

Mesoderm formation in amphibian embryos occurs during blastula stages as the result of an inductive interaction between cells of the vegetal and animal hemispheres of the embryo (Nieuwkoop, 1969; see Sive, 1993; Slack, 1994). Candidates for mesoderm-inducing signals include members of the fibroblast growth factor (FGF) family such as bFGF (Kimelman and Kirschner, 1987; Slack *et al.*, 1987) and eFGF (Isaacs *et al.*, 1992), as well as members of the transforming growth factor β (TGF-β) family such as activins A and B (Smith *et al.*, 1990; Thomsen *et al.*, 1990), Vg1 (Dale *et al.*, 1993; Thomsen and Melton, 1993) and the bone morphogenetic proteins BMP-2 and -4 (Köster *et al.*, 1991; Dale *et al.*, 1992; Jones *et al.*, 1992).

Of these factors, activin is a particularly strong candidate for an endogenous mesoderm-inducing signal. Activin protein is present in *Xenopus* oocytes and blastulae (Asashima *et al.*, 1991a; Fukui *et al.*, 1994) and different concentrations of activin can specify at least five different cell states, including dorsal structures such as notochord (Green *et al.*, 1990, 1992). Furthermore, microinjection of activin mRNA into *Xenopus* embryos causes partial axis duplication (Thomsen *et al.*, 1990). Recently, however, some of these features have been shown to be shared by Vg1 (Dale *et al.*, 1993; Thomsen and Melton, 1993), a gene encoding a maternal mRNA which is localized to the vegetal region of the oocyte, egg and early embryo (Rebagliati *et al.*, 1985; Weeks and Melton, 1987). Although it is not known whether Vg1 is secreted and processed *in vivo* (Tannahill and Melton, 1989; Dale *et al.*, 1989, 1993), proteins consisting of the pro-regions of BMP-2 or BMP-4 fused to the C-terminal mature region of Vg1 (termed BVg1) have been shown to be biologically active. Animal caps derived from embryos which have been injected with RNA encoding BVg1 differentiate as mesoderm and injection of BVg1 RNA 'rescues' embryos made ventral by UV irradiation of their vegetal hemispheres before first cleavage (Dale *et al.*, 1993; Thomsen and Melton, 1993).

Truncated growth factor receptors provide one of the few tools for studying the roles of mesoderm-inducing factors during early *Xenopus* development. Overexpression of a truncated FGF receptor, for example, causes loss of posterior structures in *Xenopus* embryos (Amaya *et al.*, 1991). The recently characterized activin receptors are transmembrane serine/threonine kinases and they fall into two classes: type I (Attisano *et al.*, 1993) and type II (Mathews and Vale, 1991; Attisano *et al.*, 1992; Mathews *et al.*, 1992). The type II receptors can be further divided into type II and type IIB subclasses (reviewed by Massagué, 1992). In *Xenopus*, XAR1 (Hemmati-Brivanlou *et al.*, 1992) is a representative of the type IIB class, while XSTK9 belongs to the type II class (Nishimatsu *et al.*, 1992). At the RNA level, both are distributed uniformly at the mid-blastula stage, when mesoderm induction occurs (Hemmati-Brivanlou *et al.*, 1992; H.V.New and J.C.Smith, submitted for publication). Overexpression of a truncated form of XAR1, lacking most of the cytoplasmic portion of the receptor (which includes the serine/threonine kinase domain), inhibits mesoderm formation and this has been taken as strong evidence that activin signalling is required for mesoderm induction in *Xenopus* (Hemmati-Brivanlou and Melton, 1992).

In this paper we first examine the specificity with which truncated forms of XAR1 and XSTK9 inhibit signalling by members of the TGF-β family and demonstrate that they abolish signalling by both BVg1 and activin. We next study the effects of follistatin on the mesoderm-inducing activities of these factors. Follistatin is an activin binding protein (Nakamura *et al.*, 1990) which blocks activin activity in several assays, including mesoderm

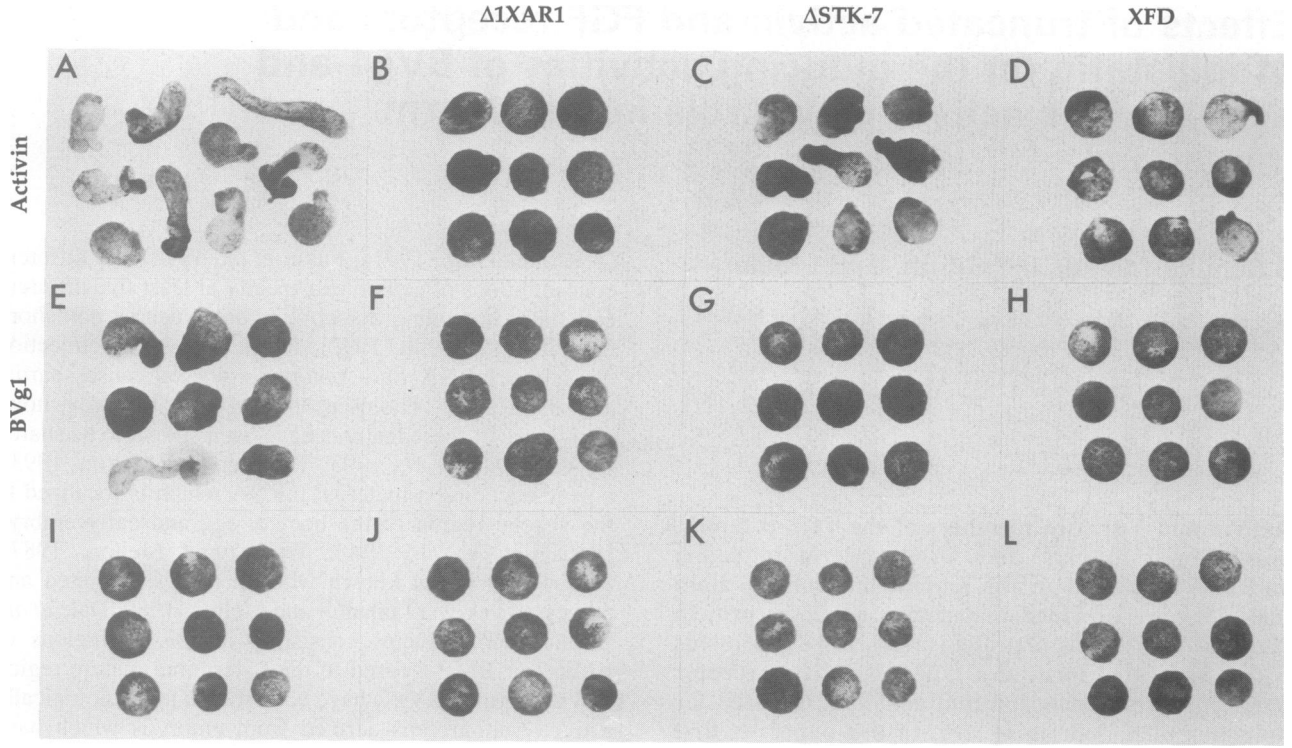

Fig. 1. Elongation of animal cap explants in response to injection of activin or BVg1 mRNA is inhibited by co-injection of truncated activin or FGF receptors. Embryos at the one-cell stage received injections of mRNA into the animal hemisphere and explants were dissected at stage 8 (mid-blastula). Photographs were taken at stage 15 and are of caps from embryos injected with (**A**) activin mRNA, (**B**) activin and Δ1XAR1 mRNA, (**C**) activin and ΔSTK−7 mRNA, (**D**) activin and XFD mRNA, (**E**) BVg1 mRNA, (**F**) BVg1 and Δ1XAR1 mRNA, (**G**) BVg1 and ΔSTK−7 mRNA, (**H**) BVg1 and XFD mRNA, (**J**) Δ1XAR1 mRNA, (**K**) ΔSTK−7 mRNA, (**L**) XFD mRNA and from uninjected cap explants (**I**). Note the complete block of elongation in (F−H).

induction (Asashima *et al.*, 1991b; Slack, 1991; Nakamura *et al.*, 1992), but we find that it has no effect on the activity of BVg1. The effects of overexpression of truncated activin receptor or follistatin mRNAs in *Xenopus* embryos are consistent with the idea that Vg1, and not activin, acts as an endogenous mesoderm-inducing factor in *Xenopus*.

Results

BVg1 activity is blocked by truncated activin and FGF receptors

Fusion of the C-terminal domain of Vg1 to the pre-proregion of *Xenopus* BMP-4 causes it to become biologically active, thus animal caps derived from embryos injected with this 'BVg1' RNA differentiate as mesodermal cell types rather than epidermis (Dale *et al.*, 1993). We have tested the abilities of two truncated activin receptors and a truncated FGF receptor to inhibit the mesoderm-inducing activity of BVg1 as well as that of activin.

The receptors used were Δ1XAR1 (Hemmati-Brivanlou and Melton, 1992), ΔSTK−7 and ΔSTK+10 (H.V.New and J.C.Smith, submitted for publication) and XFD (Amaya *et al.*, 1991). Δ1XAR1 is a truncated derivative of XAR1, a type IIB activin receptor (Hemmati-Brivanlou *et al.*, 1992). Injection of Δ1XAR1 mRNA at the one−two-cell stage blocks mesoderm formation in intact *Xenopus* embryos and inhibits the response of animal caps to activin (Hemmati-Brivanlou and Melton, 1992). ΔSTK−7 and ΔSTK+10 are derivatives of XSTK9 (Nishimatsu *et al.*, 1992), a type II activin receptor. ΔSTK+10, which retains 10 amino acids of the cytoplasmic domain, has effects on mesoderm formation similar to those of Δ1XAR1, although inhibition of ventral cell types is more complete than that of dorsal (H.V.New and J.C.Smith, submitted for publication). ΔSTK−7 is truncated within the membrane-spanning domain of XSTK9. It does not inhibit induction of mesoderm by soluble activin and does not interfere with mesoderm formation in intact embryos (H.V.New and J.C.Smith, submitted for publication), probably because it is not retained in the membrane. XFD is a truncated form of the *Xenopus* FGF receptor (Musci *et al.*, 1990) which lacks the entire cytoplasmic tyrosine kinase domain (Amaya *et al.*, 1991). Injection of XFD mRNA into early *Xenopus* embryos causes extreme trunk deficiencies without affecting head development (Amaya *et al.*, 1991).

Mouse activin A or BVg1 mRNA was injected into the animal poles of *Xenopus* embryos at the one-cell stage in the presence or absence of RNA encoding one of the three mutant receptors. Animal caps were isolated at the mid-blastula stage and then cultured. Analysis of cap morphology at neurula stages showed that both activin and BVg1 induce elongation (Figure 1A and E), although subsequent analysis showed that explants induced by our BVg1 construct contained no notochord or neural tissue and only little muscle (Figure 2A and E and Figure 3; see Dale *et al.*, 1993). Co-expression of the truncated activin receptors Δ1XAR1 (Figure 1B and F) and ΔSTK+10 (not shown) inhibits induction by both activin and BVg1.

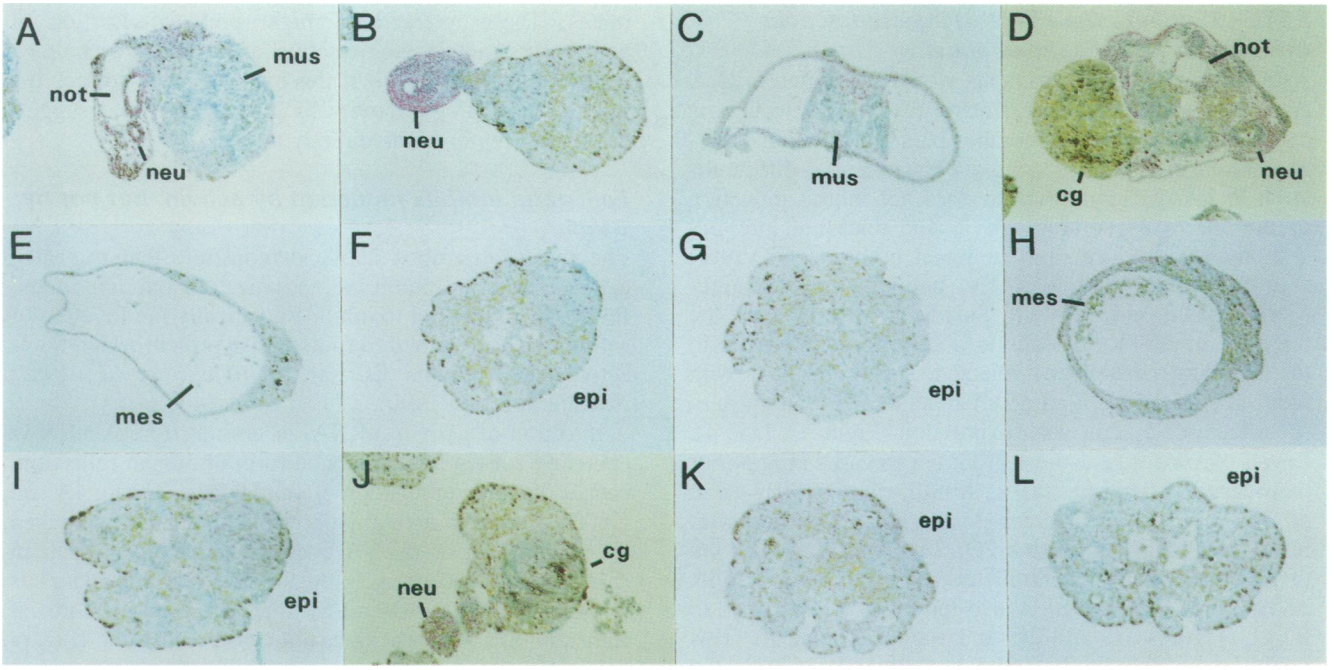

Fig. 2. Histological sections of animal cap explants cultured to stage 40, derived from embryos injected with (**A**) activin mRNA, (**B**) activin and Δ1XAR1 mRNA, (**C**) activin and ΔSTK−7 mRNA, (**D**) activin and XFD mRNA, (**E**) BVg1 mRNA, (**F**) BVg1 and Δ1XAR1 mRNA, (**G**) BVg1 and ΔSTK−7 mRNA, (**H**) BVg1 and XFD mRNA, (**J**) Δ1XAR1 mRNA, (**K**) ΔSTK−7 mRNA, (**L**) XFD mRNA and from uninjected cap explants (**I**). Mesenchyme is denoted by mes, muscle by mus, notochord by not, neural tissue by neu, cement gland by cg and epidermis by epi. Note the presence of notochord, neural tissue and cement gland in (D).

Inhibition of BVg1 activity is particularly strong; caps expressing either truncated activin receptor are indistinguishable from uninjected control caps (Figure 1I).

Surprisingly, the truncated FGF receptor XFD also inhibits activin- and BVg1-induced elongation (Figure 1D and H). While BVg1 induction was completely blocked, explants derived from activin-injected embryos elongated slightly and formed pigmented protrusions, which may represent neural tissue (see below). The inhibitory effect of XFD on induction by activin was also recently reported by LaBonne and Whitman (1994) and Cornell and Kimelman (1994).

RNase protection assays confirmed the inhibitory effects of the three truncated receptors on activin and BVg1 activity. Expression of both *Xbra*, a gene expressed in all mesoderm cells of the *Xenopus* gastrula (Smith *et al.*, 1991), and of cardiac actin, a marker for somitic muscle (Mohun *et al.*, 1988), was greatly reduced if embryos had received either XFD or Δ1XAR1 mRNA (Figure 3, lanes 1–3 and 5–7). Similar results were obtained with ΔSTK+10 (not shown). Interestingly, while XFD reduced the expression of mesodermal markers in explants which had been co-injected with activin, significant N-CAM expression remained (Figure 3, lane 2). Histological sections of such explants revealed neural tissue and, less frequently, cement gland and notochord (Figure 2D). As XFD mRNA injection causes loss only of posterior structures in intact embryos (Amaya *et al.*, 1991), we attribute this phenomenon to the inhibition of signalling pathways required for formation of posterior mesoderm, while induction of more anterior structures by activin remains unaffected. As reported previously (Hemmati-Brivanlou and Melton, 1992), injection of Δ1XAR1 mRNA

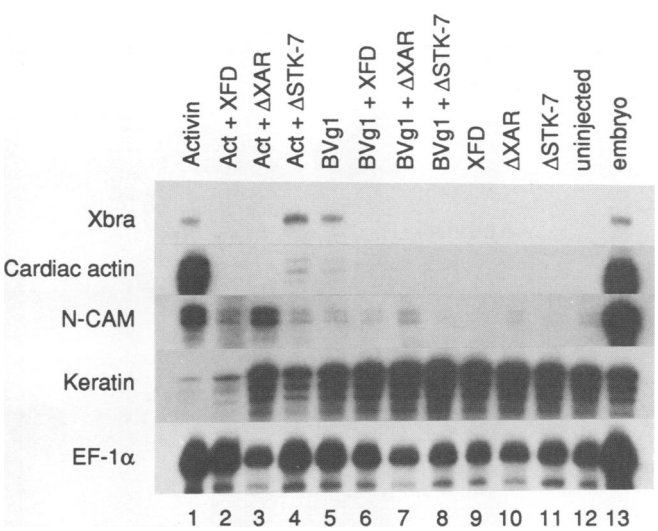

Fig. 3. Expression of mesodermal markers such as *Xbra* and actin in response to BVg1 is blocked by co-injection of Δ1XAR1, ΔSTK−7 or XFD mRNAs. RNase protection assays were carried out on animal cap explants derived from embryos injected with the indicated combinations of mRNA and then cultured to the equivalent of stage 21. EF1-α is used as a loading control.

leads to expression of N-CAM (Figure 3, lane 10) and to differentiation of neural tissue and cement gland (Figure 2J). Levels of N-CAM expression in these experiments appear to be weaker than those obtained by Hemmati-Brivanlou and Melton (1992); this may be because these authors injected 4 ng of mRNA whereas we injected only 1 ng.

ΔSTK−7 inhibits the effect of injected activin mRNA, but not of soluble activin

As a control in our experiments we have injected mRNA encoding ΔSTK−7, a derivative of XSTK9 which is truncated within the membrane-spanning domain. Overexpression of ΔSTK−7 does not cause neural differentiation in isolated animal caps, does not inhibit induction of mesoderm by soluble activin and does not interfere with mesoderm formation in intact embryos (H.V.New and J.C.Smith, submitted for publication; S.Schulte-Merker, data not shown). Surprisingly, however, the presence of ΔSTK−7 leads to a significant reduction in the inducing capacity of injected activin mRNA, with reduced elongation (Figure 1C) and a decrease in muscle-specific cardiac actin gene expression (Figure 3, lane 4). Levels of *Xbra*, a gene most strongly expressed in posterior mesoderm at neurula stages (Smith *et al.*, 1991; Gont *et al.*, 1993) were increased and this, together with histological analysis (Figure 2C), suggested that injection of ΔSTK−7 mRNA ventralizes the response to activin. ΔSTK−7 mRNA injection also inhibited the response to BVg1 (Figures 1G and 2G and Figure 3, lane 8). This inhibition appeared to be complete, although, as indicated above, the BVg1 construct used in our experiments induces only ventral mesoderm.

The ability of ΔSTK−7 mRNA to inhibit the function of activin mRNA is likely to be due to intracellular interactions between ΔSTK−7 and activin, perhaps in the endoplasmic reticulum or in one of the Golgi compartments. These interactions might prevent secretion of activin or result in the secretion of an inactive complex of activin and ΔSTK−7. If this explanation is correct, the inhibition of BVg1 activity by ΔSTK−7 would suggest that this receptor binds directly to Vg1.

Follistatin inhibits induction by activin, but not by BVg1

The results described above demonstrate that truncated activin receptors inhibit the activities of both activin and BVg1. In an attempt to inhibit specifically the function of activin we have turned to follistatin, a protein which binds activin and inhibits its function (Ueno *et al.*, 1987; Nakamura *et al.*, 1990).

Injection of activin mRNA, or co-injection of mRNAs encoding activin and a mutated form of human follistatin, causes dramatic elongation of animal caps (Figure 4A and C). This effect of activin is, however, completely blocked by co-injection of mRNA encoding wild-type follistatin (Figure 4B). In contrast, elongation induced by BVg1 is unaffected (Figure 4D and E). Injection of either form of follistatin mRNA alone does not cause elongation (Figure 4H and I).

Histological analysis (Figure 5) and RNase protection assays (Figure 6) confirm that follistatin blocks induction by activin, but not by BVg1. Induction of *Xbra* and N-CAM by activin mRNA is inhibited by co-injection of wild-type follistatin mRNA (Figure 6, lane 2) and levels of keratin are high compared with explants injected with

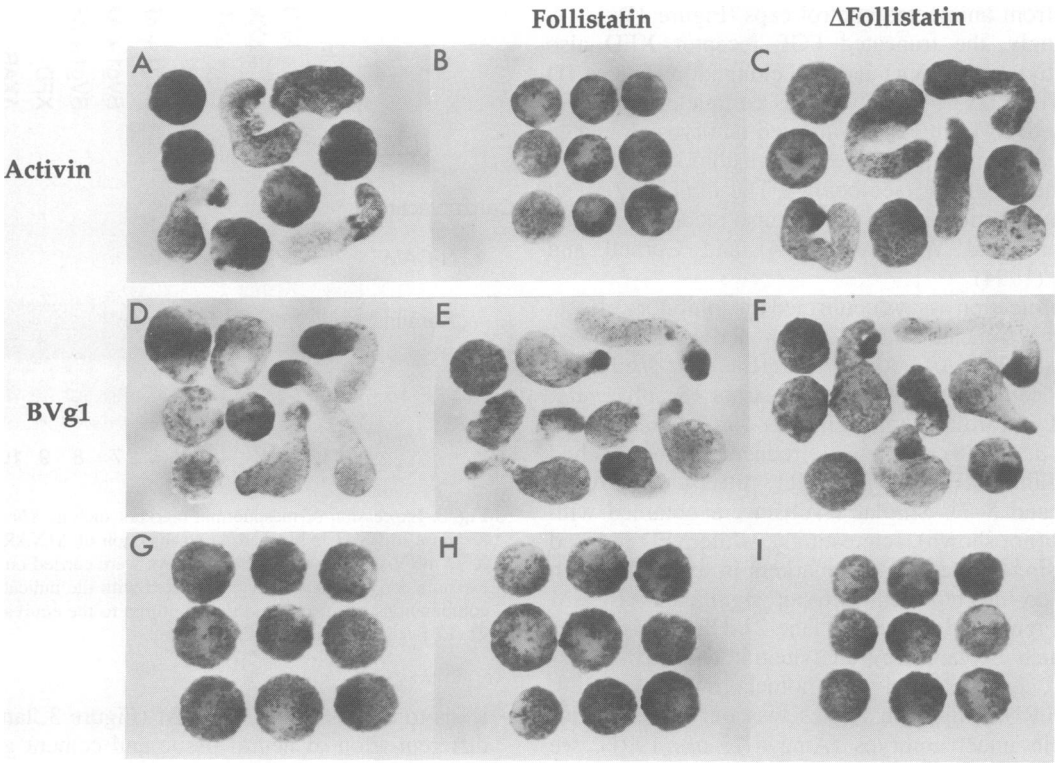

Fig. 4. Injection of follistatin mRNA inhibits activin-induced elongation of animal cap explants, but does not affect elongation due to BVg1. Embryos at the one-cell stage received injections of mRNA into the animal hemisphere and explants were dissected at stage 8 (mid-blastula). Photographs were taken at stage 15 and are of caps from embryos injected with (**A**) activin mRNA, (**B**) activin and follistatin mRNA, (**C**) activin and mutant follistatin mRNA, (**D**) BVg1 mRNA, (**E**) BVg1 and follistatin mRNA, (**F**) BVg1 and mutant follistatin mRNA, (**H**) follistatin mRNA, (**I**) mutant follistatin mRNA and from uninjected cap explants (**G**).

activin mRNA alone (Figure 6, lane 1) or with activin mRNA plus mutant follistatin mRNA (Figure 6, lane 3). At the histological level, explants expressing activin alone or activin plus mutated follistatin formed notochord, muscle and neural tissue (Figure 5A and C), whereas explants expressing activin and wild-type follistatin formed atypical epidermis (Figure 5B). Follistatin did not inhibit induction by BVg1 (Figure 5D–F and Figure 6, lanes 4–6).

A weak but reproducible increase in N-CAM expression was observed in caps injected with follistatin mRNA alone or with BVg1 and follistatin (Figure 6, lanes 5 and 7), although histological analysis did not reveal differentiation of neural tissue (Figure 5E and H). We assessed the relative efficiencies of follistatin, noggin (Lamb *et al.*, 1993; Cunliffe and Smith, 1994) and Δ1XAR1 (Hemmati-Brivanlou and Melton, 1992) to induce expression of N-CAM and found follistatin to be a much weaker inducer than noggin or Δ1XAR1 (data not shown).

Follistatin does not block mesoderm formation in whole embryos

The above results indicate that follistatin blocks the mesoderm-inducing activity of activin, but not of BVg1. In order to block the function of maternally supplied activin protein in the embryo (Asashima *et al.*, 1991a; Fukui *et al.*, 1994), it was necessary to show that injection of follistatin mRNA, unlike injection of ΔSTK−7 mRNA (see above), inhibits the effects of soluble activin. To investigate this, animal cap explants derived from embryos injected with wild-type or mutant follistatin mRNAs were incubated in medium containing 4, 8, 16 or 32 units/ml activin. Injected follistatin mRNA (1 ng) was found to be sufficient to inhibit between 8 and 16 units/ml activin (Table I), demonstrating that follistatin can block externally supplied activin.

To investigate the role of activin in normal development, we then injected wild-type follistatin mRNA into the equatorial region of early cleavage stage *Xenopus* embryos. In a series of seven experiments, over 500 embryos were injected with amounts of follistatin ranging from 100 pg to 2 ng. Higher concentrations of follistatin were toxic, but even these did not inhibit the onset of gastrulation (Figure 7A), indicating that mesoderm induction had occurred. Lower amounts of RNA, such as 100 pg, had no visible effect on development (Figure 7B). We note that 100 pg of follistatin mRNA is sufficient to block an amount of mammalian (Figure 5A and B) or *Xenopus* (not shown) activin mRNA which induces formation of notochord, muscle and neural tissue in animal caps.

To our surprise, mutant follistatin also inhibited the action of *Xenopus* activin B. While this finding does not affect the conclusions of the above experiment, it raises the question of why we did not observe N-CAM expression

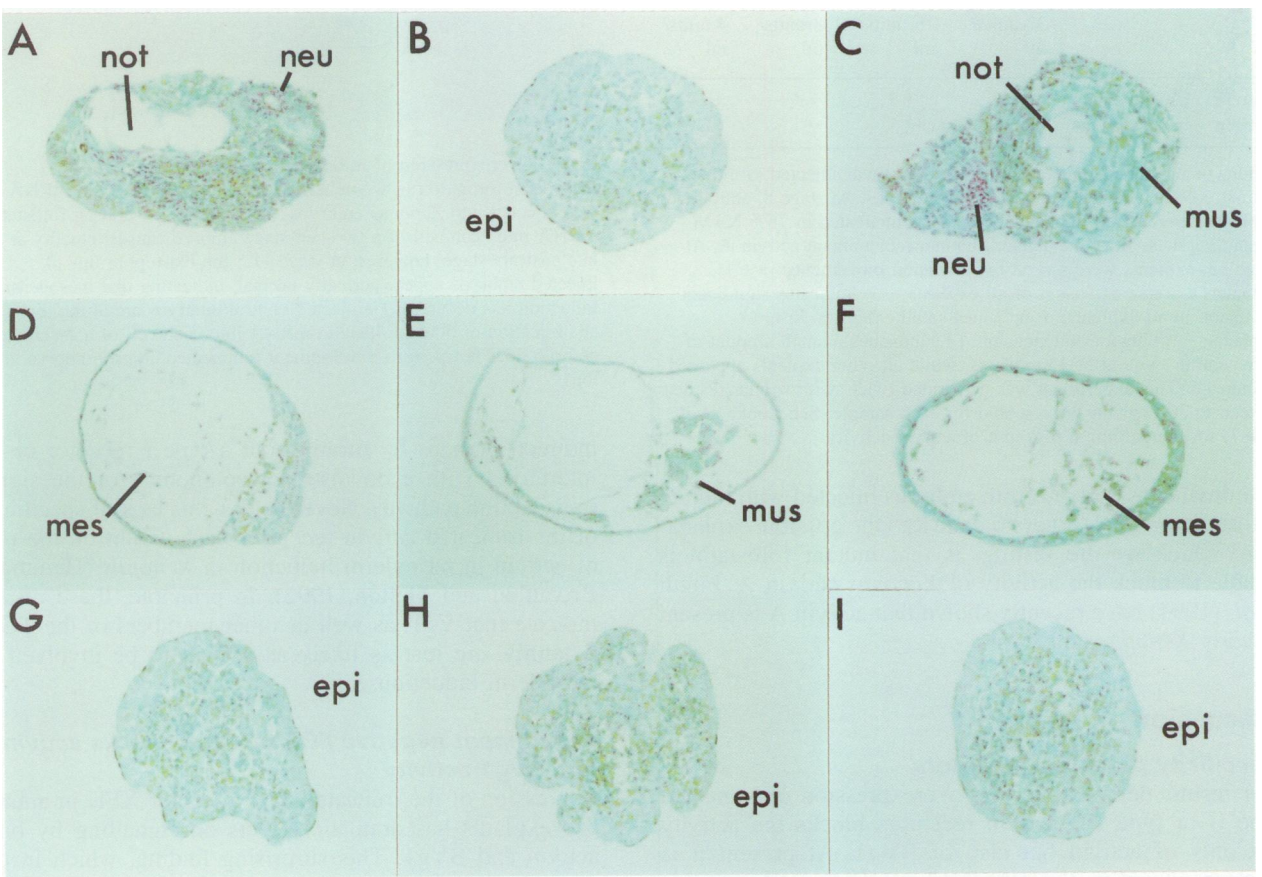

Fig. 5. Histological sections of animal cap explants cultured to stage 40, derived from embryos injected with (**A**) activin mRNA, (**B**) activin and follistatin mRNA, (**C**) activin and mutant follistatin mRNA, (**D**) BVg1 mRNA, (**E**) BVg1 and follistatin mRNA, (**F**) BVg1 and mutant follistatin mRNA, (**H**) follistatin mRNA, (**I**) mutant follistatin mRNA and from uninjected cap explants (**G**). Mesenchyme is denoted by mes, muscle by mus, notochord by not, neural tissue by neu and epidermis by epi.

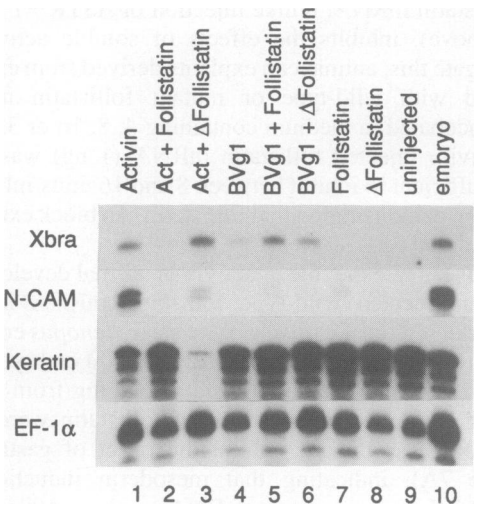

Fig. 6. Follistatin suppresses the expression of mesodermal and neural markers in activin-, but not in BVg1-induced caps. RNase protection assays were carried out on animal cap explants (whole embryo stage 21) derived from embryos injected with activin or BVg1 mRNA, with wild-type or mutant follistatin mRNA or with a combination thereof. EF1-α is the probe used as a loading control. Note the slight increase in levels of N-CAM in lanes 5 and 7.

Table I. Follistatin mRNA injection inhibits the effect of soluble activin

	32 units/ml	16 units/ml	8 units/ml	4 units/ml
Activin + Δfollistatin	+++	+++	++	+
Activin + follistatin	+	(+)	−	−

Wild-type or mutant follistatin mRNA (1 ng) was injected into the animal pole of one-cell stage *Xenopus* embryos. At stage 8, animal cap explants derived from these embryos were incubated in 75% NAM containing 4, 8, 16 or 32 units/ml human recombinant activin A. At stage 15, explants were scored for extension movements: +++ indicates extreme extension of all explants; ++ indicates significant extension in all explants; + indicates small extension in most explants; − indicates no extension; (+) indicates a small amount of extension in two out of 15 explants, while all other explants appeared uninduced. This experiment was performed twice, with similar results. Fifteen to 20 explants were scored for each sample. See Cooke *et al.* (1987) for the definition of a unit of activin activity.

in animal caps derived from embryos injected with mutant follistatin mRNA (Figure 6, lane 8). One possible explanation, which we are testing, is that mutant follistatin is unable to block the activity of *Xenopus* activin A. Fukui *et al.* (1994) have recently shown that activin A is present in early *Xenopus* embryos.

Discussion

Specificity of activin receptors

Our results demonstrate that overexpression of truncated type II or type IIB activin receptors blocks the activity not only of activin but also of BVg1. At present it is unclear where in the signal transduction pathway this inhibition occurs. Soluble Vg1 protein is not yet available, so it is not known whether Vg1 interacts directly with Δ1XAR1 and ΔSTK+10 or whether the inhibition is

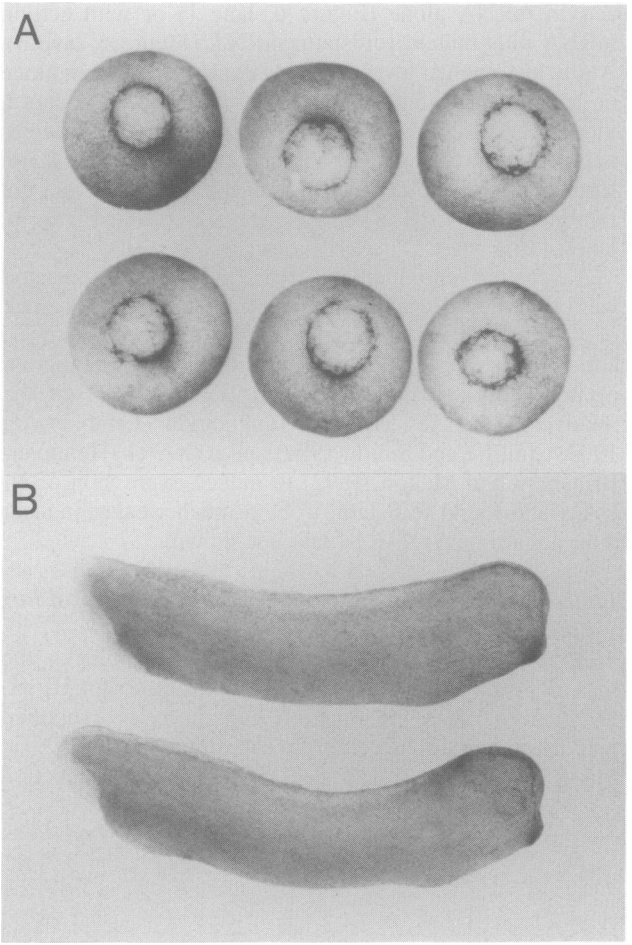

Fig. 7. Overexpression of follistatin mRNA does not perturb mesoderm formation in whole *Xenopus* embryos. Follistatin mRNA was injected into *Xenopus* embryos. (**A**) Injection of 500 pg follistatin mRNA into each cell of a two-cell stage embryo causes lethality at the late gastrula stage, however, at stage 11.5 the blastopore lips of injected embryos appear perfectly normal, indicating that mesoderm induction has occurred. Uninjected control embryos are at the top. (**B**) Injection of 50 pg follistatin mRNA into each cell of a 2-cell stage embryo does not perturb development (uninjected control embryo at top).

indirect, perhaps by titrating out a type I receptor or by interfering with a downstream component of the signal transduction pathway. Nevertheless, this lack of specificity of the truncated activin receptors casts doubt on the role of activin in mesoderm induction in *Xenopus* (Hemmati-Brivanlou and Melton, 1992). In principle, these results indicate that Vg1, as well as other members of the TGF-β family, are just as likely as activin to be involved in mesoderm induction.

A dominant negative FGF receptor blocks activin and BVg1 activity

Expression of the truncated FGF receptor XFD in animal cap explants has dramatic effects on signalling by both activin and BVg1. This surprising finding, which in the case of activin has recently been made by others (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994), is consistent with the observation that in intact embryos XFD blocks formation only of posterior tissues (Amaya

et al., 1991). Thus the presence of XFD does not inhibit formation of anterior structures in response to activin (see Figure 3D), but does suppress differentiation of muscle and more posterior tissues. In contrast, XFD completely blocks the effects of BVg1 (Figures 1H and 2H and Figure 3, lane 6), overexpression of which leads to the formation exclusively of posterior mesoderm.

In contrast to Cornell and Kimelman (1994), we observe N-CAM expression (Figure 3, lane 2) and neural tissue (Figure 2D) in animal caps derived from embryos injected with both activin and XFD mRNA. This difference may be due to the use of soluble activin protein by Cornell and Kimelman (1994), rather than injection of activin mRNA.

Follistatin can distinguish between activin and BVg1

Follistatin is a specific inhibitor of activin, but not of any other member of the TGF-β family that has been tested (Nakamura *et al.*, 1990). The experiments described in this paper confirm that the activity of mouse activin A and of *Xenopus* activin B is inhibited by follistatin and go on to demonstrate that, in contrast, the activity of BVg1 is unaffected (Figures 4–6). Use of follistatin can therefore distinguish between signalling due to activin and that due to Vg1 or other members of the TGF-β family.

Follistatin: a neural-inducing factor?

Overexpression of follistatin in *Xenopus* animal pole regions results in a small, but reproducible, increase in levels of expression of N-CAM (Figure 6, lane 7). A similar increase is also observed in animal pole regions co-expressing BVg1 and follistatin (Figure 6, lane 5). Although neural tissue is not visible in histological sections, this increase in N-CAM expression may nevertheless be significant. Overexpression of the truncated activin receptors ΔIXAR1 and ΔSTK+10 leads to formation of neural tissue (Figure 2J; Hemmati-Brivanlou and Melton, 1992; H.V.New and J.C.Smith, submitted for publication) and if this effect occurs exclusively through inhibition of activin signalling, then one would expect follistatin also to induce neural tissue. Indeed, a recent study by Hemmati-Brivanlou *et al.* (1994) has shown that the short form of *Xenopus* follistatin acts as a neural-inducing agent, as monitored by the expression of neural markers such as N-CAM and β-tubulin isotype II. The much weaker level of N-CAM expression observed in response to the long form of human follistatin used in our studies might reflect a difference in the neural-inducing capacities of the long and the short forms of the protein, or might be due to the species difference.

Follistatin mRNA is expressed in the dorsal marginal zone, or Spemann's organizer, of the *Xenopus* embryo (Hemmati-Brivanlou *et al.*, 1994) and this is consistent with a role for follistatin in neural induction. In the early mouse embryo, however, follistatin mRNA is expressed throughout the primitive streak and transcripts are absent from the ventral portion of the node (Albano *et al.*, 1994), the region which corresponds to Spemann's organizer (see Beddington, 1994). It will be important to study the expression patterns of follistatin protein, as well as mRNA, in these two organisms in order to resolve this apparent discrepancy.

Overexpression of follistatin mRNA does not interfere with mesoderm formation

The experiments discussed above demonstrate that overexpression of follistatin mRNA inhibits the action of activin, but not that of the mature region of Vg1. Follistatin therefore provides a useful tool for distinguishing between signalling by these two factors. In an attempt to study the role of activin in early *Xenopus* development, embryos were therefore injected with follistatin mRNA. This treatment inhibited the effects of exogenous *Xenopus* activin B, but caused no significant perturbation of development, suggesting that activin does not play a major role in early *Xenopus* development and that the dramatic effects of ΔIXAR1 (Hemmati-Brivanlou and Melton, 1992) and ΔSTK+10 (H.V.New and J.C.Smith, submitted for publication) are due to inhibition of other signalling molecules, such as Vg1. These results are consistent with those of Slack (1991), who has used a transfilter apparatus to demonstrate that follistatin does not inhibit the natural mesoderm-inducing signal derived from the vegetal pole region of the embryo. In addition, Vassalli *et al.* (1994) have recently shown that mice deficient in the activin β_B subunit nevertheless form mesoderm and exhibit no gastrulation phenotype.

Conclusion

The data presented in this paper suggest that activin plays a minor role in early *Xenopus* development and that the effects of truncated activin receptors might better be explained by the inhibition of other members of the TGF-β family, such as Vg1. Vg1 mRNA is localized to the appropriate region of the embryo at the right time to play a role in mesoderm induction (Rebagliati *et al.*, 1985; Weeks and Melton, 1987) and its mature region has the required inducing activity (Dale *et al.*, 1993; Thomsen and Melton, 1993). Future work should address the question of whether small amounts of Vg1 are secreted and processed *in vivo* (Dale *et al.*, 1989, 1993; see Tannahill and Melton, 1989) and, if so, how this is controlled.

Material and methods

Embryo culture and manipulation

Xenopus embryos were obtained by artificial fertilization as described by Smith and Slack (1983) and staged according to Nieuwkoop and Faber (1967). They were allowed to develop in 75% NAM (Slack, 1984) and dejellied with 2% cysteine hydrochloride (pH 7.8–8.1) after they had rotated. Animal caps were dissected at the mid-blastula stage and cultured as previously described (Cunliffe and Smith, 1994).

In vitro transcription

DNA templates used for *in vitro* transcription were as follows.

(i) Mouse activin A (Albano *et al.*, 1993) and *Xenopus* activin B (Thomsen *et al.*, 1990) were inserted into the vector pSP64T (Krieg and Melton, 1984). We thank Harvey Isaacs of Jonathan Slack's laboratory (ICRF, Oxford) for supplying the *Xenopus* clone.

(ii) Plasmids pSV2HF315 and 449 (wild-type and mutant human follistatin; Inouye *et al.*, 1991) were the kind gift of S.Shimasaki. Both were digested with *Stu*I and *Bam*HI and the fragments encoding follistatin were cloned into pSP64T to generate pSP64T-hfol and pSP64T-Δhfol respectively. Both plasmids were linearized with *Eco*RI prior to *in vitro* transcription. The mutant form of follistatin, which is unable to bind activin in a ligand-blotting assay, results from an in-frame insertion of two amino acids at the N-terminus (Inouye *et al.*, 1991).

(iii) ΔIXAR1 (Hemmati-Brivanlou and Melton, 1992) was a kind gift of Ali Hemmati-Brivanlou and Doug Melton.

(iv) The two truncated versions of XSTK9, ΔSTK−7 and ΔSTK+10, have been described by H.V.New and J.C.Smith (submitted for publication). ΔSTK+10 lacks the serine/threonine kinase domain but retains 10 amino acids of the cytoplasmic domain followed by a stop codon. In ΔSTK−7 the truncation occurs seven amino acids before the start of the cytoplasmic domain.

(v) BVg1, inserted into the vector pSP64T, has been described by Dale *et al.* (1993).

(vi) XFD (Amaya *et al.*, 1991) was a kind gift of Enrique Amaya and Marc Kirschner.

In vitro transcription was as described by Smith (1993), after which RNA was precipitated three times with ethanol in the presence of 5 mM EGTA and resuspended in DEPC-treated water.

Microinjection

Embryos to be microinjected were kept at 14°C and cultured in 75% NAM containing 4% Ficoll Type 400 (Sigma). Injection was carried out as previously described (Smith, 1993). Unless otherwise stated, the following quantities of RNA were injected into the animal pole region of a one-cell embryo, in a volume of ~14 nl: 2 pg mouse activin A mRNA, 2 pg *Xenopus* activin B mRNA, 500 pg BVg1 mRNA, 1 ng XFD mRNA, 1 ng ΔSTK−7 mRNA, 1 ng ΔSTK+10 mRNA, 1 ng Δ1XAR1 mRNA or 100 pg of wild-type or mutant follistatin mRNA.

RNA isolation and RNase protection assays

RNA from animal cap explants and whole embryos was isolated as described by Cho and De Robertis (1990) with minor modifications. Briefly, frozen tissue (up to 40 animal caps or five embryos) was homogenized in 300 μl 40 mM Tris–HCl (pH 7.5), 4 mM EDTA, 1.7% SDS, 209 mM NaCl and 2 mg/ml proteinase K. After incubation at 37°C for 15 min, two extractions with phenol:chloroform:isoamyl alcohol (25:24:1) were carried out and 10 μg tRNA and 1 vol 6 M LiCl were added to the aqueous phase. RNA was then precipitated overnight at −20°C. The precipitate was washed with cold 70% ethanol and the RNA redissolved in DEPC-treated water.

RNase protections were carried out essentially as described by Cho and De Robertis (1990). Probes were: N-CAM (as in Cunliffe and Smith, 1994), *Xbra* (Smith *et al.*, 1991), EF-1α (as in Sargent and Bennett, 1990), actin (Mohun *et al.*, 1988) and keratin (as in Cunliffe and Smith, 1992). Control hybridizations with 10 μg tRNA were always negative and are not shown in Figures 3 and 6.

Histological procedures

Animal cap explants were fixed overnight at 4°C in 3.7% formaldehyde, 50% ethanol, 2% acetic acid, 40% NAM. Following further fixation in 3.7% formaldehyde/phosphate-buffered saline (for at least 4 h), the caps were gradually dehydrated and embedded in paraffin wax. Sections (7 μm) were cut, stained and analysed as described by Green *et al.* (1990).

Acknowledgements

We are grateful to Wendy Hatton for expert histology, the NIMR photographic department for help with figures, members of the Smith Laboratory for discussions and Ansgar Schulte for babysitting. We thank everyone who sent us cDNAs and plasmids. S.S.-M. was supported by an EMBO long-term fellowship and by a fellowship from HFSPO. J.C.S. is a Howard Hughes International Research Scholar.

References

Albano,R.M., Groome,N. and Smith,J.C. (1993) *Development*, **117**, 711–723.

Albano,R.M., Arkell,R., Beddington,R.S.P. and Smith,J.C. (1994) *Development*, **120**, 803–813.

Amaya,E., Musci,T.J. and Kirschner,M.W. (1991) *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. (1991a) *Proc. Natl Acad. Sci. USA*, **88**, 6511–6514.

Asashima,M., Nakano,H., Uchiyama,H., Sugino,H., Nakamura,T., Eto,Y., Ejima,D., Davids,M., Plessow,S., Cichocka,I. and Kinoshita,K. (1991b) *Wilhelm Roux's Arch. Dev. Biol.*, **200**, 4–7.

Attisano,L., Wrana,J.L., Cheifetz,S. and Massagué,J. (1992) *Cell*, **68**, 97–108.

Attisano,L., Carcamo,J., Ventura,F., Weis,F.M.B., Massagué,J. and Wrana,J.L. (1993) *Cell*, **75**, 671–680.

Beddington,R.S.P. (1994) *Development*, **120**, 613–620.

Cho,K.W.Y. and De Robertis,E.M. (1990) *Genes Dev.*, **4**, 1910–1916.

Cooke,J., Smith,J.C., Smith,E.J. and Yaqoob,M. (1987) *Development*, **101**, 893–908.

Cornell,R.A. and Kimelman,D. (1994) *Development*, **120**, 453–462.

Cunliffe,V. and Smith,J.C. (1992) *Nature*, **358**, 427–430.

Cunliffe,V. and Smith,J.C. (1994) *EMBO J.*, **13**, 349–359.

Dale,L., Matthews,G., Tabe,L. and Colman,A. (1989) *EMBO J.*, **8**, 1057–1065.

Dale,L., Howes,G., Price,B.M.J. and Smith,J.C. (1992) *Development*, **115**, 573–585.

Dale,L., Matthews,G. and Colman,A. (1993) *EMBO J.*, **12**, 4471–4480.

Fukui,A., Nakamura,T., Uchiyama,H., Sugino,K., Sugino,H. and Asashima, M. (1994) *Dev. Biol.*, **163**, 279–281.

Gont,L.K., Steinbeisser,H., Blumberg,B. and De Robertis,E.M. (1993) *Development*, **119**, 991–1004.

Green,J.B.A., Howes,G., Symes,K., Cooke,J. and Smith,J.C. (1990) *Development*, **108**, 173–183.

Green,J.B.A., New,H.V. and Smith,J.C. (1992) *Cell*, **71**, 731–739.

Hemmati-Brivanlou,A. and Melton,D.A. (1992) *Nature*, **359**, 609–614.

Hemmati-Brivanlou,A., Wright,D.A. and Melton,D.A. (1992) *Dev. Dyn.*, **194**, 1–11.

Hemmati-Brivanlou,A., Kelly,O.G. and Melton,D.A. (1994) *Cell*, **77**, 283–295.

Inouye,S., Guo,Y., Ling,N. and Shimasaki,S. (1991) *Biochem. Biophys. Res. Commun.*, **179**, 352–358.

Isaacs,H.V., Tannahill,D. and Slack,J.M.W. (1992) *Development*, **114**, 711–720.

Jones,C.M., Lyons,K.M., Lapan,P.M., Wright,C.V.E. and Hogan,B.L.M. (1992) *Development*, **115**, 639–647.

Kimelman,D. and Kirschner,M.W. (1987) *Cell*, **51**, 869–877.

Köster,M., Plessow,S., Clement,J.H., Lorenz,A., Tiedemann,H. and Knöchel,W. (1991) *Mech. Dev.*, **33**, 191–199.

Krieg,P.A. and Melton,D.A. (1984) *Nucleic Acids Res.*, **12**, 7057–7070.

LaBonne,C. and Whitman,M. (1994) *Development*, **120**, 463–472.

Lamb,T.M., Knecht,A.K., Smith,W.C., Stachel,S.E., Economides,A.N., Stahl,N., Yancopolous,G.D. and Harland,R.M. (1993) *Science*, **262**, 713–718.

Massagué,J. (1992) *Cell*, **69**, 1067–1070.

Matthews,L.S. and Vale,W.W. (1991) *Cell*, **65**, 973–982.

Matthews,L.S., Vale,W.W. and Kintner, C.R. (1992) *Science*, **255**, 1702–1705.

Mohun,T.J., Garrett,N., Stutz,F. and Spohr,G. (1988) *J. Mol. Biol.*, **202**, 67–76.

Musci,T.J., Amaya,E. and Kirschner,M.W. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 8365–8369.

Nakamura,T., Takio,K., Eto,Y., Shibai,H., Titani,K. and Sugino,H. (1990) *Science*, **247**, 836–838.

Nakamura,T., Hasegawa,Y., Sugino,K., Kogawa,K., Titani,K. and Sugino,H. (1992) *Biochim. Biophys. Acta*, **1135**, 103–109.

Nieuwkoop,P.D. (1969) *Wilhelm Roux's Arch EntwMech Org.*, **162**, 341–373.

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

Nishimatsu,S.-I., Oda,S., Murakami,K. and Ueno,N. (1992) *FEBS Lett.*, **303**, 81–84.

Rebagliati,M.R., Weeks,D.L., Harvey,R.P. and Melton,D.A. (1985) *Cell*, **42**, 769–777.

Sargent,M.G. and Bennett,M.F. (1990) *Development*, **109**, 967–973.

Sive,H.L. (1993) *Genes Dev.*, **7**, 1–12.

Slack,J.M.W. (1984) *J. Embryol. Exp. Morphol.*, **80**, 289–319.

Slack,J.M.W. (1991) *Development*, **113**, 661–669.

Slack,J.M.W. (1994) *Curr. Biol.*, **4**, 116–126.

Slack,J.M.W., Darlington,B.G., Heath,J.K. and Godsave,S.F. (1987) *Nature*, **326**, 197–200.

Smith,J.C. (1993) In Hartley,D. (ed.), *Cellular Interactions in Development—A Practical Approach*. Oxford University Press, Oxford, pp. 181–204.

Smith,J.C. and Slack,J.M.W. (1983) *J. Embryol. Exp. Morphol.*, **78**, 299–317.

Smith,J.C., Price,B.M.J., Van Nimmen,K. and Huylebroeck,D. (1990) *Nature*, **345**, 729–731.

Smith,J.C., Price,B.M.J., Green,J.B.A., Weigel,D. and Herrmann,B.G. (1991) *Cell*, **67**, 79–87.

Tannahill,D. and Melton,D.A. (1989) *Development*, **106**, 775–785.
Thomsen,G. and Melton,D.A. (1993) *Cell*, **74**, 433–441.
Thomsen,G., Woolf,T., Whitman,M., Sokol,S., Vaughan,J., Vale,W. and Melton,D.A. (1990) *Cell*, **63**, 485–493.
Ueno,N., Ling,N., Ying,S.-Y., Esch,F., Shimasaki,S. and Guillemin,R. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 8282–8286.
Vassalli,A., Matzuk,M.M., Gardner,H.A.R., Lee,K.-F. and Jaenisch,R. (1994) *Genes Dev.*, **8**, 414–427.
Weeks,D.L. and Melton,D.A. (1987) *Cell*, **51**, 861–867.

Received on April 25, 1994; revised on May 31, 1994