Development 115, 355-369 (1992)
Printed in Great Britain © The Company of Biologists Limited 1992

Secretory and inductive properties of *Drosophila wingless* protein in *Xenopus* oocytes and embryos

ARTHUR CHAKRABARTI, GLENN MATTHEWS, ALAN COLMAN and LESLIE DALE

School of Biochemistry, University of Birmingham, Po Box 363, Birmingham B15 2TT, UK

Summary

Like its vertebrate homologues, *Xenopus* wnt-8 and murine wnt-1, we find that *Drosophila wingless* (*wg*) protein causes axis duplication when overexpressed in embryos of *Xenopus laevis* after mRNA injection. In many cases, the secondary axes contain eyes and cement glands, which reflect the induction of the most dorsoanterior mesodermal type, prechordal mesoderm. We show that the extent of axis duplication is dependent on the embryonic site of expression, with ventral expression leading to a more posterior point of axis bifurcation. The observed duplications are due to de novo generation of new axes as shown by rescue of UV-irradiated embryos. The true dorsal mesoderm-inducing properties of *wg* protein are indicated by its ability to generate extensive duplications after mRNA injection into D-tier cells of 32-cell embryos. As revealed by lineage mapping, the majority of these D cell progeny populate the endoderm; injections into animal blastomeres at this stage are far less effective in inducing secondary axes. However, when expressed in isolated animal cap explants, *wg* protein induces only ventral mesoderm, unless basic fibroblast growth factor is added, whereupon induction of muscle and occasionally notochord is seen. We conclude that in intact embryos, *wg* acts in concert with other factors to cause axis duplication.

Immunolocalisation studies in embryos indicate that *wg* protein remains localised to the blastomeres synthesizing it and has a patchy, often perinuclear distribution within these cells, although some gets to the surface. In oocytes, the pool of *wg* protein is entirely intracellular and relatively unstable. When the polyanion suramin is added, most of the intracellular material is recovered in the external medium.

Key words: *wingless*, Wnt-1, mesoderm induction, *Xenopus*, oocytes, embryos

Introduction

One of the earliest developmental decisions during amphibian embryogenesis is the specification of mesoderm in the marginal zone of the blastula. This process, known as mesoderm induction (Nieuwkoop, 1969), is believed to be triggered by diffusible factors produced by the underlying vegetal pole cells (reviewed by Smith, 1989 and Slack, 1991). The induced mesoderm subsequently differentiates into a variety of different tissues, which occupy distinct positions along the dorsoventral and anteroposterior axes of the embryo. The initial subdivision of the prospective mesodermal cell fates within the marginal zone results from the production by dorsal vegetal cells of a mesoderm-inducing factor(s) which is qualitatively or quantitatively different from that produced by ventral and lateral vegetal cells (Gimlich and Gerhart, 1984, Dale et al., 1985; Dale and Slack, 1987b). Subsequently the induced dorsal marginal zone (DMZ) "organizes" the adjacent ventral marginal zone (VMZ) by dorsalizing it in a graded fashion according to the relative positions of DMZ and VMZ cells (Spemann and Mangold, 1924; Smith and Slack, 1983, Dale and Slack, 1987b; Stewart and Gerhart, 1990). It is this dorsalization that lays the foundation of axis determination in the developing embryo.

Understandably there has been much interest in identifying the endogenous mesoderm inducers, and a great deal of progress has been achieved recently by analysing the response of isolated animal cap cells to exogenous polypeptide growth factors. In this assay, animal cap cells excised from *Xenopus* blastulae can be respecified from an ectodermal fate (epidermis) to a range of mesodermal fates (e.g. notochord, muscle, kidney etc) by incubation with purified growth factors of the transforming growth factor-beta (TGF-β) or fibroblast growth factor (FGF) superfamilies (Slack et al., 1987; Kimelman and Kirschner, 1987; Smith, 1987; Rosa et al., 1988; Paterno et al., 1989, Asashima et al., 1990; Smith et al., 1990; Sokol et al., 1990). Apart from mesoderm-inducing activity, an absolute requirement for an endogenous mesoderm-inducing factor is that it be present within the embryo at the right time and place. At present, the main candidates that have been suggested and that meet at least some of these requirements are basic FGF (Slack and Isaacs, 1989; Kimelman et al , 1989), activin (Thomsen et al., 1990), BMP4 (Koster et al., 1991; Dale et al., 1992) and Vg1 (Weeks and Melton, 1987; Dale et al , 1989, Tannahill and

Melton, 1989). Recently, using a different approach, evidence has been obtained for the participation in mesoderm induction of another class of growth factor, that encoded by the wnt gene superfamily (McMahon and Moon, 1989; Christian et al., 1991b; Smith and Harland, 1991; Sokel et al., 1991).

The murine proto-oncogene wnt-1 encodes a 370 amino acid, cysteine-rich glycoprotein (van Ooyen and Nusse, 1984), which is secreted very poorly from cultured cells into the surrounding media (Papkoff et al., 1987, Papkoff, 1989) Recent evidence suggests that secreted wnt-1 is associated with the extracellular matrix, an interaction that can be prevented by the addition of negatively charged polyanions such as heparin or suramin (Bradley and Brown, 1990; Papkoff and Schryver, 1990). In *Drosophila* there is compelling genetic and biochemical evidence that the wnt-1 homologue, *wingless* (*wg*, Rijsewijk et al.,1987), is secreted and acts on neighbouring cells (Morata and Lawrence, 1977; van den Heuvel et al., 1989; Gonzalez et al., 1991). A large family of wnt-related genes have now been identified in both mouse and frog (Gavin et al., 1990, Christian et al., 1991a), although their roles remain to be defined. In mice, wnt-1 appears to be involved in the development of the central nervous system (McMahon and Bradley, 1990; Thomas and Capecchi, 1990), whilst in *Drosophila* it is implicated in segment polarity and imaginal disc development (Morata and Lawrence, 1977; Nusslein-Volhard and Weischaus, 1980; Baker, 1988). Expression of murine wnt-1 (McMahon and Moon, 1989) and overexpression of *Xenopus* wnt-8 (Christian et al., 1981b) in early *Xenopus* embryos results in a duplicated dorsal axis. These latter results suggest that wnt proteins may act to establish a secondary organizer within the VMZ of early *Xenopus* embryos, although splitting of the primary organizer cannot be ruled out.

In this paper, we extend the experiments of McMahon and Moon (1989), using the *Drosophila wg* gene which differs from other known wnt-1 genes in encoding a protein containing an extra hydrophilic domain (Rijsewijk et al., 1987) We show that the *wg* gene is capable of generating a complete duplicate dorsal axis following injection of its RNA into ventral blastomeres of the *Xenopus* embryo. Furthermore, it is able to completely rescue UV-irradiated embryos, which would otherwise fail to develop dorsal mesoderm. These effects can be executed by *wg* without the protein being expressed in the responding cells, suggesting that *wg* acts by inducing non-expressing cells into alternative pathways. We also show that, although *wg* is able to induce ventral mesoderm in isolated animal caps, the presence of FGF is required for it to induce dorsal mesoderm. These inductive effects of *wg* are discussed in relation to the tendency reported by others (Bradley and Brown, 1990; Papkoff and Schryver, 1990) and observed here in oocytes and embryos, for wnt proteins to remain within, or closely associated with, the cells in which they are synthesized.

Materials and methods

Oocyte microinjection and culture

Oocytes were obtained from females of the frog *Xenopus laevis*, maintained and where necessary microinjected as described by Colman (1984). Following injection, oocytes were incubated in modified Barths' saline (MBS) supplemented with 1% fetal calf serum and an antibiotic/antimycotic solution (GIBCO/BRL). They were labelled by addition of 1 mCi/ml of [^{35}S]methionine (1415 Ci/mmol, Amersham), incubated for 18 hours at 20°C, and then chased in medium supplemented with 2 mM cold methionine. When required, suramin (Mobay Chemical Company) at 5 mM or heparin (Sigma) at 50 μg/ml were added to the media For analysis, media was only taken from incubations containing healthy oocytes. The preparation of oocyte homogenates, *wg* and chick oviduct protein immunoprecipitation (1 μl antisera per 40 μl homogenate or medium) and electrophoresis on reducing, 12 5% SDS-polyacrylamide gels have been described previously (Dale et al , 1989) The rabbit anti-wingless antisera was a kind gift of Drs M. van den Heuvel and R Nusse, Stanford, USA Anti-chick egg white antiserum is described in Ceriotti and Colman (1988)

Embryo preparation and microinjection

Eggs were obtained from females injected with 500-750 i u of human chorionic gonadotrophin ('Chorulon' Intervet) the previous evening and fertilized artificially using a macerated testis. Embryos were dejellied in 2% cysteine hydrochloride (pH 7 8-8 1), thoroughly washed and transferred into 10% MBS All embryos were staged according to Nieuwkoop and Faber (1967) Where necessary, dorsoventral polarity was ascertained at the 4-cell stage when dorsal blastomeres are often less pigmented than ventral blastomeres The dorsal marginal zone was then stained with a crystal of Nile Red (Kirschner and Hara, 1980) At the 1-, 4- or 32-cell-stage, single blastomeres were injected with 5-15 nl of synthetic RNA of the required concentration in water, in some cases RNA was injected as a 50:50 mixture with rhodamine-dextran-amine (RDA, Gimlich and Braun, 1985) at a final concentration of RDA of 50 mg/ml During and after injection, embryos were kept in 10% MBS containing 3% Ficol (Type 400. Sigma) to prevent leakage of cytoplasm from injected embryos In some cases, embryos were placed on a quartz slide and irradiated at the vegetal pole, 35-40 minutes after fertilisation (1st cleavage approx 90 minutes) using a Mineralite UV source (approx 260 nm wavelength), the length of exposure was determined empirically to couple good survival with a high proportion of severly defective embryos.

Scoring of UV-irradiated embryos

UV-irradiated embryos were scored using the dorsoanterior index (DAI) of Kao and Elinson (1988) Briefly, DAI=0 indicates no dorsoanterior structures and radially symmetric embryos composed entirely of ventroposterior structures; DAI=1-4 indicates an increasing degree of dorsoanterior structures and a concommitant reduction of ventroposterior structures; DAI=5 indicates normal morphology, DAI=6-9 indicates an increasing degree of dorsoanterior enhancement, coupled with increasing severity of ventroposterior trunk reduction, DAI=10 indicates a radially symmetric embryo composed almost exclusively of dorsoanterior structures, particularly in neural and notochordal tissues

Embryo dissection

Animal caps were isolated from embryos at stage 8 in full-strength MBS using ground forceps and electrolytically sharpened tungsten needles They were incubated individually in 96-well plates lined with agar and containing 100 μl of MBS plus 1 mg/ml BSA and either 0, 5 or 50 ng/ml of *Xenopus* basic Fibroblast Growth Factor (FGF; Kimelman and Kirschner, 1987). When control embryos had reached stage 40, animal caps were fixed (see below)

Histology and microscopy

(a) *Whole mount microscopy*

Embryos were prepared for confocal microscopy as described by Dent and Klymkowsky (1989) with the following modification: 0.1% Tween 20 was added to the Tris-buffered saline, pH 7.5 (TTBS). The first antibody, rabbit anti-wg, was used at 1/100 dilution, the second antibody used was a goat affinity-purified, biotinylated anti-rabbit IgG (Vector Laboratories) and was used at 1/200 dilution. Finally the embryos were stained with fluorescein Avidin D (Vector Laboratories at 1/1000 dilution. In some experiments, this procedure was then repeated using a cocktail of eight anti-haemagglutinin mouse hybridoma culture media (each at 1/125 dilution), followed by rhodamine (TRITC)-labelled goat anti-mouse antisera (Sigma) at 1/100 dilution. The hybridoma supernatants were a kind gift of Dr J. Skehel, NIMR, London, UK. Whole mounts were examined using a Biorad MRC 600 confocal microscope.

(b) *Preparation and observation of thin sections*

Stained, whole-mount embryos were taken out of Murray's medium into methanol and then embedded in Paramat paraffin wax (Gurr). 10 μm sections were dewaxed with Histoclear (Cellpath plc), rehydrated, and counterstained with Hoechst 33258 (1 μg/ml, Calbiochem) for 1 minute. They were viewed in a Leitz Diaplan microscope equipped with epifluorescent optics, and photographed with Kodak T MAX 400 film.

Embryos were fixed at 4°C with 4% paraformaldehyde in phosphate-buffered saline, pH 7.5, whilst animal caps were fixed in Susa's fixative. Samples were dehydrated and embedded in Paramat. 10 μm sections containing RDA lineage label were mounted on gelatin-subbed slides, stained with Hoechst 33258, and observed and photographed as above. All other sections were stained with periodic Schiff's, then 0.5% naphthalene black and finally 1% methyl green.

RNA preparation

A full-length cDNA encoding *wg*, obtained from A. Martinez Arias (Cambridge University, UK) as a 3kb *Bam*HI fragment, was inserted into the *Bgl*II site of the transcription vector pSP64T (Krieg and Melton, 1989). pSP64T clones containing influenza haemagglutinin [pSP64HA], and bovine prolactin (BP4) have been described previously (Ceriotti and Colman, 1989). All DNAs were transcribed with SP6 polymerase (Pharmacia) using standard protocols (Krieg and Melton, 1989). After transcription, the reaction mix was digested with 20 μg/ml DNAase 1 (Calbiochem) before phenol:chloroform extraction and ethanol precipitation. Poly(A)$^+$ RNA was prepared from chick oviducts as described by Ceriotti and Colman (1988). For injection the RNA was resuspended in sterile, deionized water at the required concentrations.

Results

Drosophila wg *induces double axes and the extent of duplication is dependent upon the site of injection*

Previous reports (McMahon and Moon, 1989; Christian et al., 1991b) have shown that injections of *Xenopus* embryos with murine wnt-1 or *Xenopus* wnt-8 (Xwnt-8) mRNAs result in the duplication of the dorsal axis. Our preliminary experiments were designed to test whether a similar phenotype resulted from *Drosophila wg* mRNA injections. In these experiments we injected *wg* mRNA into either dorsal or ventral blastomeres of the 4-cell *Xenopus* embryo, and the results confirm that this molecule, like its murine and *Xenopus* homologues, is able to induce a secondary dorsal axis (Fig. 1; Table 1). Co-injection of *wg* mRNA and the lineage label rhodamine-dextran-amine (RDA) shows that progeny of the injected blastomeres make a substantial contribution to this new axis (Fig. 2A,B). Embryos were injected with either 125 pg or 5 ng of *wg* mRNA and although the results are broadly similar, the highest concentration of RNA tended to give the most extreme phenotype. However, in other experiments as little as 5 pg of *wg* mRNA proved effective in producing multiple dorsal axes, many with completely formed heads. As controls, embryos were injected with synthetic mRNA encoding bovine prolactin (BP4), a secretory protein, and duplications of the dorsal axis were never observed (Fig. 1A; Table 1).

In the experiments described above, ventral injections proved to be the most effective at inducing secondary dorsal axes (Table 1) and the earliest sign of this induction is the appearance of a second dorsal lip in the ventral quadrant of the early gastrula (Fig. 3). In many cases and at both RNA concentrations, bifurcation occurred in the hind trunk with the embryos developing an almost complete secondary dorsal axis (Fig. 1C). This is particularly the case following injection of 5 ng of RNA. Occasionally, triplications were also observed (Fig. 1D). Histological examination of sectioned material has shown that these secondary axes usually possessed the correct spatial array of mesodermal, as well as neural and endodermal tissues (Fig. 2A). However, embryos were occasionally observed in which the secondary axis pos-

Table 1. *The extent of axial duplication is dependent upon the site of injection: dorsal v ventral injections*

RNA injected	Amount	Site of injection	Number injected	Position of bifurcation (% of injected embryos)LL				
				Normal	Forebrain	Hindbrain/ ant trunk	Mid trunk	Hind trunk
BP4	125 pg		13	100	0	0	0	0
wingless	125 pg	Dorsal	54	85	15	0	0	0
		Ventral	77	13	23	27	25	12
	5 ng	Dorsal	23	9	48	22	0	22
		Ventral	61	8	8	15	25	44

Synthetic RNA encoding *Drosophila wg* was injected into a single dorsal or ventral blastomere at the 4-cell stage, either 125 pg or 5 ng of RNA was injected. As controls 125 pg of bovine prolactin (BP4) RNA was injected into a single blastomere at the 2-cell stage. The resulting embryos were subsequently examined for the appearance of secondary axes at stages 30-36, and the position at which they bifurcated from the primary axis scored. Although the completeness of the induced secondary axes were variable, secondary axes with fully formed heads were frequently obtained and this bore no relation to the rostrocaudal position of bifurcation.

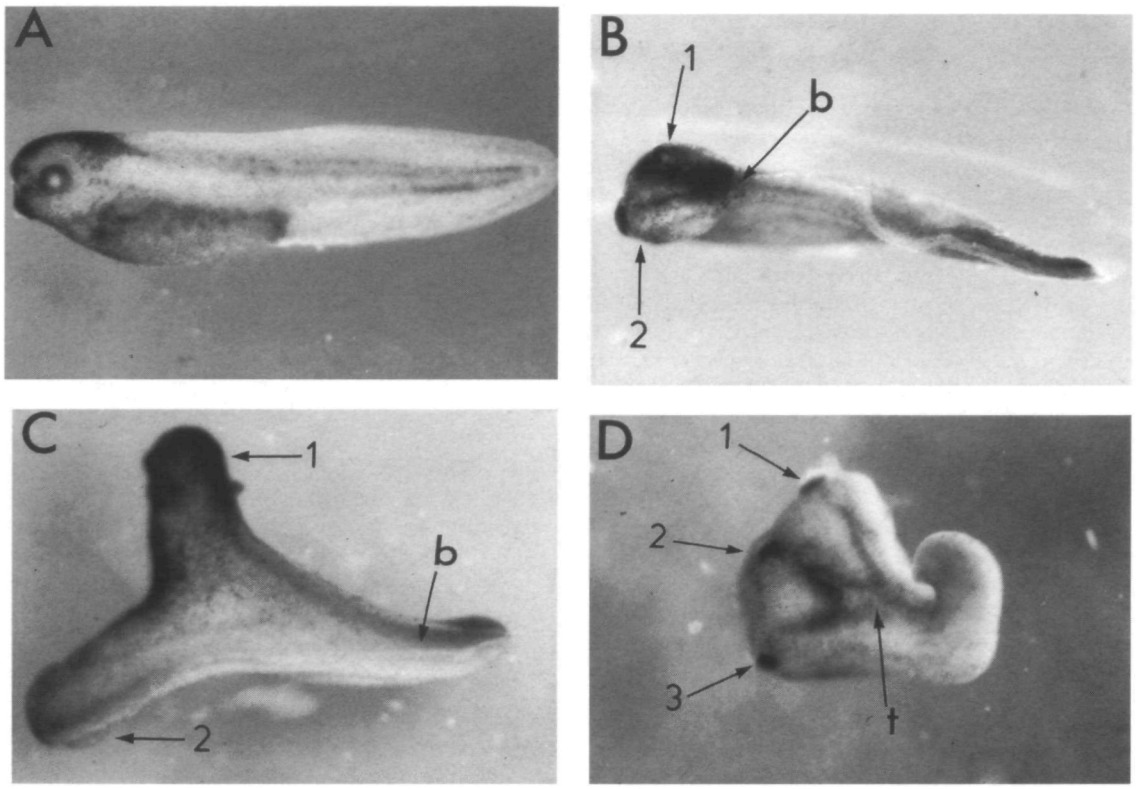

Fig. 1. The induction of multiple dorsal axes in *Xenopus* embryos by the *wg* gene is dependent upon the site of injection A single blastomere at the 2- to 4-cell stage was injected with either bovine prolactin (BP4) or *wg* synthetic mRNAs, and examined for axial duplications at stages 30-36. (A) An embryo injected with 125 pg of BP4 mRNA at the 4-cell stage develops a single dorsal axis. (B) An embryo injected with 5 ng of *wg* mRNA into a dorsal blastomere at the 4-cell stage has a duplicated head (heads are marked 1 and 2), the point of bifurcation (b) is in the hindbrain/anterior trunk. Although no control embryo injected with 5 ng BP4 mRNA is shown, such embryos develop only one dorsal axis (e g. see Fig. 3A) (C) An embryo injected with 125 pg of *wg* mRNA into a ventral blastomere at the 4-cell stage develops an almost complete secondary dorsal axis (axes are marked 1 and 2), the point of bifurcation (b) is in hind trunk. (D) An embryo injected with 5 ng of *wg* mRNA into a ventral blastomere at the 4-cell stage has two additional dorsal axes (axes are marked 1, 2 and 3), the point of trifurcation (t) is in the hindbrain - anterior trunk.

sessed two parallel notochords (see Fig. 2B,C), indeed similar duplications of the notochord were observed in embryos that were apparently quite normal externally. In contrast, following the injection of 125 pg of *wg* mRNA into dorsal blastomeres, duplicated axes were rarely obtained, and all of these either bifurcated in the head or possessed enlarged or duplicated cement glands. The frequency and extent of duplicated axes were much greater following injection of 5 ng of *wg* mRNA into dorsal blastomeres (see Table 1), although once again in the majority of cases (70%) the duplications bifurcated in the head (Fig. 1B). These results suggest that injection of *wg* mRNA does indeed induce a secondary organizer in recipient embryos, and that the site at which the primary and secondary axes fuse is determined by the angle separating the two organizers. They also clearly demonstrate that as a result of *wg* RNA injection and presumably its translation, ventral blastomeres have changed their fate to that expected of their dorsal counterparts, and suggests the induction of an ectopic dorsal organizer(s) in these blastomeres.

wg *RNA injections can rescue UV-irradiated embryos*

From the experiments described above, we have concluded that *wg* expression changes the fate of injected blastomeres, such that a second dorsal organizer is formed in the lateral or ventral marginal zone of the gastrula. We wished to confirm this conclusion by rescuing embryos which as a result of experimental intervention, lack an organizer. This is achieved by irradiating embryos at the vegetal pole with UV light (260 nm) early in the first cell cycle. These embryos subsequently develop with reduced dorsoanterior structures, many developing as radially symmetric "ventral belly pieces" (Scharf and Gerhart, 1980). If *wg* protein were inducing a secondary organizer, we would expect that injection of its mRNA would rescue these UV-irradiated embryos. To test this, embryos were irradiated at the vegetal pole soon after fertilization and then injected with either *wg*, influenza haemagglutinin (HA), or BP4 mRNA prior to the first cleavage division The results were scored using the dorsoanterior index (DAI) of Kao and Elinson (1988; see materials and methods) Following injection of either HA or BP4 mRNAs into UV-irradiated embryos, no increase in dorsoanterior development was seen over uninjected embryos (DAI < 2 in both cases, Table 2), many remained completely ventralized (Fig. 4B). In contrast, injection of *wg* mRNA greatly increases the extent of dorsoanterior development (DAI > 5,

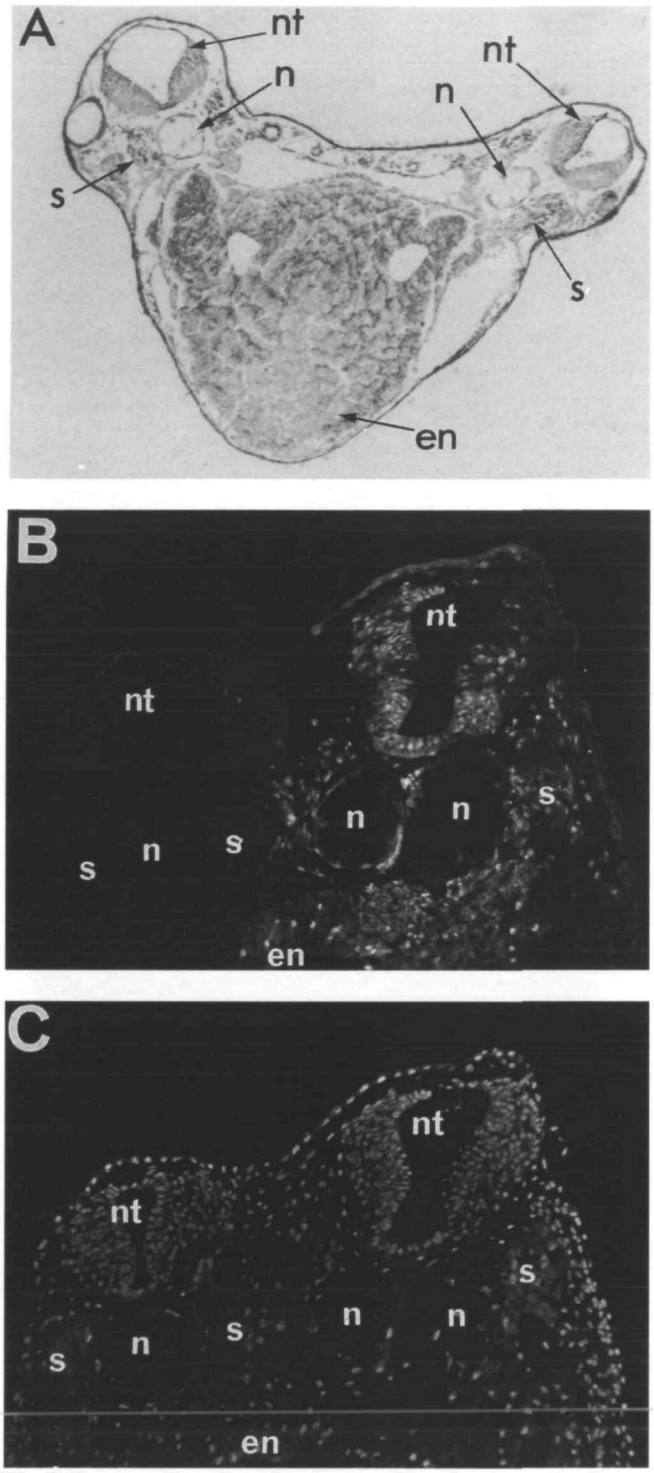

Fig. 2. Cross sections through two stage-32 embryos previously injected with 125 pg of *wg* mRNA at the 4-cell stage, ventral blastomeres were injected in both cases (A) Section through the duplicated embryo shown in Fig. 1C, each dorsal axis is composed of a neural tube (nt), notochord (n) and somite (s), note the endoderm (en) is also partially duplicated. (B) Section through a duplicated embryo that had been coinjected with lineage label (RDA) as well as *wg* mRNA, in this case the secondary axis possesses two parallel notochords, both of which are rhodamine labelled. Also labelled within this secondary axis are the somites, neural tube and endoderm, and all these are unlabelled within the primary axis (C) Same section as in B counterstained with Hoechst to illuminate nuclei.

Table 2 and Fig. 4C) with some of the resulting tadpoles near normal in appearance (Fig. 4C), in others dorsoanterior tissues were over-represented in the final body pattern (Fig. 4D). The range of body plans observed following *wg* mRNA injection into these embryos is similar to those previously described for rescue of UV-irradiated embryos following treatment with lithium (Kao et al., 1986). Coinjection of RDA with *wg* mRNA has confirmed that the injected blastomere makes an extensive contribution to the developing dorsal axis in UV-irradiated embryos (data not shown). These results confirm our conclusion that *wg* protein is capa-

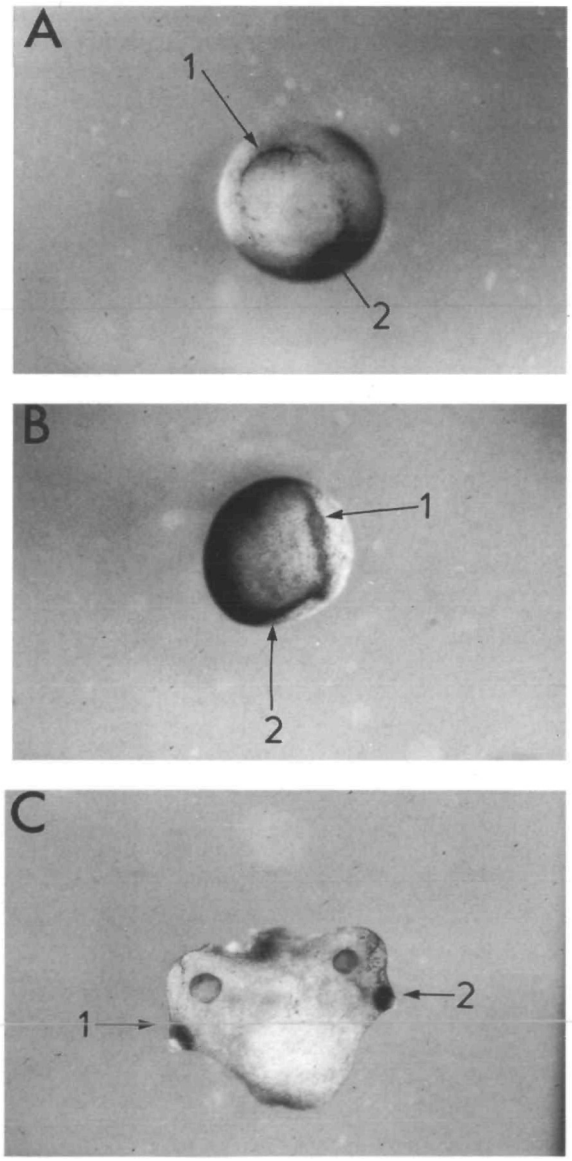

Fig. 3. Duplication of the dorsal axis following the injection of 125 pg of *wg* RNA into a ventral C-tier blastomere at the 32-cell stage (A) An early gastrula with two dorsal lips separated by an angle of nearly 160°. (B) The same embryo at a neurula stage with two neural plates, the point of fusion is very close to the blastopore (C) The same embryo as a tadpole possess two well formed heads that includes both eyes and cement gland, only the most anterior abdomen was formed the remainder being deleted In all three figures the two axes are marked 1 and 2

ble of inducing an organizer in competent marginal zone cells.

wg *mRNA induces secondary axes to a greater extent when injected into vegetal cells than into animal cells*

From the results described above, it is not clear if *wg* needs to be expressed in the marginal zone cells that will ultimately form the organizer, or whether expression in neighbouring animal or vegetal cells is sufficient. To test this we have injected its mRNA into progressively smaller portions of the embryo, localizing it to either inducing or responding cells. In the first of these experiments *wg* mRNA was injected into ventral blastomeres at the 8-cell stage, when the 3rd cleavage plane has divided the animal and vegetal hemispheres, and the results presented in Table 3 show that injections into vegetal blastomeres were much more effective at inducing duplicated dorsal axes than similar injections into animal blastomeres. Following animal injections duplicated axes were rarely obtained, and anterior structures, such as the head, were never observed. Coinjection of lineage label, demonstrated that *wg* expression had no effect on the fate of injected animal blastomeres (data not shown). In contrast, following vegetal injections, well developed secondary axes were usually obtained, and in most cases these included complete, or nearly complete, secondary heads.

To further clarify the most effective site of *wg* expression for axial duplication we have injected single blastomeres of the 32-cell embryo with *wg* mRNA. At this stage blastomeres are frequently arranged as four tiers of eight cells, tiers A-D

Table 2. *Rescue of UV irradiated embryos by injection of* wingless *RNA*

Expt	RNA inj	Number inj	Mean DAI
1	Uninjected	76	1 21
	HA	11	0 64
	wingless	76	5 20
2	Uninjected	50	1 09
	HA	12	0 63
	wingless	34	5 09
3	BP4	127	1 85
	wingless	72	5 74
4	BP4	70	2 1
	wingless	57	5 75

Xenopus embryos were irradiated at the vegetal pole with UV light (260 nm), 35-40 minutes post-fertilization, and subsequently injected with 5 ng of either influenza haemagglutinin (HA), bovine prolactin (BP4) or *wg* synthetic RNAs prior to the completion of the first cleavage division The dose of UV light used in these experiments was determined empirically to give a severe phenotype coupled with maximum survival Embryos were scored, at control stages 35-40, using the dorsoanterior index (DAI) of Kao and Elinson (1988), and the mean scores for four separate experiments are given A brief description of the DAI scale is given in the Materials and methods

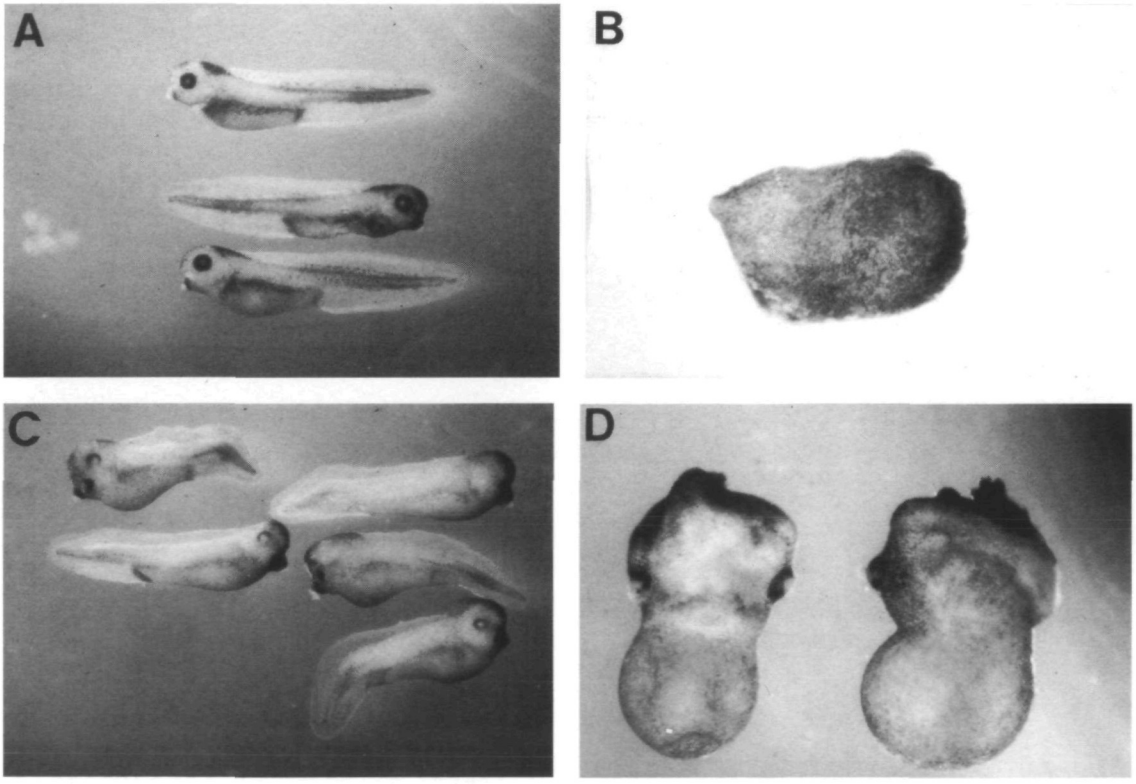

Fig. 4. Injection of *wg* mRNA can rescue UV-irradiated embryos, demonstrating the de novo induction of dorsal organizer material Embryos were irradiated with UV light (see Materials and methods) 35-40 minutes post-fertilization to inhibit the formation of the dorsal organizer, they were subsequently injected with 5 ng of either BP4 or *wg* mRNA prior to the completion of the first cleavage division (A) Unirradiated embryos injected with BP4 mRNA develop normally (DAI = 5) (B) UV-irradiated embryo injected with BP4 mRNA develops no dorsoanterior structures (DAI = 0) (C) UV-irradiated embryos injected with *wg* mRNA develop nearly normal dorsoanterior structures (DAI = 5 & 6) (D) UV-irradiated embryos injected with *wg* mRNA develop enhanced dorsoanterior structures (DAI = 7 & 8)

running from the animal to the vegetal pole (Nakamura and Kishiyama, 1971), and we have concentrated on injections into ventrovegetal blastomeres of tiers C and D. Fate maps have shown that, whereas C-tier blastomeres contribute to both mesoderm and endoderm, D-tier blastomeres contribute most of their volume to endoderm, making little contribution to mesoderm (Dale and Slack, 1987a; Moody, 1987). Following injection of *wg* mRNA into either tier, duplicated axes were observed with equal frequency (Table 3), and in both cases complete secondary heads were obtained. By coinjecting lineage label, followed by histological examination of the resulting embryos, it was clear that *wg* mRNA-injected C-tier blastomeres made a substantial contribution to mesoderm of the second dorsal axis, most of the progeny populating the notochord and somite of this axis (Fig. 5B). In contrast, *wg* mRNA-injected D-tier blastomeres contributed most of their volume to endoderm of the second axis, and it was clear that the fate of this endoderm had been respecified to a more dorsoanterior form (Fig. 5D). Our failure to observe a consistent contribution of injected D-tier blastomeres to the mesoderm, suggests that expression of *wg* within this germ layer is not necessary for the observed duplications. We conclude that injection of *wg* RNA changes the inductive properties of ventral D-tier blastomeres; normally these blastomeres would only induce ventral mesoderm in the adjacent marginal zone (Gimlich and Gerhart, 1984; Dale et al., 1985, Dale and Slack, 1987b), but following *wg* mRNA injection they are able to induce dorsal 'organizer' mesoderm.

wg *requires the presence of FGF to induce dorsal mesoderm in isolated animal caps*

All our results so far suggest that *wg* protein acts to induce dorsal mesoderm in the ventral and/or lateral marginal zone of injected embryos. One way to test this point further is to apply *wg* protein to animal caps isolated from blastulae, an assay that has identified molecules such as basic FGF and activin as mesoderm-inducing factors (Slack et al., 1987; Kimelman and Kirschner, 1987; Asashima et al, 1990; Smith et al., 1990; Sokol et al., 1990). Unfortunately, purified, active *wg* protein is not available, so we have had to adopt the alternative strategy of isolating animal caps from embryos previously injected in the animal hemisphere with *wg* RNA. Embryos were injected with 3 ng of either BP4 or *wg* RNA before the first cleavage division and cultured until stage 8 (mid blastulae), at which point animal caps were isolated and incubated in a simple salt solution until control embryos had reached stage 40, before being examined histologically. Whereas control, BP4 mRNA-injected animal caps differentiated as atypical epidermis (Fig. 6A), *wg* mRNA-injected animal caps differentiated as fluid-filled epidermal vesicles containing a few mesenchyme-like cells and occasionally mesothelium (Fig. 6B). Such cases are usually regarded as weak ventral inductions, suggesting that *wg* protein can be classified as a ventral mesoderm-inducing factor alongside molecules such as FGF (Slack et al., 1987; Kimelman and Kirschner, 1987) and BMP4 (Koster et al , 1991; Dale et al , 1992), as opposed to a dorsal mesoderm-inducing factor such as activin (Asashima et al., 1990; Smith et al., 1990, Sokol et al., 1990).

Since no dorsal mesoderm, i.e. notochord and muscle, was found in the experiments described above, the inductive potency of *wg* protein alone cannot account for duplications of the dorsal axis that we have described above; in these cases, notochord as well as muscle was clearly differentiated in the secondary dorsal axis (see Figs 2A, 5). This suggests that other factors, perhaps FGF or members of the TGF-β family, are required to act in concert with *wg* protein to induce dorsal mesoderm. To test this point, we have isolated animal caps from embryos previously injected with 1-5 ng of either *wg* or BP4 mRNA and incubated them in media containing either 0, 5 or 50 ng/ml of *Xenopus* bFGF. Animal caps were incubated until control stage 40 before being analysed histologically and, in three separate experiments, *wg* mRNA-injected animal caps differentiated dorsal mesoderm in the presence of bFGF (Fig. 6D). Although in most cases only muscle was differentiated, in one experiment, utilising the highest concentration of both RNA and bFGF, a few caps (2/11) also differentiated a small amount of notochord (Fig. 6F). In these same experiments, animal caps injected with BP4 mRNA only differentiated small amounts of

Table 3. *The extent of axial duplication is dependent upon the site of injection: animal v vegetal injections*

RNA inj	Amount	Site of inj	Number	Normal	Duplicated
BP4	250 pg	4-Cell	22	100%	0%
wingless	250 pg	8-Cell Animal	63	73%	27%
	250 pg	8-Cell Vegetal	65	0%	100%
	250 pg	32-Cell C-Tier	45	27%	73%
	250 pg	32-Cell D-Tier	49	31%	69%
BP4	250 pg+RDA	4-Cell	14	100%	0%
	250 pg+RDA	8-Cell Animal	4	100%	0%
wingless	250 pg+RDA	8-Cell Animal	20	95%	5%
	250 pg+RDA	8-Cell Vegetal	26	42%	58%
BP4	125 pg+RDA	32-Cell D-Tier	25	100%	0%
wingless	125 pg+RDA	32-Cell D-Tier	31	87%	13%
BP4	250 pg+RDA	32-Cell D-Tier	16	100%	0%
wingless	250 pg+RDA	32-Cell D-Tier	52	56%	44%
BP4	500 pg+RDA	32-Cell D-Tier	11	100%	0%
wingless	500 pg+RDA	32-Cell D-Tier	35	20%	80%

Synthetic RNA encoding either *wg* or bovine prolactin (BP4) was injected into a single animal- or vegetal-ventral blastomere at the 4- to 32-cell stages, and the resulting embryos scored for axial duplication at stage 35/36 Between 125 and 500 pg of RNA was injected, and in later experiments RNA was premixed with the lineage label rhodamine-dextran-amine (RDA, final concentration 50 mg/ml)

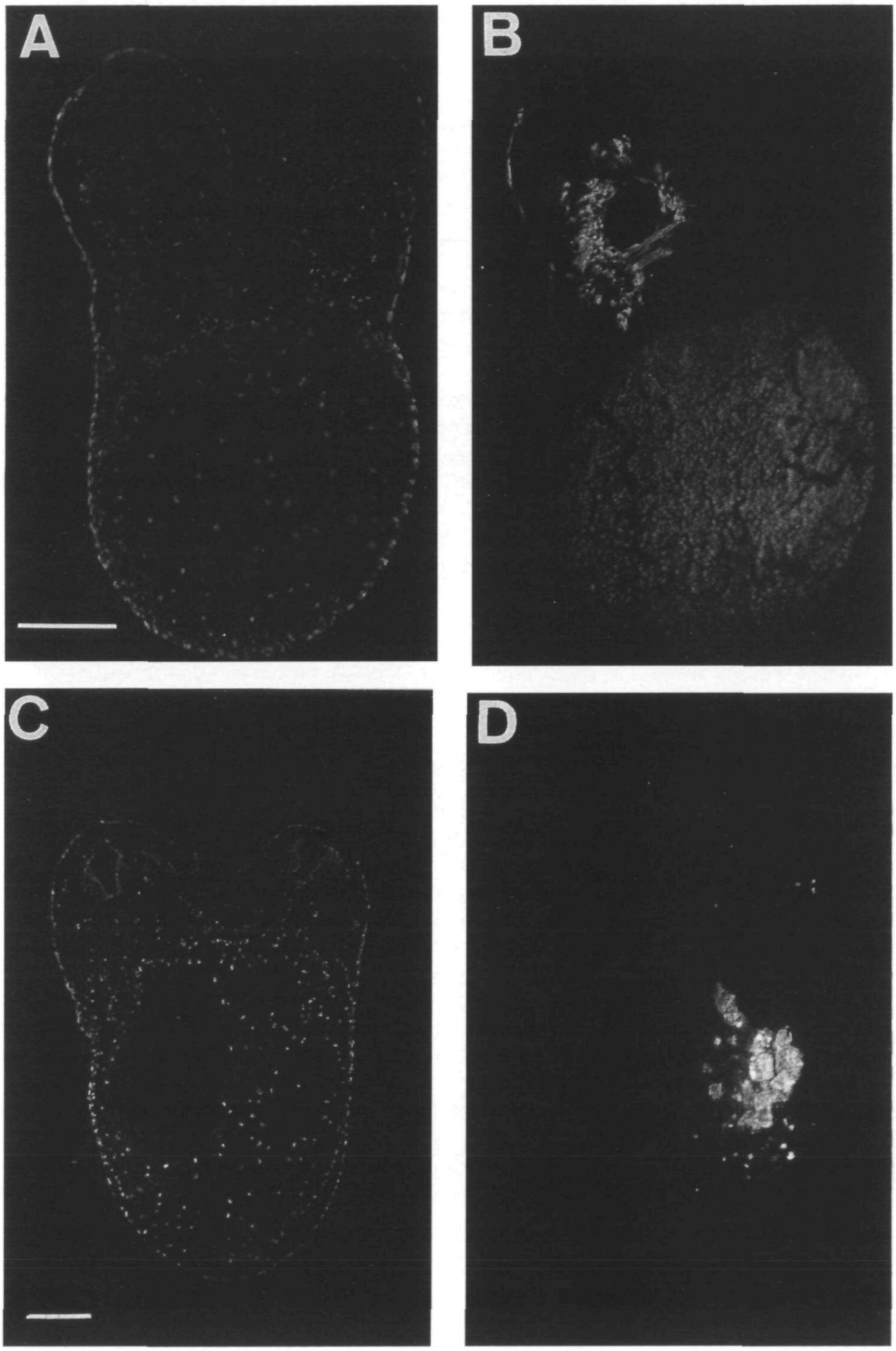

Fig. 5. Lineage analysis of *wg* injected blastomeres at the 32-cell stage Ventrovegetal blastomeres of either the C- or D-tier were coinjected with lineage label and 500 pg of *wg* mRNA, allowed to develop until stage 30, then fixed, sectioned, and counterstained with Hoechst to show the distribution of nuclei. (A, B) Cross section through the trunk of a C-tier injected embryo, B shows the typical distribution of rhodamine-labelled cells and A shows the same section counterstained with Hoechst Note that the rhodamine is restricted to the notochord, somite and neural tube of only one axis. (C, D) Cross section through the trunk of a D-tier injected embryo; C shows the typical distribution of rhodamine-labelled cells, and D shows the same section counterstained with Hoechst Note that in this case the rhodamine is largely restricted to the endoderm underlying only one of the two dorsal axes, only a few labelled cells are found within the somitic mesoderm and none within the notochord. Scale bars, 50 μm

muscle in the presence of 50 ng/ml bFGF (Fig. 6E). The difference between these animal caps was evident as early as control stage 10.5-11. In the presence of bFGF, *wg* mRNA-injected animal caps underwent a greater degree of convergent extension than BP4 mRNA-injected animal caps. These results show, that whereas *wg* protein alone cannot act as a dorsal mesoderm-inducing factor, it can act synergistically with a ventral mesoderm-inducing factor such as bFGF to induce dorsal mesoderm. However, the near absence of notochord differentiation in these experiments might suggest a requirement for yet another factor(s) to explain complete axial duplication in *wg* mRNA-injected embryos. This factor(s) would be present in either the marginal zone or vegetal pole, but not in the animal cap.

wg *protein is expressed on the surface of* Xenopus *embryonic blastomeres*

Results described above suggest that *wg* protein can act to alter the fate of blastomeres not previously injected with its

mRNA. This is not surprising since it is known to be secreted by *Drosophila* embryonic cells and is frequently found 2-4 cell diameters from its site of synthesis (Gonzalez et al , 1991). In previous studies (McMahon and Moon, 1989, Christian et al , 1991b), the spatial distribution of the mammalian or amphibian Wnt proteins in *Xenopus* embryos was not reported, probably because of the unavailability of the appropriate antisera. To analyse the distribution of *wg* protein in *Xenopus* embryos, we have injected 5 ng of its RNA into single blastomeres at the 8-cell stage and examined the

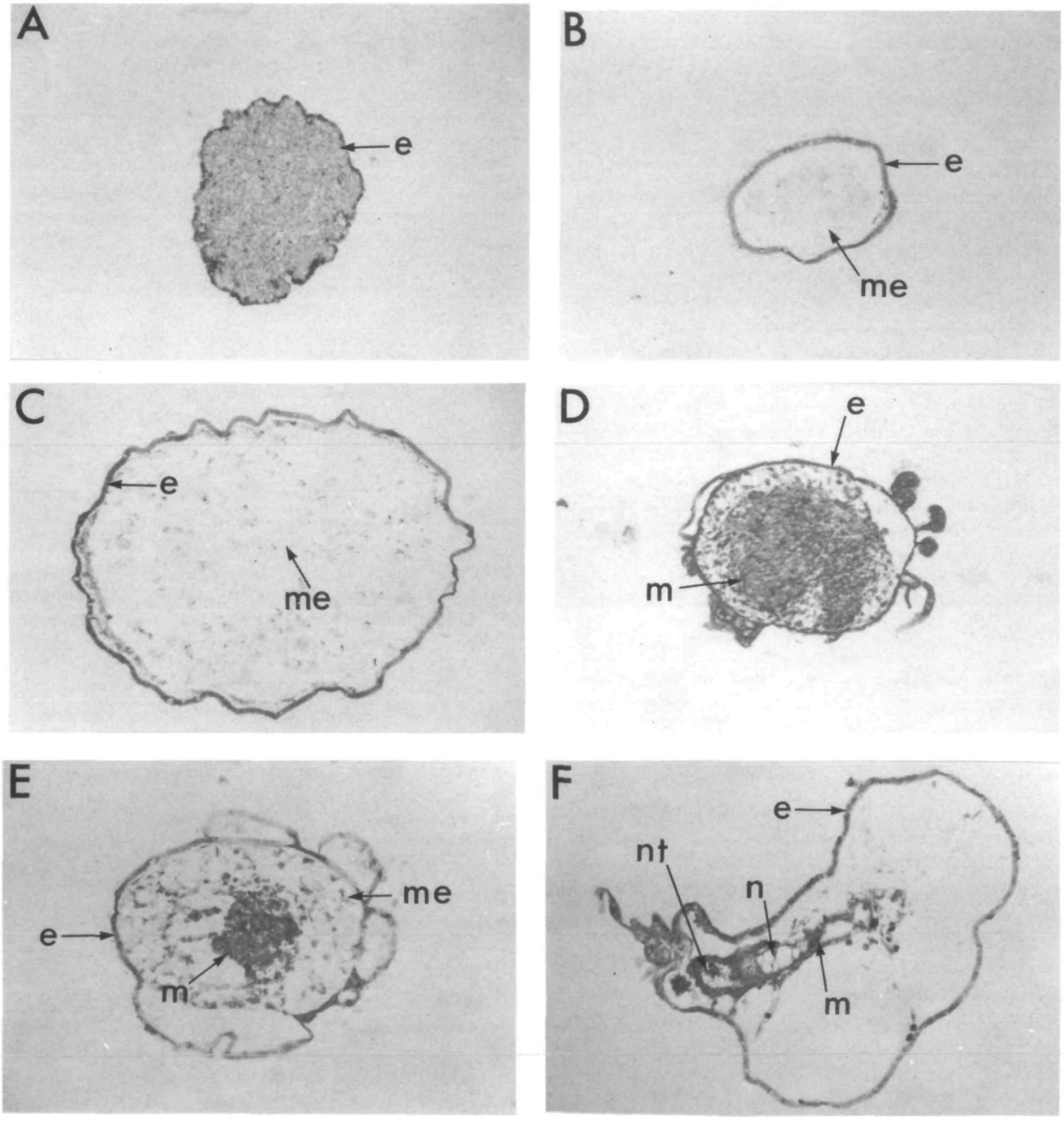

Fig. 6. Mesoderm induction in isolated animal caps Embryos were injected at the 1-cell stage with 5 ng of either BP4 or *wg* mRNA, and animal caps isolated at stage 8 (approx 1064 cells) and incubated in either 0, 5 or 50 ng/ml of *Xenopus* basic FGF. Animal caps were fixed at stage 40 and processed for histological examination. (A) A BP4 mRNA-injected animal cap differentiates atypical epidermis (e). (B) A *wg* mRNA-injected animal cap differentiates as epidermis (e) enclosing a fluid-filled vesicle that frequently contains mesenchyme-like cells (me). (C) A BP4 mRNA-injected animal cap incubated in 5 ng/ml of FGF differentiates as epidermis (e) enclosing a fluid-filled vesicle filled with mesenchyme-like cells (me) and mesothelium (D) A *wg* mRNA-injected animal cap incubated in 5 ng/ml of FGF differentiates as epidermis (e) enclosing a large mass of muscle (m). (E) A BP4 mRNA-injected animal cap incubated in 50 ng/ml of FGF differentiates as epidermis (e) enclosing a fluid-filled vesicle filled with mesenchyme-like cells (me) and occasionally a small block of muscle (m). (F) A *wg* mRNA-injected animal cap incubated in 50 ng/ml of FGF differentiates as epidermis (e) enclosing a muscle (m) and occasionally a small amount of notochord (n) and neural tube (nt). Although very little muscle is present in the section shown, in other sections from this cap large muscle masses were observed.

embryos by immunomicroscopy at the early gastrula stage after staining with anti-*wg* antibodies. Initially, embryos were examined using whole-mount confocal microscopy and Fig. 7B shows a typical optical section seen in such an embryo, whilst Fig. 7A shows a superimposition of all the optical sections that contained any *wg* protein signal. The fluorescing region shown represents approximately the volume occupied by the blastomere injected initially. It is clear that *wg* protein remains localised to the region of injection and some is found close to the plasma membrane of individual blastomeres.

Sections from injected embryos were also examined by conventional epifluorescence microscopy (Fig 7C). Analysis of this section indicates that most of the *wg* protein expressed by injected blastomeres is found intracellularly although some is at the cell surface (also see Fig. 7 D). The perinuclear localisation and punctate appearance of the signal suggests that intracellular *wg* is localised to the endoplasmic reticulum and other components of the secretory pathway. In *Drosophila*, *wg* protein is found associated with cells in which it is not synthesised (Gonzalez et al., 1991). In order to establish whether this occurs in the frog embryos we have co-injected *wg* mRNA along with haemagglutinin mRNA, as an integral membrane protein, haemagglutinin will act as a cell autonomous marker. We find (Fig. 7 D,E) that *wg* protein is only seen in cells expressing haemagglutinin and that it has a predominantly intracellular distribution which is in contrast to the surface distribution of haemagglutinin. We conclude that all detectable *wg* protein remains associated with the cells in which it is synthesised.

Wg *protein is synthesised but not normally secreted from* Xenopus *oocytes*

The results above indicate that some, albeit a very small proportion, of *wg* protein can get to the cell surface of blastomeres, behaviour consistent with its putative role in mesoderm induction. Unfortunately more direct answers on *wg* protein secretion in embryos are difficult to obtain due to the fragility of the early blastomeres. Instead, we have examined *wg* protein secretion in oocytes since this cell type has proved a useful system for testing the intrinsic properties of a variety of non-frog secretory and membrane proteins (Colman and Morser, 1979; Colman et al., 1981a; Cerriotti and Colman, 1988). In addition, the behavior of the *wg* protein in oocytes might provide a useful guide to its behavior in the cleaving embryo.

Initially, oocytes were coinjected with mRNAs encoding *wg* protein and several chick oviduct secretory proteins, including ovalbumin and lysozyme Oocytes were then cultured with radioactive methionine for various times before immunoprecipitating oocyte homogenates and culture media with the relevant antisera. It is clear from Fig. 8 (A and B) that whereas the oviduct proteins are secreted into the media, *wg* protein is not. Thus *wg* protein, like its murine counterpart wnt-1, is poorly secreted by expressing cells. Of course these results do not tell us where the *wg* protein isolated from homogenates is localized in the oocyte; it may be intracellular as suggested for the embryo above, or it may be associated with components of the extracellular matrix as suggested for murine wnt-1 (Bradley and Brown, 1990; Papkoff and Schryver, 1990).

To analyse this point further, we have incubated oocytes injected with *wg* mRNA in media containing either 5 mM suramin or 50 μg/ml heparin. It has previously been shown that some murine wnt-1 is secreted if transformed cells are incubated in media containing these reagents (Bradley and Brown, 1990; Papkoff and Schryver, 1990). When we included these reagents in oocyte incubation media, we found that suramin, but not heparin treatment, led to the appearance of *wg* in the medium (Fig. 8C), and this occurred whether suramin was present during or after synthesis of radioactive *wg* protein. In fact, more protein was found in the media than in the Triton extract of the oocyte prepared at the end of the incubation. Since the amount of extracted material was the same, regardless of treatment, we conclude that suramin treatment allows the escape from the cell of protein which would otherwise be degraded The possibility that a significant proportion of *wg* protein is lost in the Triton X-100-insoluble oocyte debris was excluded by experiments where the Triton-insoluble material was extracted with 1% SDS at 80°C prior to immunoprecipitation, conditions observed to solubilise matrix-associated murine wnt-1 (Bradley and Brown, 1990); less than 10% of the radiolabelled *wg* protein was recovered by these procedures (data not shown). The reasons for the failure of heparin to release *wg* protein into the media are unclear; unlike murine wnt-1 it is not known if *wg* protein binds to heparin, in which case its release would not be expected. Alternatively heparin and suramin may release murine wnt-1 from different compartments of the cell, suggesting the absence of *wg* protein from the heparin release compartment in oocytes.

Suramin treatment may induce secretion of wg *protein from oocytes*

In mammalian cells, it is not clear how suramin acts to release proteins from cells into the media; whereas it may displace or interfere with the binding of proteins to the extracellular matrix, as suggested for murine wnt-1 (Papkoff and Schryver, 1991), it has been claimed that suramin can enter cells by endocytosis promoting the secretion of intracellular material (Huang and Huang, 1988). We have attempted to resolve the situation in oocytes in two ways: First oocytes expressing *wg* in the absence of suramin, were exposed to trypsin at 4°C; we have previously shown that the conditions used resulted in the proteolysis of surface-associated, but not intracellular, oocyte proteins (Ceriotti and Colman, 1989). As shown in Fig. 8A, trypsin treatment had no effect on oocyte-associated *wg* protein, although after disruption of the oocytes by homogenisation, all the *wg* protein was sensitive to trypsin (data not shown) Second, oocytes expressing *wg* were incubated with suramin at 4°C, conditions known to severely inhibit both endocytosis and secretion. Fig. 8D shows that at 4°C no *wg* appears in the medium and also that oocyte-associated *wg* is stabilised; in controls incubated at 20°C, *wg* protein is efficiently secreted into the media in the presence of suramin. If suramin can displace extracellular *wg* from the surface, we would have expected to have seen this occur at 4°C. These results suggest that the majority of *wg* protein is located intracellularly and its release is triggered

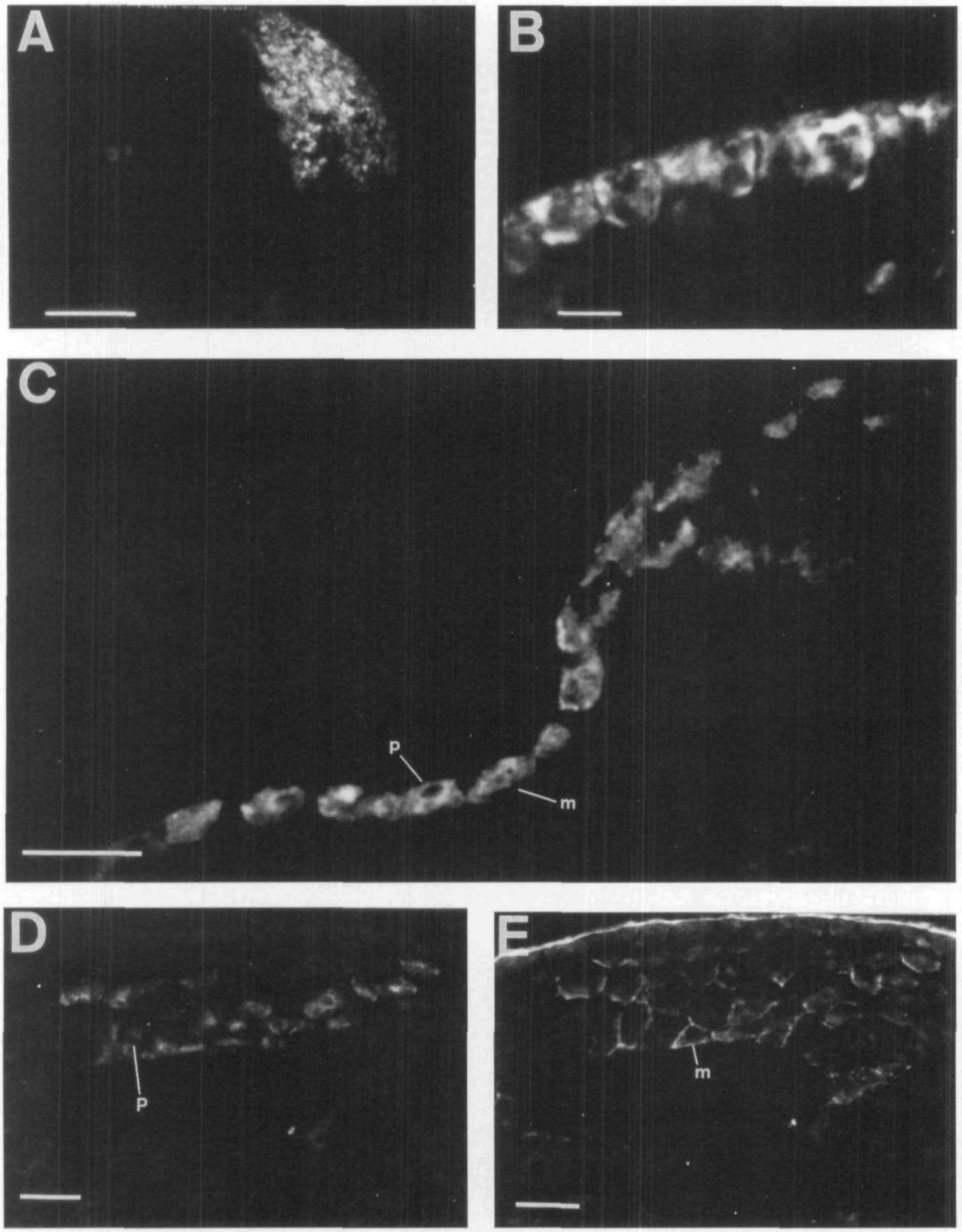

Fig. 7. Immunolocalisation of *wg* in blastomeres. 5 ng *wg* mRNA alone or 5 ng each of *wg* and haemagglutinin mRNAs were injected into animal blastomeres at the 8-cell stage and development continued until stage 10.5, the early gastrula stage. The embryos were then processed for whole-mount confocal microscopy as described in Materials and methods. Panel A shows the superimposition of all the optical signals obtained from an embryo injected with *wg* mRNA and stained with anti-*wg* antisera (scale bar, 250 μm), whilst panel B shows one optical section only (scale bar 25 μm). After confocal microscopy, embryos were prepared for conventional sectioning and the sections (panels C, D and E) observed with standard epifluorescent optics. Panel C shows the distribution of *wg* protein in cells in the roof of the blastocoel (scale bar, 50 μm). Panels D and E (scale bars, 50 μm) show the same section stained with anti-*wg* antisera (panel D) and anti-haemagglutinin antibodies (panel E); the strong signal from the vitelline envelope was also seen in control embryos stained with anti-haemagglutinin antibodies. Arrows point to perinuclear (p) and plasma membrane-associated (m) signal.

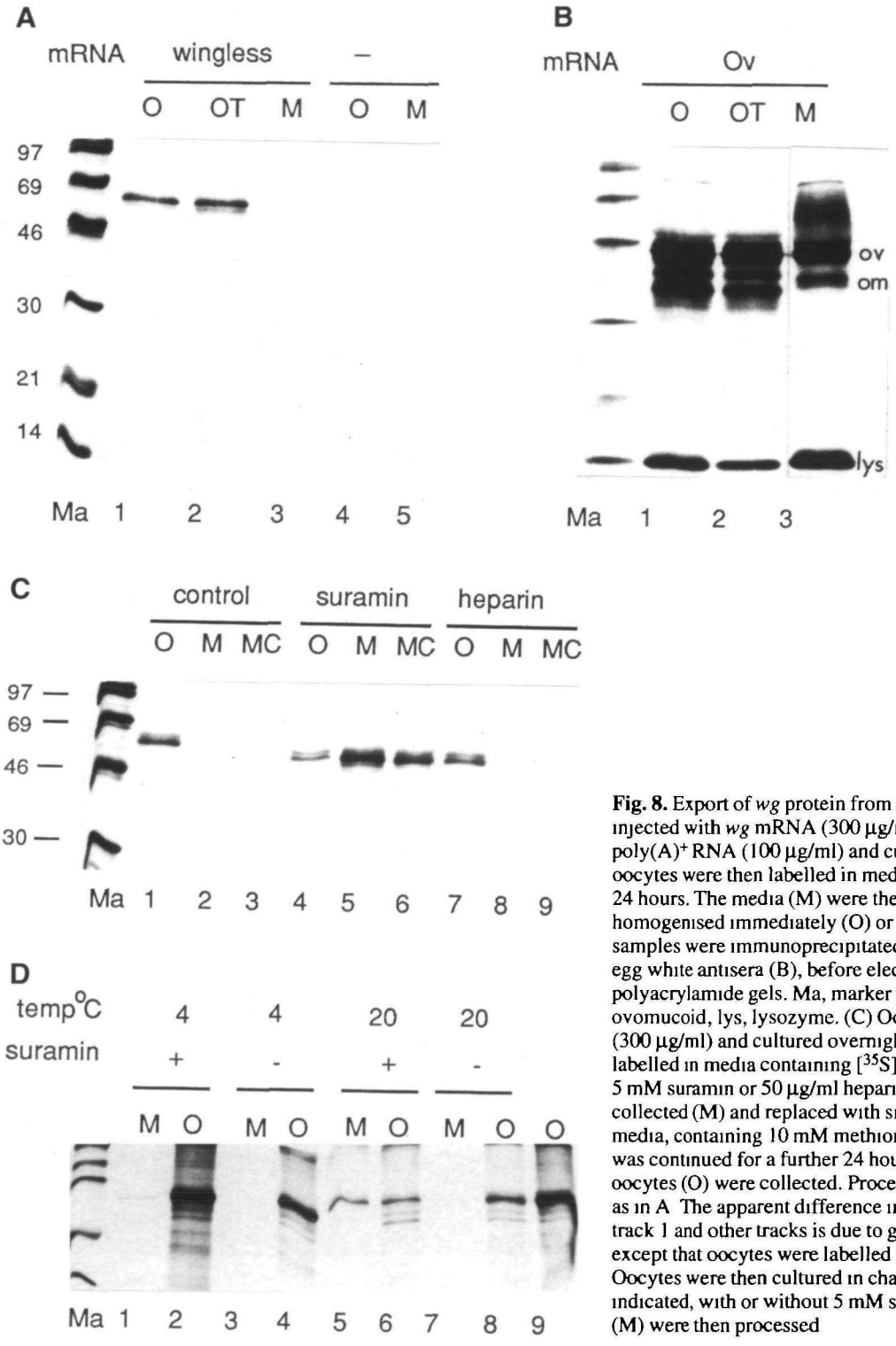

Fig. 8. Export of *wg* protein from oocytes (A,B) Oocytes were co-injected with *wg* mRNA (300 μg/ml) and chick oviduct (Ov) poly(A)$^+$ RNA (100 μg/ml) and cultured overnight Healthy oocytes were then labelled in media containing [^{35}S]methionine for 24 hours. The media (M) were then removed and the oocytes were homogenised immediately (O) or after trypsin treatment (OT). All samples were immunoprecipitated with anti-*wg* (A) or anti-chick egg white antisera (B), before electrophoresis on 12 5% polyacrylamide gels. Ma, marker proteins; ov, ovalbumin, om, ovomucoid, lys, lysozyme. (C) Oocytes injected with *wg* mRNA (300 μg/ml) and cultured overnight. Healthy oocytes were then labelled in media containing [^{35}S]methionine with or without either 5 mM suramin or 50 μg/ml heparin for 24 hours Media were then collected (M) and replaced with similar, though non-radioactive media, containing 10 mM methionine (chase medium) Culture was continued for a further 24 hours before media (MC) and oocytes (O) were collected. Processing for the presence of *wg* was as in A The apparent difference in mobility between the band in track 1 and other tracks is due to gel distortion (D) As panel C except that oocytes were labelled in the absence of suramin. Oocytes were then cultured in chase medium at the temperatures indicated, with or without 5 mM suramin Oocytes (O) and media (M) were then processed

by the presence of externally added suramin, although we cannot rule out the possibility that, in the absence of suramin, *wg* protein is constitutively secreted but immediately degraded extracellularly.

Discussion

The *Drosophila wg* gene was first identified as a mutation that transformed the adult wing into a duplication of the notum, a part of the thorax derived from the same imaginal disc (Sharma and Chopra, 1976; Morata and Lawrence, 1977). Subsequently, mutations which inactivate *wg* function were shown to be embryonic lethals of the segment polarity class (Nusslein-Volhard and Wieschaus, 1980), the posterior part of each segment being replaced by a mirror-image duplication of the anterior part (Baker, 1988). The

gene encodes a protein with homology to the murine proto-oncogene wnt-1 (Risjewijk et al., 1987), and is expressed in a narrow stripe of cells within each segment (van den Heuvel et al., 1989; Gonzalez et al., 1991). It is then secreted and moves into adjacent cells, in particular it enters a narrow stripe of cells posterior to the *wg* stripe where it controls expression of the *engrailed* gene (Gonzalez et al., 1991; Bejsovec and Martinez Arias, 1991, Heemskerk et al., 1991). The gene is therefore an important regulator of cell fate during *Drosophila* embryogenesis.

Our results show that *wg*, like its vertebrate homologues, murine wnt-1 and Xwnt-8 (McMahon and Moon, 1989; Christian et al., 1991b; Smith and Harland, 1991; Sokol et al., 1991), causes the formation of a second dorsal axis following injection of synthetic RNA into early *Xenopus* embryos, demonstrating that the ability of wnt proteins to induce organizer activity is conserved across a broad phylogenetic spectrum. Prior to these experiments it was not immediately obvious that this would be the case, *wg* differs from other wnt proteins in that it possess an additional domain of 85 amino acids which are remarkably hydrophilic (Risjewijk et al., 1987). Furthermore, not all wnt proteins induce secondary axes following injection into *Xenopus* embryos, it has been reported that Xwnt-5 does not have this property (Christian et al., 1991b).

In agreement with others (Sokol et al., 1991; Smith and Harland, 1991; Christian et al., 1992), our results suggest that wnt proteins achieve this axial duplication by acting as a dorsal mesoderm inducing signal. During normal development this signal is only emitted by dorsovegetal blastomeres of the blastula (Gimlich and Gerhart, 1984; Dale and Slack, 1987b; Kageura, 1990), inducing the overlying marginal zone to form Spemann's organizer Our evidence is threefold: First, *wg* can rescue embryos that had previously been irradiated at the vegetal pole with ultraviolet light, a treatment known to inhibit the formation of the dorsovegetal inducing centre and consequently Spemann's organizer (Gerhart et al., 1989). Identical results have been obtained for both wnt-1 and Xwnt-8 (Sokol et al., 1991; Smith and Harland, 1991; Christian et al., 1992), and argues against a mechanical explanation in which the normal organizer is split. Second, a secondary dorsal axis is reliably formed only following the injection of *wg* mRNA into ventrovegetal blastomeres, injections into the animal hemisphere have little or no effect. At the 32-cell stage injections into the D-tier induce a secondary dorsal axis without any substantial contribution to the mesoderm from the injected blastomere, which differentiates as dorsal endoderm This is the normal fate of dorsal D-tier blastomeres (Dale and Slack, 1987a; Moody, 1987) and suggests that *wg* expressing ventral D-tier blastomeres have changed their fate towards that of their dorsal counterparts, acquiring the ability to induce an organizer in the adjacent C-tier. The same conclusion was reached by Smith and Harland (1991) following similar experiments with Xwnt-8.

Our third line of evidence is that animal caps isolated from *wg* expressing blastulae differentiate mesoderm, suggesting that *wg* protein is a mesoderm inducing factor. However, in contrast to the ability of *wg* protein to induce dorsal mesoderm in the marginal zone of an embryo, it only induces ventral mesoderm in isolated animal caps. The simplest explanation for this is that *wg* protein normally acts in concert with other factors that are not present in our animal caps. We have shown that this other factor could be FGF, in its presence *wg* expressing animal caps differentiate dorsal mesoderm. Interestingly, it has recently been shown that whereas FGF is found within the marginal zone of blastulae it is absent from animal caps (Shiurba et al., 1991). Similar conclusions to ours have also been reached by Christian et al. (1992) using Xwnt-8 expressing animal caps. These authors also showed that when larger animal caps were taken, some dorsal mesoderm was found in absence of exogenous factors, a result that might explain the observations of muscle within Xwnt-8 expressing animal caps by Sokol et al. (1991).

We have shown that the fate of cells not expressing *wg* protein can be influenced by its production elsewhere in the embryo, yet our immunohistological (embryos) and biochemical (oocyte) data appear to indicate an inability of this protein to spread beyond the producer cells. Although this behavior is consistent with what has been observed for this family of proteins in mammalian systems (Bradley and Brown, 1990; Papkoff and Schryver, 1990), in *Drosophila* embryos, *wg* protein has been found 2-4 cells away from its site of synthesis (Gonzalez et al., 1991). In cultured cell lines, suramin or heparin treatment leads to the secretion/release of the murine wnt-1 protein, although the proportion of protein found in the culture medium is quite variable and can be very low (Bradley and Brown, 1990; Papkoff and Schryver, 1990). In oocytes, suramin, though not heparin, treatment leads to the almost quantitative recovery of oocyte-associated material in the medium. This salvaged protein does not seem to have been associated with the cell surface, as evidenced by its trypsin-insensitivity or its resistance to suramin-induced release at 4°C In this way, it differs from its mammalian counterpart, some of which clearly accumulates in the extracellular matrix. The observation that the intracellular steady-state concentration of *wg* is unaffected by suramin treatment indicates that secretion or degradation are mutually exclusive choices for this protein, a situation in oocytes that we have described previously for murine immunoglobulins (Colman et al., 1981b). Although suramin probably can interact with various classes of secretory protein, it will be interesting to see whether it affects the formation of the second dorsal lip (see Fig. 3) in *wg* expressing embryos, since this lip is the first phenotypic sign of *wg* action and reflects the presence of a new organizer region. Previous use of suramin has been restricted to later stages of gastrulation, where arrest of gastrulation movements follows its injection (Gerhart et al., 1989).

Although the seeming lack of movement of *wg* in our experiments could be just an issue of sensitivity, a small amount of *wg* protein diffusing away from its site of secretion, an alternative explanation is that this molecule acts primarily upon adjacent cells. There are precedents for paracrine factors being retained at the surface of the cells that produce them; in the case of platelet-derived growth factor (PDGF) B chain dimer, this retention enhances its cell transforming ability (Beckman et al., 1988) and is mediated by a C-terminal stretch of amino acids with claimed homology to sequences in the C termini of murine and human Wnt 1 homologues (La Rochelle et al., 1991). Whatever the explanation for *wg* protein immobility, it remains difficult to reconcile completely the molecular properties of *wg* with our

perceived understanding of mesoderm induction, where diffusion of inducing factors throughout the prospective mesoderm is anticipated. Of particular interest are the contrasting effects of expressing *wg* in the animal hemisphere and the D-tier of the vegetal hemisphere; although the injected blastomeres are adjacent to the C-tier in both cases, only D-tier injections reliably change the fate of the neighbouring blastomeres towards that of their dorsal counterparts. We might reconcile these results if we assume that *wg* protein is indeed restricted to the vicinity of its secretion, perhaps by an interaction with extracellular matrix materials, and that only cells close to the prospective mesoderm-endoderm border are capable of responding. This is unlikely to be the result of the restricted location of wnt-1 receptors, since animal cap cells are competent to respond to *wg*, but is more likely the result of the restricted location of some accessory factor(s) required for *wg* function (see above).

Finally, the molecule that we have studied in our experiments, *wg* protein cannot normally be involved in the development or function of the amphibian organizer. Like others (McMahon and Moon, 1989; Christian et al., 1991b), our hypothesis is that this molecule activates the receptor for an endogenous member of the wnt family. The endogenous activator is unlikely to be *Xenopus* wnt-1 which appears to be expressed only from neurula stages onwards (Nordermeer et al., 1989), after the organizer's functions are over. A more credible candidate would be Xwnt-8 which Sokol et al. (1991) and Smith and Harland (1991) have shown to have the properties of a dorsal mesoderm inducer, using some of the approaches described in this paper. However, although this molecule is present at the right time in development, it is localised to the non-organizer ,ventral and lateral marginal zone (Christian et al., 1991b; Smith and Harland, 1991). Despite the fact that *wg* cannot be the endogenous inducer, we feel justified in pursuing our studies using it for two reasons: First, the provision of good antisera allow the patterns of *wg* location and phenotypic effect to be compared, and second, the availability of various *wg* null mutants, especially a temperature-sensitive one (Gonzalez et al., 1991), may generate further insight into how wnt proteins act during development and over what time period they are effective

We particularly thank A. Martinez Arias for constant encouragement, guidance, and criticism of the manuscript. We wish to thank the following for provision of reagents: A. Martinez Arias, *wg* cDNA, J. Slack, bFGF, M. van den Heuvel and R. Nusse, anti-*wingless* antisera; J Skehel for anti-haemagglutinin hybridoma supernatants. We thank S Bhamra for the confocal microscopy he performed at the University of Warwick. A Colman is indebted to the British Medical Research Council and Wellcome Trust for the support of this work

References

Asashima, M., Nakano, H., Shimada, K., Ishii, K., Shibai, H. and Ueno, N. (1990) Mesodermal induction in early amphibian embryos by activin A (erythroid differentiation factor) *Roux's Arch. Dev Biol* **198**, 330-335

Baker, N. (1988) Embryonic and imaginal requirements for *wingless* gene in whole *Drosophila* embryos *Devl Biol.* **125**, 96-108

Beckman, M. P., Betsholtz, C., Heldin, C-H., Westermark, B., Di Marco, E., Di Fiore, P. P., Robbins, K. C. and Aaronson, S. A . (1988) Comparison of the biological properties and transforming potential of Human PDGF-A and PDGF-B chains *Science* **241**, 1346-1349

Bejsovec, A. and Martinez Arias, A. (1991) Roles of *wingless* in patterning the larval epidermis of *Drosophila Development* **113**, 471-486

Bradley, R. S. and Brown, A. M. C. (1990) The proto-oncogene int-1 encodes a secreted protein associated with the extracellular matrix *EMBO J* **9**, 1569-1575

Ceriotti, A. and Colman, A. (1988) Binding to membrane proteins within the endoplasmic reticulum cannot explain the retention of the glucose regulated protein GRP 78 in *Xenopus* oocytes *EMBO J* **7**, 633-638

Ceriotti, A. and Colman, A. (1989) Protein transport from endoplasmic reticulum to the Golgi complex can occur during meiotic metaphase in *Xenopus* oocytes *J Cell Biol* **109**, 1439-1444

Christian, J. L., Gavin, B. J., McMahon, A. P. and Moon, R. T. (1991a) Isolation of cDNAs partially encoding four *Xenopus* Wnt-1/int-1-related proteins and characterization of their transient expression during embryonic development *Devl Biol* **143**, 230-234

Christian, J. L., McMahon, J. A., McMahon, A. P. and Moon, R. T. (1991b) Xwnt-8, a *Xenopus* wnt-1/int-1-related gene responsive to mesoderm inducing growth factors, may play a role in ventral mesodermal patterning during embryogenesis *Development* **111**, 1045-1055

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

Colman, A. (1984) Translation of eucaryotic mRNA in *Xenopus* oocytes In *Transcription and Translation A Practical Approach* (eds, B D Hames and S. J Higgins) pp 271-300 Oxford IRL Press

Colman, A. and Morser, J. (1979). Export of proteins from oocytes of *Xenopus laevis Cell* **17**, 517-526

Colman, A., Lane, C. D., Craig, R., Boulton, A., Mohun, T. and Morser, J. (1981a) The influence of topology and glycosylation on the fate of heterologous secretory proteins made in *Xenopus* oocytes *Eur J Biochem.* **113**, 339-348

Colman, A. Morser, J., Lane, C. D., Besley, J., Wylie, C. and Valle, G. (1981b) Fate of secretory proteins trapped in oocytes of *X laevis* by disruption of the cytoskeleton or by imbalanced subunit synthesis *J Cell Biol* **91**, 770-780

Cooke, J. (1972) Properties of the primary organization field in the embryo of *Xenopus laevis* II Positional information for axial organization in embryos with two head organizers *J Embryol exp Morph* **28**, 27-46

Dale, L., Howes, G., Price, B. M. J. and Smith, J. C. (1992) Bone morphogenetic protein 4 A ventralising factor in early *Xenopus* development. *Development* **115**, in press

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. and Slack, J. M. W. (1987a) Fate map for the 32-cell stage of *Xenopus laevis Development* **99**, 527-551

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

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

Dent, J. A. and Klymkowsky, M. W. (1989) Whole mount analyses of cytoskeletal reorganisation and function during oogenesis and early embryogenesis in *Xenopus* In *The Cell Biology of Fertilization* (eds H Schatten and G Schatten) pp 64-103 New York Academic Press

Gavin, B. J., McMahon, J. A. and McMahon, A. P. (1990) Expression of multiple novel wnt-1/int-1-related genes during fetal and adult mouse development *Genes Dev* **4**, 2319-2332

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 Supplement**, 37-51

Gimlich, R. L. and Braun, J. (1985) Improved fluorescent compounds for tracing lineage *Devl Biol* **109**, 509-514

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

Gonzalez, F., Swales, L., Bejsovec, A., Skaer, H. and Martinez Arias, A. (1991) Secretion and movement of *wingless* protein in the epidermis of the *Drosophila* embryo *Mech. Devl* **35**, 43-54.

Heemskerk, J., Dinardo, S., Kostriken, R. and O'Farrell, P. H. (1991) Multiple modes of engrailed regulation in the progression toward cell fate determination *Nature* **352**, 404-410

Huang, S. S. and Huang, J. S. (1988) Rapid turnover of the PDGF receptor in *sis*-transformed cells and reversal by suramin *J Biol Chem.* **263**, 12608-12618

Kageura, H. (1990) Spatial distribution of the capacity to initiate a secondary embryo in the 32-cell embryo of *Xenopus laevis Devl. Biol* **142**, 432-438

Kao, K. R. and Elinson, R. P. (1988) The entire mesodermal mantle behaves as Spemann's organizer in dorso-anterior enhanced *Xenopus laevis* embryos *Devl Biol.* **127**, 64-77

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

Kimelman, D., Abraham, J. A., Haaparanta, T., Palisi, T. M. and Kirschner, M. (1989). The presence of fibroblast growth factor in the frog egg. its role as a natural mesoderm inducer *Science* **242**, 1053-1056

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

Kirschner, M. and Hara, K. (1980) A new method for local vital staining of amphibian embryos using Ficoll and crystals of Nile Red. *Mikroskopie* **36**, 12-15

Koster, M., Plessow, S., Clement, J. H., Lorenz, A., Teidemann, H. and Knochel, W. (1991) Bone morphogenetic protein 4 (BMP4), a member of the TGF β family in early embryos of *Xenopus laevis* analysis of mesoderm inducing activity *Mech. Dev* **33**, 191-199

Krieg, P. and Melton, D. A. (1987) In vitro RNA synthesis with SP6 RNA polymerase *Methods in Enzymology* **155**, 397-415

La Rochelle, W. J., May-Siroff, M., Robbins, K. C. and Aaronson, S. A. (1991) A novel mechanism regulating growth factor association with the cell surface. identification of a PDGF retention domain *Genes Dev* **5**, 1191-1199

McMahon, A. P. and Moon, R. T. (1989) Expression of the proto-oncogene *int-1* in *Xenopus* embryos leads to duplication of the embryonic axis *Cell* **58**, 1075-1084

McMahon, A. P. and Bradley, A. (1990) The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain *Cell* **62**, 1073-1085.

Moody, S. (1987) Fates of the blastomeres of the 16-cell stage *Xenopus* embryo *Devl Biol* **119**, 560-578

Morata, G. and Lawrence, P. A. (1977) The development of *wingless*, a homeotic mutation of *Drosophila Devl Biol* **56**, 227-240

Nakamura, O. and Kishiyama, K. (1971) Prospective fates of blastomeres at the 32-cell stage of *Xenopus laevis Proc Japan Acad* **47**, 407-412

Nieuwkoop, P. D. (1969) The formation of the mesoderm in urodelean amphibians I The induction by the endoderm *Wilhelm Roux' Arch. EntwMech. Org* **169**, 341-373

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

Nusslein-Volhard, C. and Weischaus, E. (1980) Mutations affecting segment number and polarity in *Drosophila Nature* **287**, 795-801

Noordermeer, J., Meijlink, F., Verrijzer, P. I., Rijsewijk, F. and Destree, O. (1989) Isolation of the *Xenopus* homolog of int-1/*wingless* and expression during neurula stages of early development *Nucl Acids Res* **17**, 11-18

Papkoff, J. (1989) Inducible overexpression and secretion of int-1 protein *Mol Cell Biol* **9**, 3377-3384

Papkoff, J., Brown, A. and Varmus, H. (1987) The int-1 proto-oncogene products are glycoproteins that appear to enter the secretory pathway *Mol Cell Biol* **7**, 3978-3984

Papkoff, J. and Schryver, J. (1990) Secreted int-1 protein is associated with the cell surface *Mol Cell Biol* **10**, 2723-2730

Paterno, G. D., Gillespie, L. L., Dixon, M. S., Slack, J. M. W. and Heath, J. K. (1989) Mesoderm-inducing properties of int-2 and kFGF two oncogene encoded growth factors related to FGF *Development* **106**, 79-83

Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D. and Nusse, R. (1987) The *Drosphila* homologue of mouse mammary oncogene int-1 is identical to the segment polarity gene *wingless Cell* **50**, 649-657

Rosa, F., Roberts, A. B., Danielpour, D., Dart, L. L., Sporn, M. B. and Dawid, I. B. (1988) Mesoderm induction in amphibians: the role of TGF-beta 2-like factors *Science* **239**, 783-785

Scharf, S. and Gerhart, J. C. (1980) Determination of the dorsal-ventral axis in eggs of *Xenopus laevis*· complete rescue of UV-impaired eggs by oblique orientation before first cleavage *Devl Biol* **79**, 181-198

Sharma, R. P. and Chopra, V. L. (1976) Effect of *wingless* (*wg*) mutation on wing and haltere development in *Drosophila melanogaster Devl Biol* **48**, 461-465

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

Slack, J. M. W. (1991) *From Egg To Embryo Regional Specification in Early Development* (2nd Edition) Cambridge Cambridge University Press

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

Slack, J. M. W. and Isaacs, H. V. (1989) Presence of basic fibroblast growth factor in the early *Xenopus* embryo *Development* **105**, 147-154

Smith, J. C. (1987) A mesoderm inducing factor is produced by a *Xenopus* cell line *Development* **99**, 3-14

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

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

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

Smith, 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

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

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

Spemann, H. and Mangold, H. (1924) Uber Induktion von Embryonanlagen durch Implantation artfremder Organisatoren *Arch mikr Anat EntwMech* **100**, 599-638

Stewart, R. and Gerhart, J. (1990) The anterior extent of dorsal development of the *Xenopus* embryonic axis depends on the quality of the organiser in the late blastula *Development* **109**, 363-372

Tannahill, D. and Melton, D. A. (1989) Localised synthesis of the Vg1 protein during early *Xenopus* development. *Development* **106**, 775-786

Thomas, K. R. and Capecchi, M. R. (1990) Targeted disruption of the murine int-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature* **346**, 847-859

Thomsen, G. T., 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

van den Heuvel, M., Nusse, R., Johnston, P. and Lawrence, P. (1989) Distribution of the *wingless* gene product in *Drosophila* embryos a protein involved in cell-cell communication *Cell* **59**, 739-749

van Ooyen, A. and Nusse, R. (1984) Structure and nucleotide sequence of the putative mammary oncogene *int-1* proviral insertions leave the protein-encoding domain intact. *Cell* **39**, 233-240

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-β *Cell* **51**, 861-867

(Accepted 27 February 1992)