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Xenopus axis formation: induction of *goosecoid* by injected *Xwnt-8* and activin mRNAs

Herbert Steinbeisser[1,*], Eddy M. De Robertis[1], Min Ku[2], Daniel S. Kessler[2] and Douglas A. Melton[2]

[1]Molecular Biology Institute and Department of Biological Chemistry, University of California, Los Angeles, CA 90024-1737, USA
[2]Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138, USA

*Author for correspondence

SUMMARY

In this study, we compare the effects of three mRNAs - *goosecoid,* activin and *Xwnt-8* - that are able to induce partial or complete secondary axes when injected into *Xenopus* embryos. *Xwnt-8* injection produces complete secondary axes including head structures whereas activin and *goosecoid* injection produce partial secondary axes at high frequency that lack head structures anterior to the auditory vesicle and often lack notochord. *Xwnt-8* can activate *goosecoid* only in the deep marginal zone, i.e., in the region in which this organizer-specific homeobox gene is normally expressed on the dorsal side. Activin B mRNA, however, can turn on *goosecoid* in all regions of the embryo. We also tested the capacity of these gene products to restore axis formation in embryos in which the cortical rotation was blocked by UV irradiation. Whereas *Xwnt-8* gives complete rescue of anterior structures, both *goosecoid* and activin give partial rescue. Rescued axes including hindbrain structures up to level of the auditory vesicle can be obtained at high frequency even in the absence of notochord structures. The possible functions of *Wnt*-like and activin-like signals and of the *goosecoid* homeobox gene, and their order of action in the formation of Spemann's organizer are discussed.

Key words: *Xenopus laevis*, *goosecoid*, *Wnt*, activin, axis formation, mesoderm induction

INTRODUCTION

Formation of the body axis in the vertebrate embryo results from a series of sequential inductive cell-cell interactions. The unfertilized frog egg is radially symmetric, but within one hour after sperm entry the dorsoventral polarity is established by a cortical rotation of the egg cytoplasm (Gerhart et al., 1989). At about the 32-cell stage a signal inducing dorsal mesoderm emanates from the dorsal-vegetal blastomeres that constitute the so-called 'Nieuwkoop center' (Nieuwkoop, 1973; Gimlich and Gerhart, 1984; Jones and Woodland, 1987). This signal then induces axis-forming properties in the overlying cells, in a region known as Spemann's organizer. The organizer tissue consists of an area of about 60˚ of arc in the dorsal marginal zone and, upon transplantation, can recruit cells from the host embryo to form a new (secondary) body axis (Spemann and Mangold, 1924; Spemann, 1938; Stewart and Gerhart, 1990).

One of the recent advances in embryology is the realization that these inductive events may be mediated by growth factors of the FGF, TGF-beta (in particular activins) and *Wnt* families of cell signalling molecules (for reviews see Green and Smith, 1991; Kimelman et al., 1992; Woodland, 1993). A novel dorsalizing agent, *noggin*, which encodes a secreted protein and is expressed in the unfertilized egg and organizer region, has been reported recently (Smith and Harland, 1992). Furthermore, several transcription factors have been found to be expressed in the dorsal lip (organizer) region of the *Xenopus* gastrula (Blumberg et al., 1991; Taira et al., 1992; Dirksen and Jamrich, 1992; Knoechel et al., 1992; Ruiz i Altaba and Jessell, 1992). In this paper, we analyze the activities of three of these molecules, *Xwnt-8*, activin and the homeobox gene *goosecoid* (*gsc*), all of which are able to induce, to varying extents, the formation of secondary body axes in *Xenopus* embryos.

Wnt proteins are extracellular signaling molecules. Production of soluble, biologically active *Wnt* proteins has not been successful and therefore all studies in *Xenopus* have been carried out by injection of synthetic mRNA of either *Wnt*-1, a gene expressed in the CNS (McMahon and Moon, 1989), or *Xwnt-8*, which is normally expressed on the ventral side of the gastrula (Christian et al., 1991). Despite the fact that neither gene is expressed in the embryo at the time when the Nieuwkoop center and the Spemann organizer are first established, both have strong dorsalizing effects when injected as mRNA. Current thinking is that *Wnt*-1 and *Xwnt-8* mimic the effect of an as yet unidentified member of this family of growth factors that specifies dorsal mesoderm early in development. Microinjection of *Xwnt-8* mRNA leads to the formation of secondary axes and to the rescue of axial structures in embryos ventralized by UV irradiation (Sokol et al., 1991; Smith and Harland, 1991). UV

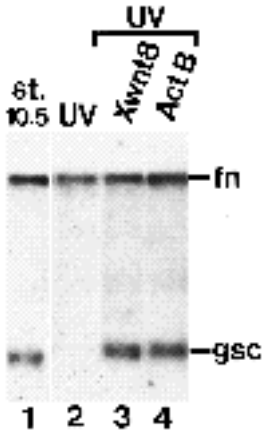

Fig. 1. *Goosecoid* RNA is induced after injection of *Xwnt-8* or activin B mRNA in UV embryos. 4-cell embryos treated with UV (lane 2) were injected with *Xwnt-8* (lane 3) or activin B mRNA (lane 4). Lane 1; uninjected embryos. RNA was extracted at stage 10.5 and the equivalents of 2.5 embryos were analyzed by northern blot hybridization using probes for *gsc* and fibronectin (fn). The UV treatment resulted in ventralized embryos with a dorsoanterior index, DAI, of less than 0.5 as defined by the method of Kao and Elinson (1988).

treatment blocks the cortical rotation and, consequently, the formation of the Nieuwkoop center (Gimlich and Gerhart, 1984). The axes induced by *Wnt* mRNA are complete, i.e., contain head (including anterior structures such as eyes), trunk and tail. Lineage tracing studies indicate that ventral blastomeres injected with *Xwnt-8* mRNA adopt the function of a Nieuwkoop center (Smith and Harland, 1991), inducing the overlying cells to form a new Spemann organizer (Sokol et al., 1991). The novel dorsalizing signal *noggin* is able to rescue complete secondary axes at high frequency in UV-treated embryos and to induce a Nieuwkoop center (Smith and Harland, 1992). While only *Xwnt-8* mRNA was used in the present study, *noggin* might be expected to behave similarly.

Activin is a potent inducer of dorsal mesoderm (Green et al., 1992). Several biological assay systems have been utilized to test the extent of anterior development induced by activin. Microinjection of synthetic activin mRNA into embryos can induce secondary axes which, however, lack anterior structures such as eyes (Thomsen et al., 1990). Similarly, axial rescue of UV-treated embryos by injection of activin mRNA is incomplete, i.e., the embryos lack anterior structures (Sokol et al., 1991).

gsc is a homeobox gene and as such encodes a DNA-binding protein (Blumberg et al., 1991). Expression of *gsc* mRNA closely follows the localization of organizer tissue in normal embryos, as well as in those treated experimentally to decrease or increase the amount of organizer (Cho et al., 1991). Synthetic goosecoid mRNA microinjected into ventral blastomeres of embryos is able to induce new dorsal lips and secondary axes which are in most cases incomplete (Cho et al., 1991; C. Niehrs, H. S., E. D. R., D. S. K. and D. M., unpublished results). In some experiments, however, secondary axes with head structures, in particular eyes, have been found, but always at very low frequencies (Cho et al., 1991; K. Cho, personal communication). This previous work did not address whether *gsc* is able to rescue axis formation in UV-treated embryos nor whether *gsc* expression is affected by the dorsal signal generated by microinjection of *Xwnt-8* or activin mRNA.

In summary, three very different genes can initiate the formation of twinned body axes to different extents, but it is not known whether they are part of the same pathway. In the present study, we attempt to define the relationship between *Wnt*, activin and *gsc*. We find that *Xwnt-8* mRNA activates *gsc* expression exclusively in a ring of cells located in the marginal zone, while activin mRNA can turn on *gsc* in all embryonic locations. When *gsc*, activin and *Xwnt-8* mRNAs were tested for the ability to rescue an axis

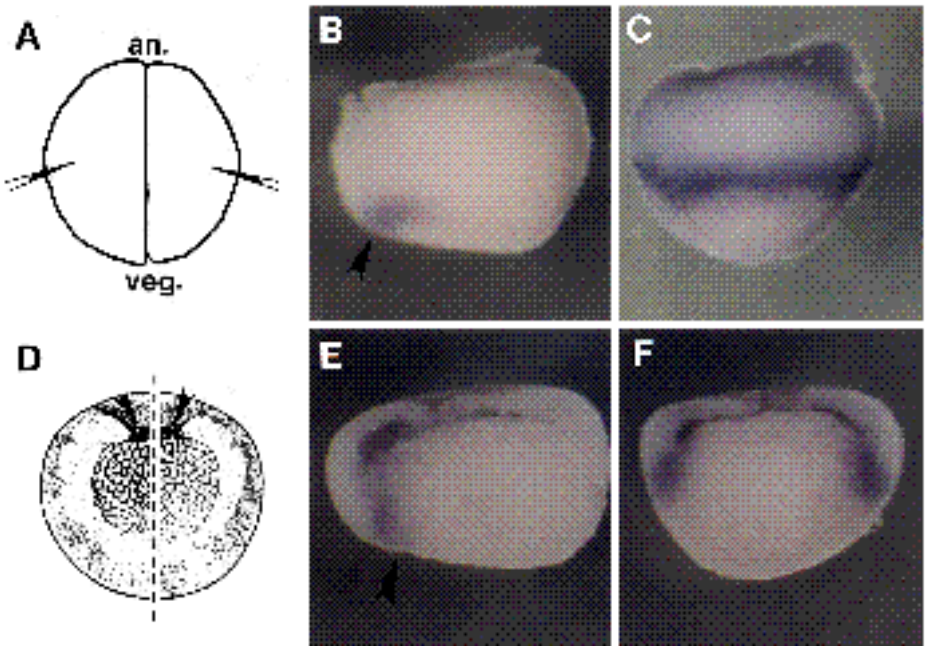

Fig. 2. Injected *Xwnt-8* mRNA induces *goosecoid* RNA exclusively in the marginal zone. Diagram of the injection experiment where both blastomeres of 2-cell embryos were injected with *Xwnt-8* mRNA (A). At stage 10.5 an in situ hybridization for *gsc* was performed using uninjected embryos (B,E) and embryos injected with *Xwnt-8* RNA (C,F). Embryos were cut through the dorsal lip as indicated in (D) and photographed to show a lateral view with the collapsed blastocoel at the top (E,F). Arrowhead indicates the dorsal lip.

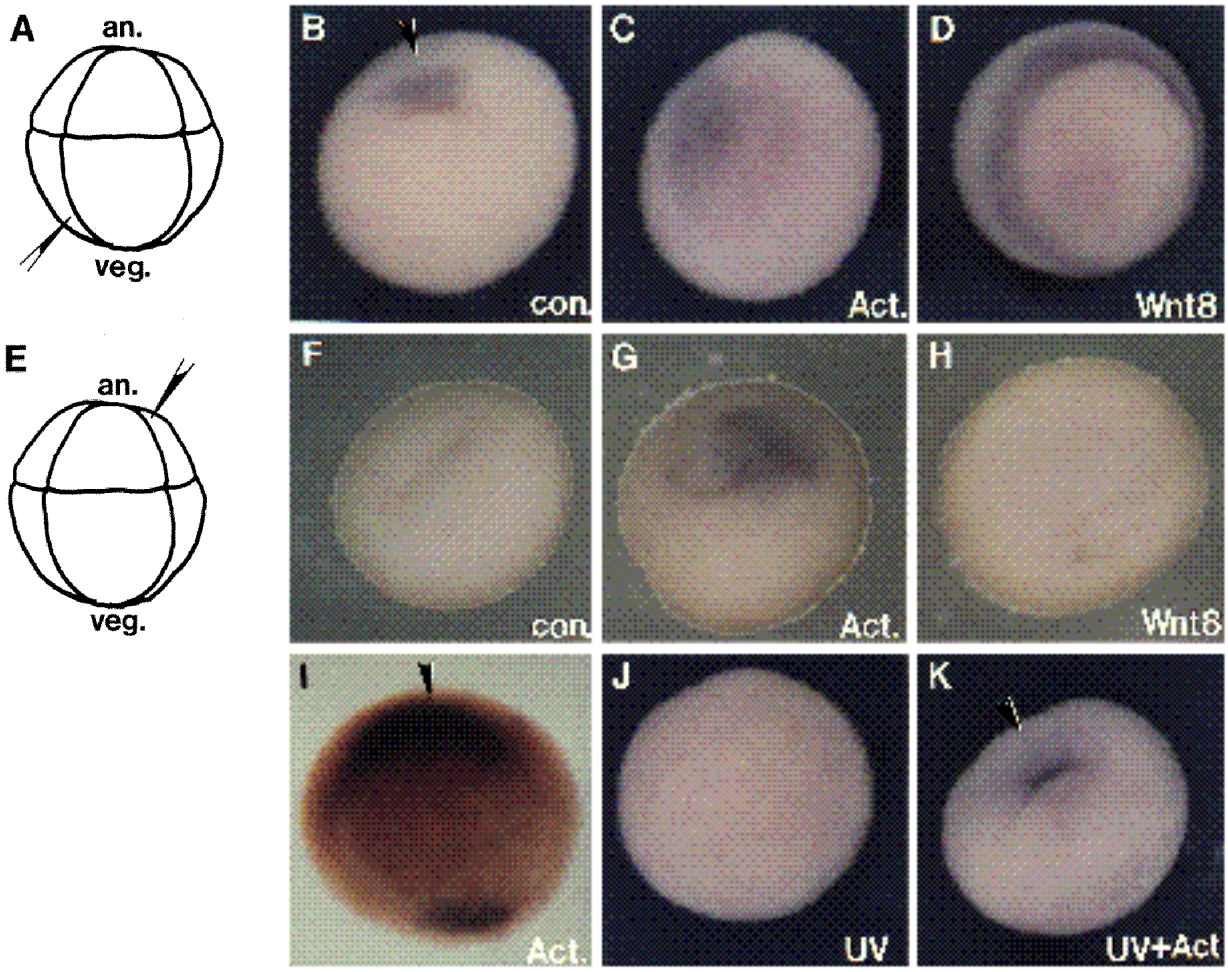

Fig. 3. Different spatial activation of *goosecoid* after injection of *Xwnt-8* and activin B mRNA. 8-cell embryos were injected into animal (an.) or vegetal blastomeres (veg.) as diagramed in (A) and (E) with activin (C,G) or *Xwnt-8* RNA (D,H). Embryos injected at opposite sides of the marginal zone display two regions of *gsc* expression (I). UV-treated embryos (J) lack *gsc* expression and dorsal lips, which can be restored by an injection of activin mRNA into the marginal zone (K). Note that in K activin mRNA induces *gsc* both above and below the blastopore lip, while in normal embryos (B) *gsc* is expressed exclusively above the dorsal lip. In situ hybridization for *gsc* was done using uninjected stage 10.5 embryos (B,F) as controls. B,C,D,I,J,K, vegetal pole view; F,G,H, animal pole view. The position of the dorsal lip is indicated by arrowheads. The embryo in I has been rendered transparent with a clearing agent.

in UV-ventralized embryos, it was found that *gsc* closely parallels the phenotypic effects of activin mRNA, leading to a partial (incomplete) axis. The possible relationship between these three signals in the formation of Spemann's organizer is discussed.

MATERIALS AND METHODS

Preparation of synthetic RNA and microinjection of embryos

Capped synthetic RNA was generated by in vitro transcription of plasmids containing full-length sequences of the *Xenopus* activin beta B (Thomsen et al., 1990), the *Xenopus Xwnt-8* (Christian et al., 1991) and the *Xenopus gsc*, as well as a truncated *gsc* construct lacking the homeobox (Cho et al., 1991). For the injection the RNA was resuspended in Gurdon's injection buffer (88 mM NaCl, 1 mM KCl, 15 mM Tris/HCl pH 7.5). Eggs fertilized in vitro were dejellied in 0.2% cysteine/HCl pH 7.8 and injected before the first cell division with 10 nl, at the 2-, 4-cell stage with 5 nl/blastomere and at the 32-cell stage with 1 nl/blastomere of RNA solution. For UV rescue experiments, pigmented embryos were injected in 1× MMR, 4% Ficoll and after 1-2 hours transferred to 0.1× MSB. For in situ hybridization experiments albino embryos were similarly treated.

UV treatment and histology of embryos

After removing the jelly with 0.2% cysteine HCl pH 7.8, embryos were transferred to 1× MMR, 5% Ficoll and irradiated 30 minutes after fertilization for 80 or 90 seconds with UV light, using a GL 25 lamp (UVP, San Gabriel), through a quartz slide or saran wrap. This treatment led to embryos with a DAI lower than 0.5, scored after 2 days (stage 30). For histology, embryos were fixed for 1.5 hours in Bouin's fixative (75 parts saturated picric acid, 25 parts formaldehyde 37%, 5 parts acetic acid) washed in 70% ethanol, embedded in histowax, sectioned and stained with hematoxylin eosin as previously described (Cho et al., 1991).

Whole-mount in situ hybridization

To detect *gsc* transcripts in the embryos, the whole-mount in situ hybridization protocol of Harland (1991) with minor modifications (Cho et al., 1991) was used. Digoxigenin-labelled sense and anti-

sense RNA were generated by *in vitro* transcription of the full-length cDNA clone (*pgsc*) (Cho et al., 1991) using the digoxigenin-labelling kit (Boehringer Mannheim) following the manufacturer's instructions.

Whole-mount immunostaining

Embryos were fixed in methanol/DMSO and bleached in 10% hydrogen peroxide. The immunostaining was performed according to Hemmati-Brivanlou and Harland (1989). The monoclonal antibody MZ 15 (Smith and Watts, 1985) was diluted 1:200 and the anti-mouse-HRP secondary antibody (Cappel) was used in a final dilution 1:500. The Tor 70 antibody was used as described by Bolce et al. (1992). After the staining the embryos were cleared in benzylbenzoate/benzylalcohol 1:1.

RNA isolation and northern blot analysis

RNA of stage 10.5 embryos was extracted using the proteinase K method. The RNA equivalent of 2.5 embryos was analyzed by northern blot using a full-length *gsc* cDNA, labelled with ^{32}P (Cho et al., 1991) and a *Xenopus* fibronectin clone (Yisraeli et al., 1990) as probes for hybridization. The filters were hybridized at 42˚C in 5× SSPE, 40% formamide and 5% SDS, and washed at 55˚C in 0.1× SSC, 0.2% SDS and exposed overnight.

RESULTS

Activin and *Xwnt-8* mRNA can induce similar levels of *goosecoid* expression in UV-treated embryos

To test whether *gsc* RNA can be induced by *Xwnt-8* protein, it was necessary to microinject synthetic mRNA because, unlike activin, *Wnt* protein is not available in soluble form. Previous work had shown that, in animal cap cells, *gsc* mRNA is rapidly induced by soluble recombinant activin, even in the absence of protein synthesis (Cho et al., 1991), at concentrations that induce the formation of organizer tissue (Green et al., 1992). In order to test and quantitate the effects of *Xwnt-8* and activin mRNA microinjection, we irradiated embryos before the first cell division with UV light. This treatment ventralizes the embryos and abolishes *gsc* expression (Cho et al., 1991). The use of UV-treated embryos provides a way of eliminating endogenous *gsc* mRNA (Fig. 1, compare lanes 1 and 2). As shown by northern blot analysis, microinjection of *Xwnt-8* and activin mRNA into UV-treated 4-cell embryos resulted in similar amounts of *gsc* RNA induction (Fig. 1, lanes 3 and 4). As a control for RNA recovery and quality, a probe for *Xenopus* fibronectin was included in this experiment (Yisraeli et al., 1990).

Having established that both mRNAs are equally potent inducers of *gsc*, we next investigated whether *Xwnt-8* and activin act upon the same populations of embryonic cells.

Xwnt-8 induces *gsc* only in a ring of marginal zone cells while activin is effective in the entire embryo

Fig. 2 shows an experiment in which *Xwnt-8* mRNA was delivered into the entire embryo by injecting both blastomeres of 2-cell embryos and the expression of *gsc* monitored by whole-mount in situ hybridization. *gsc* mRNA was strongly induced (compare Fig. 2B and C) but, interestingly, only in a ring corresponding to the entire marginal zone of the gastrula embryo. A section through these embryos revealed that the region expressing *gsc* coincided with the deep marginal zone (which includes large yolky cells) in both the dorsal lip of uninjected embryos (Fig. 2E) and in the *Xwnt*-induced ring (Fig. 2F).

In contrast to *Xwnt-8*, microinjection of activin mRNA induces *gsc* expression at the site of injection anywhere in the embryo. When injected bilaterally into 2-cell embryos, activin mRNA results in diffuse *gsc* staining not localized in the ring of the marginal zone (data not shown). This lack of localization is best seen in the experiment shown in Fig. 3, in which activin mRNA was introduced into single blastomeres at the 8-cell stage. Depending on the point of injection (Fig. 3A,E), activin mRNA can turn on *gsc* both in yolky vegetal cells (Fig. 3C) and in animal pole cells (Fig. 3G), at the gastrula stage. *gsc* can also be induced by activin mRNA injection into the ventral marginal zone of both normal (Fig. 3I) and UV-treated embryos (Fig. 3K).

Xwnt-8 RNA injected into vegetal cells induced *gsc* expression in the marginal zone but not in the vegetal cells (Fig. 3D). *Xwnt-8* mRNA injection into an animal blastomere was unable to induce *gsc* in the animal cap (Fig. 3H), although it was able to induce the population of marginal zone cells (not seen in the top view shown in Fig. 3H). The simplest conclusion from these results is that the *Xwnt-8* protein derived from injected mRNA expressed before mid blastula transition is not sufficient for the induction of the *gsc* gene, requiring additional factors located within the marginal zone. Activin B does not require these components and can turn on *gsc* in the entire embryo.

gsc-induced axes in UV-treated and untreated embryos

The finding that *Xwnt-8* and activin can restore *gsc* expression in UV-treated embryos (Fig. 1) raises the question of whether gsc mRNA is able to rescue axis formation in ventralized embryos. This is of particular interest because previous work has shown that the *Wnt* and activin signals act very differently in the UV rescue assay. While *Xwnt-8* rescues complete head structures including eyes, activin mRNA can only produce a partial, incomplete rescue (Sokol et al., 1991). As shown in Table 1, microinjection of *gsc* mRNA into 4- or 32-cell embryos can rescue axial structures in embryos ventralized by UV. A small, variable percentage of UV-treated embryos can escape ventralization and have weak residual axial structures including dorsal mesodermal tissue such as muscle (Table 1). This variability can be circumvented by microinjecting two diagonally opposed blastomeres at the 4-cell stage. Instead of radially ventralized embryos (Fig. 4A), one now finds twinned body axes due to the double injection (Fig. 4B). The rescued axes have well-developed dorsal fins and neural crest derivatives such as melanocytes (Fig. 4B), and end anteriorly in knob-like structures that usually include auditory vesicles (corresponding to a dorsoanterior index, DAI, of 2 in the scale of Kao and Elinson, 1988). When these UV-rescued embryos were stained with the notochord-specific antibody MZ-15 (Smith and Watt, 1985), it was unexpectedly found that not all axes contained a notochord, despite having very similar external morphology. For example, Fig. 4C shows

Table 1. Rescue of axial structures by microinjection of *goosecoid* and activin B mRNA

Sample	Survivors %	Survivors (*n*)	*DAI % 0	1	2	3	4	5	Average DAI
UV	96	(24)	63	29	8	0	0	0	0.5
UV+*gsc* 0.4 ng	56	(14)	14	71	14	0	0	0	1.0
UV+*gsc* 0.8 ng	76	(19)	5	42	53	0	0	0	1.5
UV+*gsc* 1.6 ng	52	(13)	0	46	54	0	0	0	1.5
UV+activin 0.3 ng	80	(28)	14	25	53	7	0	0	1.5
UV+*Xwnt8* 0.04 ng	64	(16)	0	0	0	31	25	44	4.1
untreated	100	(25)	0	0	0	0	8	92	4.9

*DAI, the dorsoanterior index, was estimated after 72 hours of development as described by Kao and Elinson (1988).

an embryo with twinned axes, including dorsal fins and auditory vesicles, but no notochords. Other embryos of similar morphology had one (Fig. 4D) or two notochords (Fig. 4E). Histological examination of *gsc*-rescued axes lacking notochords stained with the muscle-specific marker 12/101 (Kintner and Brockes, 1984) revealed that the somites fuse across the midline, filling the area normally occupied by notochord, as shown in Fig. 4F.

The fact that axes without notochords were present in UV embryos rescued by *gsc* led us to re-examine the frequency with which notochord structures are present in secondary axes induced by *gsc* mRNA injection into ventral blastomeres at the 4-cell stage. As shown in Table 2, the frequency of secondary axes is highly dependent on the concentration of injected RNA, as noted previously (Cho et al., 1991; Niehrs et al., 1993). Complete axes including eyes are only very rarely produced by *gsc* mRNA (two embryos in the experiments shown in Table 2, three embryos in Cho et al., 1991). The predominant phenotype is a partial axis that extends anteriorly up to the hindbrain level, including auditory vesicles. When embryos were stained with the MZ-15 marker, it was found that both secondary axes with (Fig. 4G) or without (Fig. 4H) notochords can be obtained, and this is also dependent on the amount of injected *gsc* mRNA (Table 2). Histological examination of secondary axes lacking notochords showed, as in the case of UV-rescued embryos (Fig. 4F), somites fused along the midline (data not shown). Although more extreme phenotypes can also be obtained (embryos containing three and four massive notochords have been obtained in some experiments, Cho et al., 1991; E. D. R., unpublished observations), the majority of *gsc*-induced axes do not contain notochord (Table 2). A similar frequency of notochords has been reported for secondary axes induced by activin B mRNA microinjection into ventral blastomeres (see Table 1 in Sokol et al., 1991). We conclude that injection *of gsc* mRNA into the marginal zone of ventralized embryos is sufficient to rescue a body axis that includes somites and neural tissue up to the hindbrain level, but which often lack notochords.

gsc and activin rescue axis formation in UV-treated embryos to similar extents

UV-treated embryos rescued by *gsc* differ greatly from those induced by *Xwnt-8*, which rescue the axis completely,

Table 2. Axis induction by *goosecoid* mRNA microinjection is dose-dependent

mRNA ng[1]	Total number of survivors	Partial[2] second. axes % (n)	Complete[3] second. axes % (n)	Second. axes with notochord % (n)
Exp. I				
gsc				
0.3	39[4]	5 (2)	0	0
0.6	54	39 (21)	0	5 (1)
0.8	39	53 (18)	0	9 (2)
1.2	45	69 (29)	4 (2)	22 (7)
Exp. II				
gsc				
0.2	21	0	0	n. d.
0.4	20	10 (2)	0	
0.8	24	54 (13)	0	0[5] (10)
1.6	24	54 (13)	0	
truncated *gsc*				
0.6-1.0	82	0	0	n. d.
Xwnt8				
0.04	23	17 (4)	65 (15)	100[5] (10)

[1]4 nl of synthetic mRNA per cell was injected into two ventral blastomeres at the 4-cell stage. Presence of secondary notochords was scored by whole-mount staining with the MZ 15 antibody (experiment I), or with the Tor 70 antibody (Kushner, 1984; Bolce et al.,1992) and histological sections (experiment II).

[2]Partial axis extending to otic vesicle and hindbrain.

[3]Complete axis including head structures such as eyes.

[4]Four of these embryos, which had very small secondary axes, were not stained with the MZ 15 notochord marker.

[5]Ten embryos with secondary axes were pooled from the three mRNA concentrations, five stained for notochords with the Tor 70 antibody in whole mounts and five analyzed histologically.

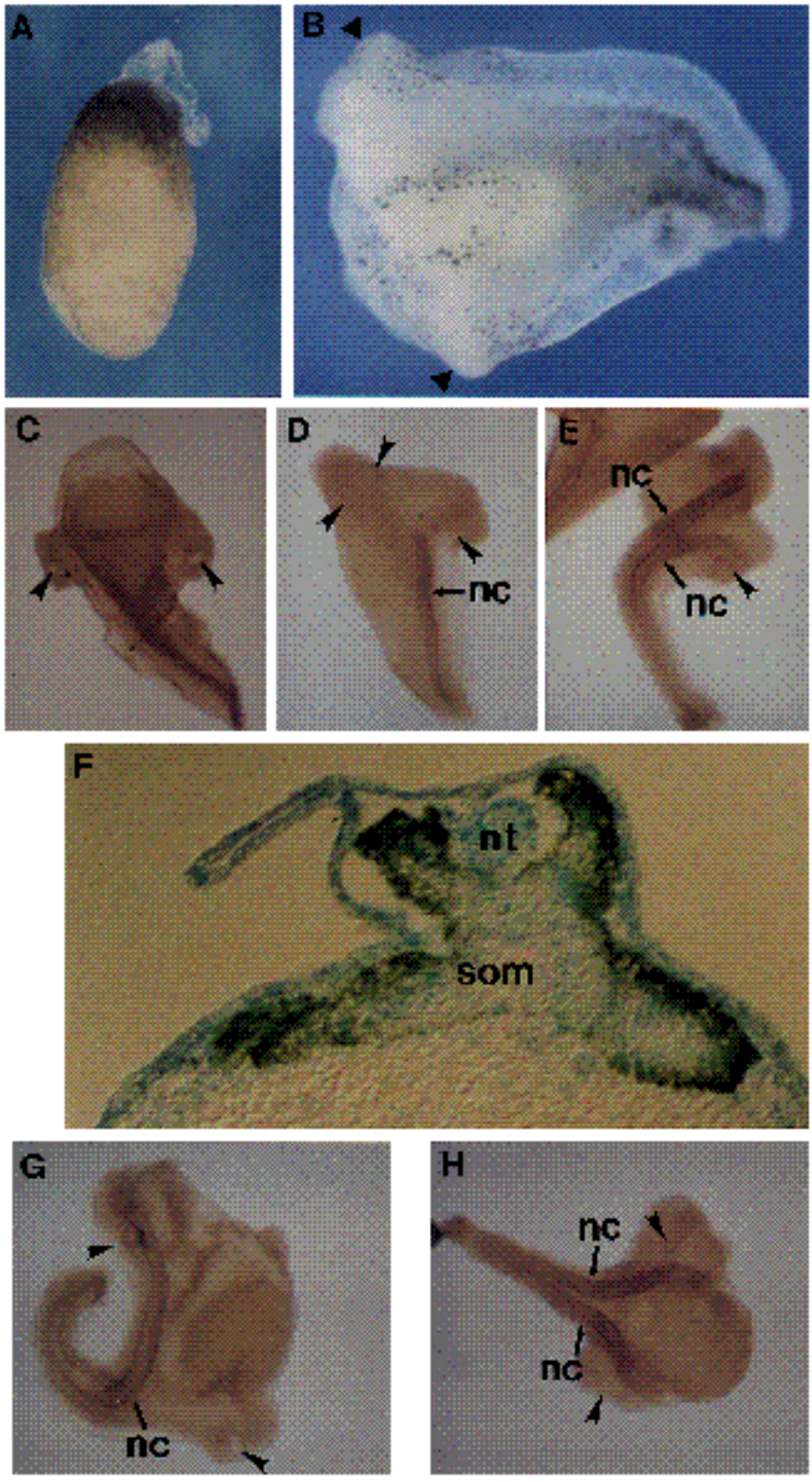

Fig. 4. Induction of axial structures by injection of *gsc* mRNA. (A-F) Rescue of UV-treated embryos; (G,H) ventral injections into untreated embryos. (A) Embryo ventralized by UV (DAI=0); (B) embryo that was microinjected into two diagonally opposed blastomeres at the 4-cell stage with *gsc* mRNA (0.5 ng per blastomere) showing the formation of twinned body axes. (C-E) Twinned embryos injected as in B, which have been stained for notochord with MZ-15 antibody showing no notochords, one notochord and two notochords, respectively. In this experiment (n=16), 9 embryos showed two rescued axes, and of these 6 had two notochords, 2 had one, and 1 (C) had none. The arrowheads indicate the anterior end of the embryo (B) and auditory vesicles (C,D,E,G,H). (F) Transverse section through a notochord-less rescued axis that was whole-mount stained with the muscle-specific antibody 12/101; note that somites are fused across the midline. (G,H) Twinned axes induced by microinjection of *gsc* mRNA (0.6 ng/cell) into two ventral blastomeres at the 4-cell stage; note the absence of notochord in the secondary axis in G, despite the presence of auditory vesicles. nt, neural tube; som, somites; nc, notochord.

including eye structures (Sokol et al., 1991; Smith and Harland, 1991). However, preliminary experiments suggested that axes rescued by activin B mRNA were very similar to those rescued by *gsc*, also ending in anterior knob-like structures (DAI=2). We therefore undertook a more detailed comparison of the extent of rescue effected by activin and *gsc* mRNA. In an attempt to deliver the mRNAs to the marginal zone, three adjacent C tier blastomeres of UV-treated 32-cell embryos were injected (Fig. 5A) with 0.15 ng of synthetic mRNA. Both external (Fig. 5B,C) and histologi-

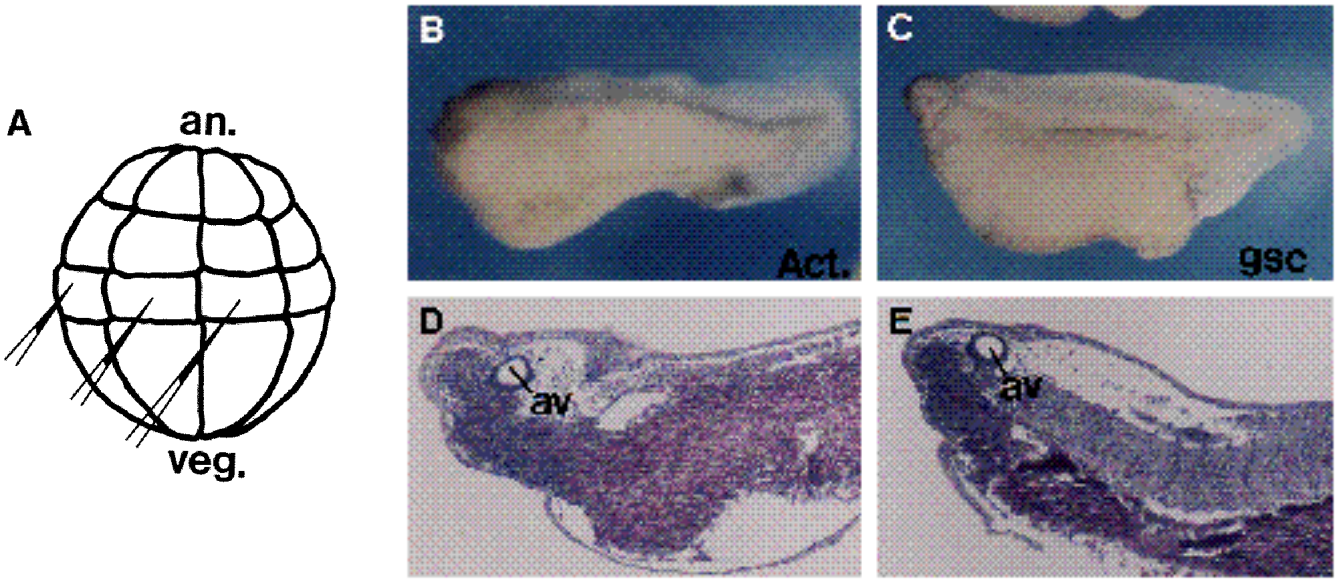

Fig. 5. Rescue of axial structures in UV embryos by activin and *gsc* mRNA. (B-E) The results of an experiment in which either activin B (100 pg/cell) or *gsc* mRNA (150 pg/cell) was injected into three adjacent blastomeres of the 'C' tier of UV-ventralized 32-cell embryos. (A) Experimental design; (B) axis rescued by activin mRNA (DAI=0.4 for uninjected UV embryos, *n*=78, 24 of the injected embryos showed axial structures, *n*=28, average DAI=1.5). (C) Axis rescued by *gsc* RNA (DAI=0.24 for uninjected UV-treated controls, *n*=104, 12 of the injected embryos showed axial structures, *n*=22, average DAI=1.1). (D,E) Histological sections of embryos rescued with activin (D) or *gsc* (E) mRNA, note that in both cases the axis extends up to the level of the auditory vesicle (av).

cal (Fig. 5D,E) examination indicated that activin and *gsc* mRNAs rescue axis structures to the same extent (as judged by comparing serial sections of all DAI 2 embryos obtained in this experiment, *gsc* *n*=8, activin *n*=14). In most of the rescued axes, the anterior knob-like ends included hindbrain structures, which could be identified by prominent auditory vesicles (Fig. 5D,E). (In very rare cases, histological analysis showed the presence of eye structures in *gsc*- and activin-injected embryos, see Table 1, but this may occur only in embryos in which the UV treatment fails to block completely the cortical rotation).

We conclude that the extent of UV rescue by *gsc* is indistinguishable from that produced by injected activin B mRNA, routinely including hindbrain and auditory vesicles as the most anterior structures, whereas *Xwnt-8* (Sokol et al., 1991; Smith and Harland, 1991) can rescue complete axes including eye structures.

DISCUSSION

We have addressed the function of three molecules able to initiate axis formation - *gsc*, activin and *Xwnt-8* - by microinjection of their synthetic mRNAs into *Xenopus* embryos. Two main conclusions emerge. First, *Xwnt-8* mRNA can only activate the *gsc* gene in a very narrow region of the embryo, the deep marginal zone. Second, there is a close parallel between the extent of axial rescue in UV-treated embryos caused by *gsc* and activin, but not by *Xwnt-8*, mRNA.

gsc activation in the marginal zone

The *Xwnt-8* dorsalizing signal acts at an early stage of the axial specification pathway, mimicking the formation of a Nieuwkoop center which in turn induces organizer tissue (Smith and Harland, 1991). The injection experiments reported here demonstrate that *gsc* expression, which marks the organizer (Cho et al., 1991), can be induced by *Xwnt-8* mRNA. Recently it has also been shown that *Wnt-1* mRNA can induce *gsc* on the ventral side of injected embryos (Christian and Moon, 1993). The results reported here suggest that the *Wnt* dorsalizing signal alone is not sufficient to induce this homeobox gene, for it is activated only in a ring of cells in the marginal zone. This underscores the importance of the marginal zone in the development of the organizer, implying that the natural *Wnt*-like dorsalizing signal will require additional components localized in the marginal zone. Candidate molecules for such components include growth factors such as fibroblast growth factor (FGF) and activin, which have been shown to cooperate with *Xwnt-8* mRNA in the formation of dorsal structures (Christian et al., 1992; Sokol and Melton, 1992) in animal cap cells. The entire ring of the deep marginal zone is able to respond to the *Wnt* stimulus (Fig. 2). Interestingly, it is within the dorsal side of this arc of cells that the organizer is formed during the course of normal development.

The relationship between *gsc* induction and the activin signal

Activin B mRNA is an equally potent inducer of *gsc* expression but, unlike *Wnt*, induction by activin can take place in all regions of the embryo. This suggests that additional region-specific factors are not required, in agreement with previous results showing that *gsc* is a primary response gene to activin induction in explanted animal caps (Cho et al., 1991).

The similarity in the activities of activin, a secreted peptide growth factor, and *gsc*, an intracellular homeodomain-containing transcription factor, is best seen in the UV rescue experiments. Activin B and *gsc* mRNAs can restore axis formation to the same extent, and axes extending up to the level of the hindbrain and auditory vesicles, with or without notochords, are observed in UV-treated embryos (Fig. 5) as well as in secondary axes induced by ventral injection of normal embryos (Fig. 4) and can be obtained at high frequencies (Table 1). The lack of notochord is not without precedent in amphibians. Axes without notochords, in which the somites are fused along the dorsal midline underneath the spinal cord, can be readily obtained in a number of experimental manipulations which result in moderately ventralized embryos (Pasteels, 1945; Waddington and Perry, 1956; Youn and Malacinski, 1981; Cooke, 1985; Shih and Keller, 1992; Christian and Moon, 1993). The observation that the axes are rescued up to the auditory vesicle is of interest, because those axes span the entire region in which the *Hox* genes are expressed (posterior to rhombomere 3; McGinnis and Krumlauf, 1992), raising the possibility that activin and *gsc* trigger the expression of *Hox* gene complexes, which are widely believed to play an important role in axial patterning of posterior regions of the body.

Why do gsc and activin fail to produce complete axes? This question is not answered by the present series of experiments, but three possibilities are worth considering.

First, the site of *gsc* expression might be critical. It may be necessary for *gsc* to be expressed precisely in the yolky cells of the deep marginal zone in order to obtain a fully functional organizer. This is what is observed after injection of *Wnt-8* mRNA, but not with activin B mRNA. Although we did attempt to target *gsc* and activin mRNA as close as possible to the deep marginal zone by microinjecting into blastomeres of tier C at the 32-cell stage, these cells do not give rise exclusively to marginal zone mesoderm and include many other cell fates as well (Dale and Slack, 1987; Moody, 1987).

Second, the timing and the concentration of the expressed proteins might be critical. While the *Wnt* signal presumably acts very early (at the 32- to 64-cell stage, when the Nieuwkoop center releases its signal, Jones and Woodland, 1987), activin B and *gsc* may act at a later stage, as suggested by the accumulation of their transcripts after mid-blastula transition (Thomsen et al., 1990; Blumberg et al., 1991). The experiments reported here utilize synthetic mRNAs injected during early cleavage stages. While large amounts of mRNA are injected, the stability of the proteins translated is unknown due to lack of antibodies for the three gene products tested. It is conceivable that the amount of protein remaining at the early gastrula stage is not adequate to evoke complete Spemann organizer activity. Inducing a Nieuwkoop center, which acts much earlier, should be easier in this experimental design and less prone to effects due to misexpression of molecules with potent biological effects before the time at which they normally act in the course of embryogenesis. While injected *Xwnt-8* mRNA has a potent dorsalizing effect, the role of this gene in normal development may be to ventralize the embryo. This is suggested by its ventral expression in the gastrula and by the effects of injected *Xwnt-8* DNA constructs that are only expressed after the mid blastula transition (Christian and Moon, 1993). The effect of *Xwnt-8* mRNA injection is thought to be due to binding of the corresponding protein to a receptor for a yet unidentified member of the *Wnt* family which would be active in the Nieuwkoop center in vivo (Sokol et al., 1991).

Third, *gsc* and activin might not be sufficient to induce head structures and may need to act in concert with other factors. Embryonic ectoderm is known to have a predisposition to adopt particular cell fates (Sharpe et al., 1987). Thus, activin can induce eye structures in the dorsal, but not ventral, halves of animal cap explants (Sokol and Melton, 1991). This prepattern is acquired very early, at the early blastula (stage 6.5-7), and requires the cortical rotation since it can be abolished by UV treatment (Sokol and Melton, 1991). *Xwnt-8* mRNA, which rescues UV-treated embryos completely (Sokol et al., 1991; Smith and Harland, 1991), is able to rescue ectodermal prepatterning (Sokol and Melton, 1992), while activin and *gsc* may be unable to do so due to the absence of a critical cofactor.

Xwnt-8, activin and *gsc* can induce axis formation in *Xenopus* embryos, and our results are consistent with these three gene products acting on the same developmental pathway. The injection of mRNAs encoding activin B (this work) or a dominant-negative activin receptor mutant that blocks *gsc* induction (Hemmati-Brivanlou and Melton, 1992) place the activin signal upstream of *gsc*, whereas a dominant negative FGF-receptor mutant does not affect *gsc* expression (Amaya et al., unpublished data). All cells of the embryo can activate *gsc* after activin mRNA injection, as is the case in early mouse embryos incubated with recombinant activin protein (Blum et al., 1992). At present there are no reports on the localization of activin-like molecules in the *Xenopus* embryo, but one might expect *gsc* to reflect the regions in which their concentration is maximal (Green et al., 1992).

Several detailed models have been recently proposed for axial specification in *Xenopus* (Moon and Christian, 1992; Sive, 1993; Woodland, 1993), and our observations are in general agreement with current views in this rapidly moving field. Signals such as *Wnt* and activin may interact to produce dorsal mesoderm (organizer tissue), which then expresses *gsc*. It is worth noting, however, that our results on vegetally injected activin mRNA do not seem to support the view that activin is a vegetally produced factor that is released and then acts on the overlying marginal zone (as is the case for injected *Xwnt-8* mRNA). Because activin can induce *gsc* everywhere in the embryo, and because *gsc* requires a high threshold of activin (Green et al., 1992), it might be predicted that an activin-like molecule should be expressed in the organizer proper. The elucidation of the biochemical pathway leading to the generation of Spemann's organizer, however, will require additional experiments, in particular loss-of-function studies.

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