Development 120, 1191-1199 (1994)
Printed in Great Britain © The Company of Biologists Limited 1994

A role for cytoplasmic determinants in mesoderm patterning: cell-autonomous activation of the *goosecoid* and *Xwnt-8* genes along the dorsoventral axis of early *Xenopus* embryos

P. Lemaire* **and J. B. Gurdon**

Wellcome/CRC Institute for Cancer and Developmental Biology, Tennis Court Road, Cambridge CB2 1QR, UK and Department of Zoology, University of Cambridge, UK

*Author for correspondence

SUMMARY

Although an induction event is required for the formation of mesoderm in *Xenopus* embryos, it is not clear that this induction is wholly sufficient to give rise to a correctly patterned mesodermal layer. We have studied the expression of the two genes, *goosecoid* and *Xwnt-8,* in *Xenopus* gastrulae in which cell-cell communication, and therefore mesoderm induction, has been prevented by frequent cell dispersion. Although neither the early pan-mesodermal marker *Xbra* nor the muscle-specific α-actin gene were activated under these conditions, *goosecoid* and *Xwnt-8* were activated in cells of dorsal and ventrolateral origin respectively, thus correctly reflecting their distribution during normal development. We also show that the spatial pattern of expression of these two genes along the animal-vegetal axis is similar in normal and in dissociated early gastrulae: *goosecoid* is mainly expressed in future mesoderm while the domain of expression of *Xwnt-8* spans the mesoderm-endoderm boundary. These results show that, during the blastula and early gastrula stages, gene activation can be controlled cell-autonomously along both the animal-vegetal and dorsoventral embryo axes. This suggests that the inheritance of localised maternal cytoplasmic determinants is a key event for the patterning of mesoderm. We present a modified model of mesoderm formation in which the different mesoderm cell types are produced as a result of cooperation between induction-dependent and induction-independent immediate-early genes.

Key words: mesoderm, induction, cytoplasmic determinants, gene expression, *Xwnt-8*, *goosecoid*, *Xbra*, *Xenopus*, axis determination, in situ hybridisation

INTRODUCTION

In early amphibian embryos, mesoderm is generated as a result of the induction of cells in the equatorial region of the early embryo by the underlying vegetal cells (reviewed by New et al., 1991). This induction can be reconstituted in vitro by conjugating vegetal tissue and animal tissue (Nieuwkoop, 1969). This results in the differentiation of some of the animal cells into mesodermal derivatives. More recently, it has been shown that members of the Fibroblast Growth Factor (FGF) and TGF-β families can efficiently mimic the action of the vegetal inducer. Although the exact composition of the signals emitted by the vegetal cells is still unknown, the disruption of mesoderm formation in embryos overexpressing dominant negative mutants of the FGF or activin receptors (Amaya et al., 1991, 1993; Hemmati-Brivanlou and Melton, 1992) strongly argues that molecules belonging to these two families of growth factors play a crucial role.

In addition to the characterisation of the signalling factors, several immediate-early response genes have been isolated that can be activated in ectoderm cells following treatment with activin or FGF and which therefore represent candidate downstream mesodermal genes. Among these, the *Xenopus* homologue of the mouse *Brachyury* gene, *Xbra*, is thought to be expressed throughout early gastrula mesoderm (Smith et al. 1991). During the gastrula and neurula stages, its expression becomes restricted to the notochord and posterior mesoderm (Smith et al., 1991; Green et al., 1992). Also belonging to this class of early expressing genes are *Xwnt-8,* encoding a secreted protein, and *goosecoid*, a homeobox gene. *Xwnt-8* is initially expressed in the entire marginal zone except for the dorsal-most cells (Christian et al., 1991; Smith and Harland, 1991; Christian and Moon, 1993). There is some uncertainty about the expression of this gene during gastrulation. Smith and Harland (1991) reported that its expression becomes restricted to the ventral and posterior mesoderm, while Christian and Moon (1993) found expression in both ventral and lateral plate mesoderm. *goosecoid* is originally expressed in the dorsal-most cells, which correspond to the organiser region (Cho et al., 1991). Recently, Green and colleagues (1992) have demonstrated that animal cap cells respond to increasing concentrations of activin by progressively activating genes normally expressed in a more dorsal location. These findings have greatly improved our understanding of the formation and pat-

terning of mesoderm and they show that an activin treatment is sufficient to turn early mesodermal genes on in animal caps. They do not however address the issue of whether mesoderm induction is necessary for the normal expression of these genes in the equatorial region of early *Xenopus* embryos, or whether some equatorial cells can activate mesodermal genes in the absence of cell interactions. Here, we address this issue by studying gene expression in embryos in which mesoderm induction has been blocked.

The formation of mesoderm can be artificially blocked by preventing cell-cell interactions between fertilisation and the early gastrula stages. This can be achieved by culturing the embryos in calcium-free medium, thus preventing cell adhesion, and dispersing the loose cells (Gurdon et al., 1984; Symes et al., 1988). If embryos are submitted to this treatment up to the end of the period of competence for mesoderm induction (stage 10.5) and subsequently reaggregated by adding back calcium, then the mesoderm fails to form as demonstrated by the absence of cardiac α-actin expression, a marker for muscle (Sargent et al., 1986).

We have studied the expression of *Xbra, goosecoid* and *Xwnt-8* in embryos whose cells were dissociated from the egg to the early gastrula (stage 10.5). We have made the surprising finding that, under these conditions, although the synthesis of zygotic *Xbra* mRNA is not detectable, both *Xwnt-8* and *goosecoid* are activated after the midblastula transition (MBT). Moreover, we find that, by the early gastrula stage, the dissociated cells expressing *goosecoid* are of dorsal origin while those expressing *Xwnt-8* are of ventrolateral origin.

MATERIALS AND METHODS

Embryos and dissociation procedures

Embryos were obtained by in vitro fertilisation, cultured in 0.1× Modified Barth Saline (MBS) as described previously (Gurdon et al., 1985a) and staged according to Nieuwkoop and Faber (1967). For the dissociation experiments, embryos were transferred to 1× MBS lacking calcium and magnesium (Ca/Mg-free MBS) during the second half of the first cell cycle. They were manually demembranated at the appropriate stage and the loose cells were cultured in 5 to 10 ml of Ca/Mg-free MBS containing 0.2% bovine serum albumin in agarose-coated plastic dishes. Cells were kept dispersed by gently agitating the dishes every 30 minutes. When indicated in the text, they were reaggregated by adding calcium and magnesium to reconstitute the normal concentration of these ions in MBS. The dissociated cells or aggregates were staged by comparison with control embryos cultured in parallel. Embryos were marked with vital dyes according to the following procedure. To label the dorsal side, a few drops of 1% nile blue sulphate (Sigma) were mixed with some drops of 0.1 M Na_2CO_3. The resulting precipitate was collected on forceps and applied to the upper side of a fertilised egg tilted so that the animal-vegetal axis is horizontal with the sperm entry pointing downwards. Embryos were inspected during the first cell division to ensure that the cleavage furrow passed through or near the blue mark. Embryos inaccurately marked were discarded. To label the ventral side during the first cleavage, embryos already labelled on the dorsal side were marked with a precipitate of neutral red and sodium carbonate opposite to the nile blue sulphate mark. Accuracy was verified by examining embryos at stage 10.5.

PCR primers

The following primers were synthesized on a Applied Biosystems 380B DNA synthesizer:

Xbra: Sense primer 5′-GCCTGTCTGTCAATGCTCCA-3′ (positions 1058 to 1077 of xt6 cDNA (Smith et al., 1991; EMBL database entry M77243)) antisense primer: 5′-TGTGCTCCATGCT-CATACAA-3′ (positions 1329 to 1348 of xt6 cDNA).

Xwnt-8: Sense primer 5′-TTAAAGATCAAGCACGACCA-3′ (positions 679 to 698 of EMBL database entry X57234), antisense primer 5′-GACTGTGCAGCACCAGTGA-3′ (positions 1005 to 1023).

goosecoid: Sense primer 5′-TGTGGAGCAGTTCAAGCTCT (positions 376 to 395 from EMBL database entry M81481), antisense primer 5′-ATCTGGGTACTTGGTTTCTT-3′ (positions 617 to 636).

FGF Receptor gene: sense primer 5′-TTGAAGTCTGATGC-GAGTGA-3′ (positions 1528 to 1547 from EMBL database entry M37201), antisense primer 5′-GGGTTGTAGCAGTACTCCAT-3′ (positions 1723 to 1742).

RT-PCR assay

Cells or tissue samples were lysed in NETS/phenol buffer (Hopwood et al., 1989) containing 50 ng/μl tRNA and quickly homogenised in a glass homogeniser. After centrifugation, the aqueous phase was recovered and subjected to a second phenol/chloroform extraction. The nucleic acids were then ethanol precipitated and resuspended in a 5-10 μl of water. Selective precipitation of RNA was achieved by two consecutive ammonium acetate precipitations as follows. 0.25 volumes of 10 M ammonium acetate were added to the nucleic acids solution. After a one hour incubation on ice, the tube was centrifuged, the pellet washed with 70% ethanol and resuspended in 5-10 μl of water. This treatment discriminated RNA from DNA as efficiently as a lithium chloride precipitation but was found to be more compatible with the subsequent reverse transcription reaction. The RNA was reverse transcribed as described by Rupp and Weintraub (1991). One tenth to one half of the cDNA was used to perform the amplifications in a 50 μl volume essentially as in Rupp and Weintraub (1991) except that we used an MJ Research, Inc thermal cycler with the following temperature profile: denaturation at 94°C for 30 seconds, primer annealing for 30 seconds at 60°C, extension for 1 minute at 72°C. We usually used 25 to 27 cycles and ran 30% of the reaction on a 6% sequencing gel. Using these conditions, the signal obtained was a linear function of the input cDNA as measured by amplification of serial dilutions of the input cDNA (data not shown). As an internal loading control, the primers for the FGF receptor gene were always included in the PCR reactions. We chose to use this gene because it has an abundance similar to that of *Xwnt-8* and *goosecoid* but it is expressed at a constant level throughout early development (our data and Musci et al., 1990). Gels from the different RT-PCR experiments were dried and autoradiographed. The signals were quantitated using a Microtek MRS-600ZS scanner linked to a Macintosh computer running the Image software (NIH). In each case, the relative abundance of *Xwnt-8* and *goosecoid* RNA in different samples was determined by comparing the signals obtained for these genes with that obtained for the FGF receptor gene. To make sure that the signals that we obtained were not due to amplification from genomic DNA that might have been left in the RNA preparation, we also performed amplifications from samples in which the reverse transcriptase had been omitted from the reverse transcription reaction. No signal was obtained under these conditions (data not shown).

RNase protection assays

RNase protection assays were performed as described previously (Gurdon et al., 1985a). The *Xbra* probe was prepared following the instructions of Smith and colleagues (1991) from the plasmid pXbra. The *goosecoid* probe was prepared from a construct called pGSC2 (a generous gift of E. De Robertis and J. C. Smith) carrying a segment of the gene extending from the 5′ end of the p*gsc* cDNA (Cho et al., 1991) to the first *Apa*I site. The *Xwnt-8* probe was synthesized from a construct containing nucleotides 684 to 1017 of EMBL database entry X57234, while the FGF receptor probe was derived from a

construct containing nucleotides 1839 to 2042 of EMBL database entry M61687.

In situ hybridisation probes

Probes were synthesized essentially as described by Harland (1991). The *Xbra* probe was obtained using the pXbra plasmid (Smith et al., 1991) linearised with *Bgl*II and transcribed from the T7 promoter. The *Xwnt-8* probe was synthesized from a full-length *Xwnt-8* cDNA independently cloned in our laboratory from a gastrula cDNA library in pBluescript SK-, linearised with *Eco*RI and transcribed from the T3 promoter. Both probes were chemically hydrolysed to fragments of an average size of 300 nucleotides.

In situ hybridizations

Albino embryos at stage 10.5 were fixed in MEMFA (Hemmati-Brivanlou et al., 1990) for 2 hours and then transfered to methanol and subsequently embedded in a mixture of Histoplast (Shandon) and bees wax (98:2). Embryos were sectioned (20 µm thickness), the sections were layered onto polylysine-coated glass slides and left to dry for 3 days on a hot plate set at 45°C. Sections were dewaxed, refixed and subjected to proteinase K, HCl and acetic anhydride treatments as described (Hopwood et al.,1989). The slides were then washed in water and prehybridised for 2 hours at 60°C with 300 µl hybridisation buffer (recipe in Hemmati-Brivanlou et al., 1990). The prehybridisation buffer was then removed and replaced with 200 µl of hybridisation buffer containing 3 µg/ml of digoxigenin-labelled probe. The sections were hybridised overnight at 60°C. The hybridisation buffer was removed and the slides were incubated successively in hybridisation buffer (60°C, 10 minutes), 50% hybridisation buffer in 2× SSPE containing 0.3% CHAPS (Boehringer, cat No 810 118) (60°C, 10 minutes), 25% hybridisation buffer in 2× SSPE, 0.3% CHAPS (room temperature, 10 minutes), 2× SSPE, 0.3%CHAPS (room temperature, 2 times 30 minutes). RNase treatment and high stringency washes were performed as described previously (Hopwood et al., 1989). All subsequent steps were performed at room temperature. To detect the digoxigenin, the slides were first washed twice for 10 minutes in 2× SSPE, 0.3% CHAPS (1 ml/slide), they were then incubated for 3 times 10 minutes in buffer 1 (100 mM Tris-HCl pH 7.5, 150 mM NaCl) and then for 30 minutes in buffer 1 containing 0.5% blocking reagent (Boehringer cat. No 1096 176). Incubation with anti-digoxigenin alkaline phosphatase conjugated fab fragments (Boehringer cat. No 1093 274 diluted 1/500) was for 1 hour in buffer 1. After three 15 minutes washes in buffer 1, the slides were washed for 10 minutes in buffer 3 (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1 % Tween-20 and 0.025% Levamisole). The colour reaction was performed at room temperature in buffer 3 containing Nitroblue tetrazolium and S-bromo-4-chloro-3-indolyl (Boehringer) following the manufacturer's instructions. When the signal was sufficiently intense, slides were fixed in MEMFA for 45 minutes washed in PBS containing 0.1% Tween-20 and mounted in 10% PBS, 90% glycerol.

RESULTS

Cell dissociation of embryos between fertilisation and stage 10.5 prevents the formation of mesoderm

Mesoderm induction takes place from the 32-cell stage (stage 6) to the early gastrula stage (stage 10.5) (Gurdon et al., 1984; Jones and Woodland, 1987). To prevent this induction, we disrupted cell-cell communication by cultivating embryos in calcium-free magnesium-free medium from shortly after cortical rotation until stage 10.5 (Fig. 1A). The vitelline membrane was removed at the 32-cell stage. The loose cells were gently dispersed and cultured as single cells until they were either reaggregated by the addition of calcium and magnesium or harvested at the appropriate stage to analyse gene expression. To ensure that daughter cells were not in contact with each other after cell division, cells were redispersed every 30 minutes during the incubation period. Comparison of the number of cells in a normal and a dissociated gastrula indicated that the dissociation treatment did not result in cell loss or in a significant modification of the division rate of the cells. Two methods were used to quantify the amount of

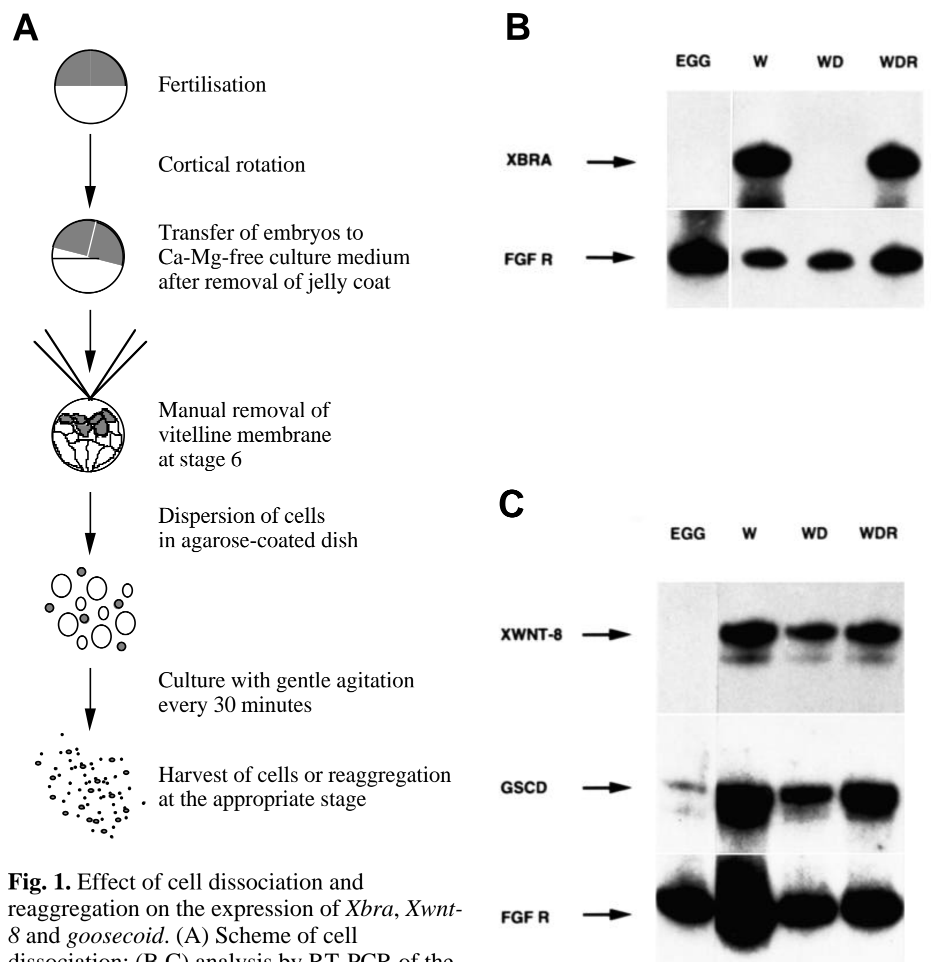

Fig. 1. Effect of cell dissociation and reaggregation on the expression of *Xbra*, *Xwnt-8* and *goosecoid*. (A) Scheme of cell dissociation; (B,C) analysis by RT-PCR of the expression of *Xbra*, *XWnt*-8, *goosecoid* (gscd) and the FGF receptor (FGFR). The latter gene is ubiquitously expressed and serves as a loading control. The samples analysed were eggs, whole embryos (W), whole embryos dissociated between the stages 6 and 10.5 (WD), or whole embryos dissociated between the stages 6 and 9 and subsequently reaggregated by the addition of calcium and magnesium (WDR). For the analysis of gene expression, all embryos and dissociated cells were lysed at the equivalent of stage 10.5.

Table 1. Effect of dissociation on the number of transcripts of *Xbra*, *Xwnt-8* and *goosecoid* in whole early gastrulae and eggs

Gene analysed	Assay	Expression in egg as % of FGF-R value	Expression at stage 10.5 as % of FGF-R value: Normal embryo	Expression at stage 10.5 as % of FGF-R value: Dissociated stage 6-10.5	Expression at stage 10.5 as % of FGF-R value: Dissociated stage 2-10.5	Expression in embryos dissoc. from stage 6-10.5 as % of normal stage 10.5	Expression in embryos dissoc. from stage. 2-10.5 as % of n stage 10.5
Xwnt-8	RNase P	<1 (3)	74±13 (4)	41±15 (5)	26±14 (2)	50±15 (4)	35±15 (2)
	RT-PCR	<1 (3)	53±19 (4)	30±18 (4)	NT	59±30 (4)	NT
Gscd	RNase P	<2 (2)	38±22 (3)	14±2 (3)	14±2 (2)	47±37 (3)	30±4 (2)
	RT-PCR	<5 (2)	53±17 (5)	26±20 (5)	NT	63±20 (5)	NT
Xbra	RNase P	<4 (6)	149±50 (6)	<4 (6)	<2 (2)	<3 (6)	<2 (2)
	RT-PCR	<2 (2)	306±62 (4)	<3 (4)	NT	<1 (4)	NT
Column number		1	2	3	4	5	6

Effect of dissociation on the number of transcripts for *Xbra*, *Xwnt-8* and *goosecoid* in whole early gastrulae and eggs. Columns 1 to 4 present a quantification of the intensities of the signals obtained for the genes *Xwnt-8*, *goosecoid* and *Xbra* expressed as a percentage of the signal obtained for the FGF receptor gene (FGFR). Columns 5 and 6 indicate the ratio gene X/FGFR value in dissociated embryos as a percentage of the gene X/FGFR value in normal embryos. In all columns, the format is: average value ± standard deviation (number of experiments). V.M., vitelline membrane; RNase P, results from the RNase protection assay; RT-PCR, results from the RT-PCR assay; NT, not tested.

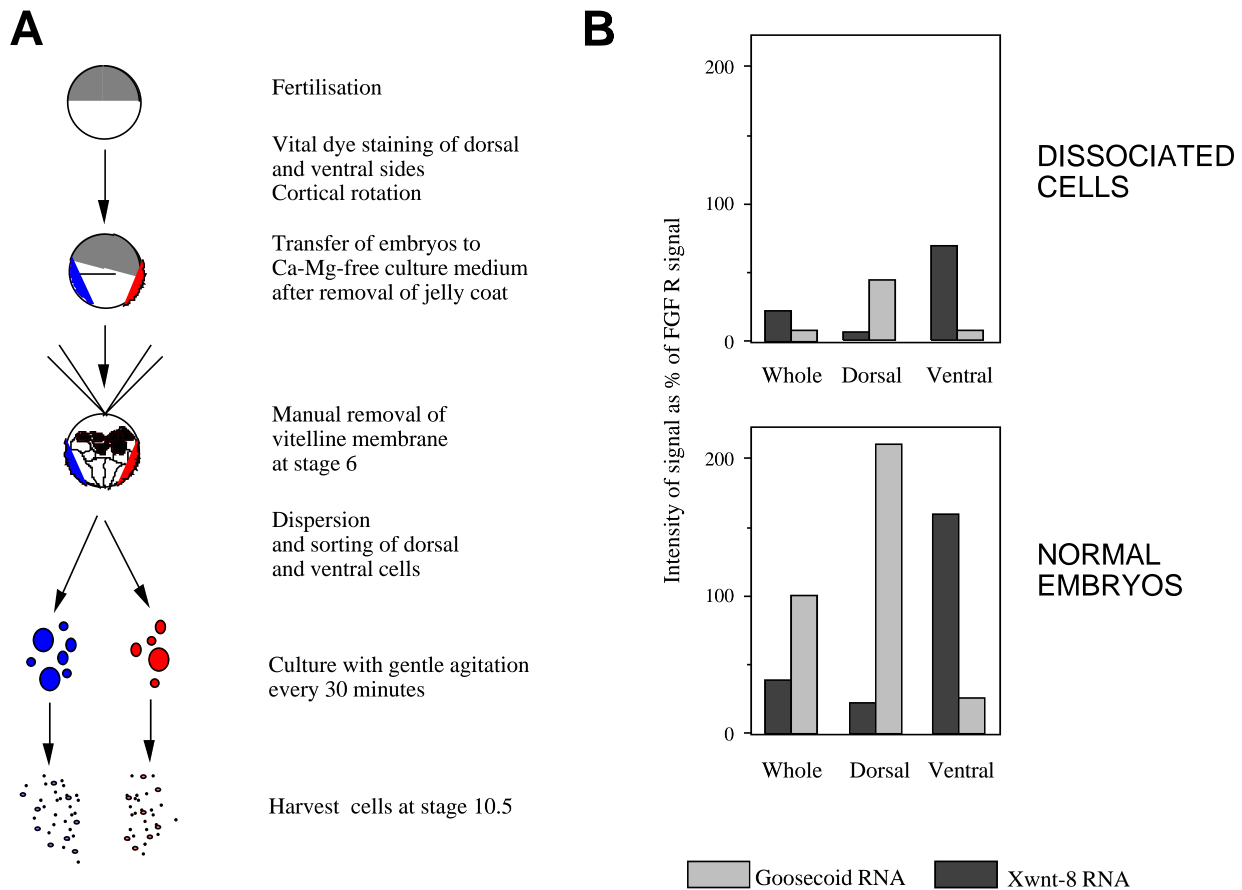

Fig. 2. Expression of *XWnt-8* and *goosecoid* in dissociated cells originating from the most dorsal or most ventral quarters of embryos. (A) Experimental design. In the embryo diagrams, the dorsal side is to the left. The dorsal and ventral dye marks are shown in blue and red respectively. (B) Histograms of the expression of *Xwnt-8* or *goosecoid* shown as a percentage of the FGF receptor gene expression. The assay used was RT-PCR. In this experiment, dissociation had an atypically strong effect on the level of expression of *goosecoid* in whole embryo cells.

transcripts for the three genes that we studied: an RNase protection assay and an RT-PCR assay. Using the FGF receptor as a standard, we showed that the variation in expression level of the three genes *Xbra*, *goosecoid* and *Xwnt-8* in normal and dissociated embryos was essentially the same by the two methods (Table 1 columns 5 and 6). This established that the RT-PCR assay could be used reliably to quantify relative amounts of transcripts. This is important because in later stages

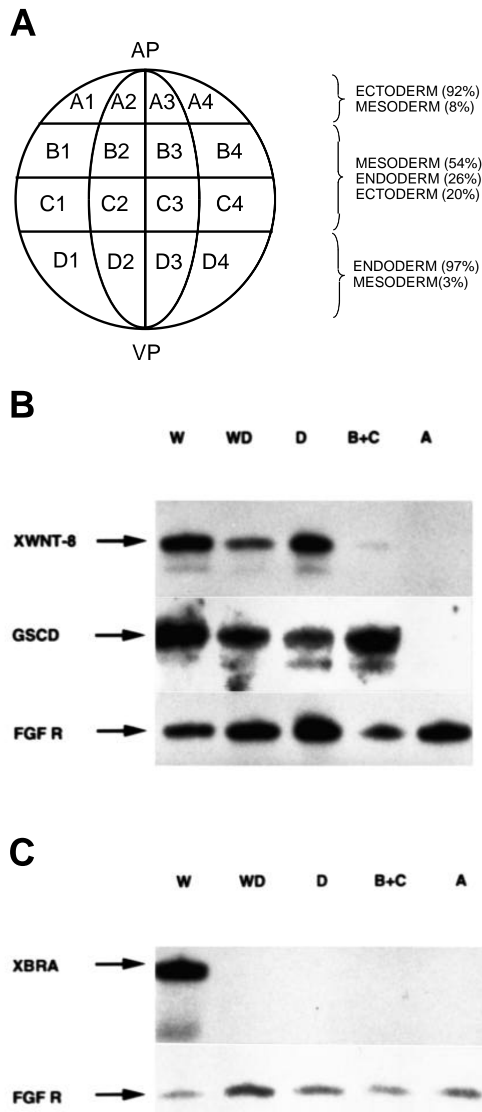

Fig. 3. Expression pattern of *XWnt-8* and *goosecoid* in stage 10.5 dissociated cells descended from the vegetal, marginal and animal parts of 32-cell embryos. (A) Fate map of the 32-cell embryo indicating the percentage of the volume of the progeny of each of the tiers A, B, C, and D that will become either mesoderm, ectoderm or endoderm (data obtained from Dale and Slack, 1987b). AP, animal pole; VP, vegetal pole. (B,C) RT-PCR analysis of the expression of *Xwnt-8*, *goosecoid* (*Gscd*), *Xbra* or the FGF receptor (FGFR) in cells originating from tier A, from pooled tiers B and C (B+C), or from tier D of a 32-cell embryo. The analysis was performed on cells aged to the equivalent of stage 10.5. W, whole stage 10.5 embryos; WD, dissociated whole embryos aged to the equivalent of stage 10.5.

of this work, the amount of RNA in our samples was too small for an RNase protection assay and we therefore had to rely on the RT-PCR assay (see below).

We first compared the expression of the *Xbra*, an early pan-mesodermal marker (Smith et al., 1991), in normal stage 10.5 embryos and in dispersed cells from embryos dissociated from stage 6 to stage 10.5 (Fig. 1B). *Xbra* expression in dispersed cells was reduced to a very low maternal level. In contrast, the activation of genes such as *EF1*α (Krieg et al., 1989) or *GS17* (Krieg and Melton, 1985), which does not require cell communication, was not affected by cell dissociation (data not shown). This indicates that dissociation prevented the activation of genes normally activated by induction. To exclude the possibility that this effect was due to the death of either responding or emitting cells during the dissociation process, we also analysed the expression of *Xbra* in dissociated embryos that had been reaggregated before the end of the competence for mesoderm induction. As expected, normal levels of *Xbra* mRNA were present in embryos dissociated at stage 6 and reaggregated from stage 9 to stage 10.5 (Fig. 1B). These results were confirmed by the analysis at stage 18 of the expression of the cardiac α-actin gene, a marker for muscle at this stage (Mohun et al., 1984). In embryos dissociated from stages 6 to 10.5 and subsequently reaggregated until stage 18, cardiac actin was not expressed but it was transcribed at a normal level if reaggregation took place at stage 9 (data not shown).

These results show that the dissociation procedure effectively disrupts cell-cell communication, and therefore prevents the formation of mesoderm.

Effect of cell dissociation on the activation of *XWnt-8* and *goosecoid*

To extend the results obtained in the previous section using early regional markers, we compared the level of expression of *Xwnt-8* and *goosecoid* in eggs and in embryos dissociated from stage 6 to stage 10.5. Surprisingly we found that dissociation, which prevented any detectable activation of *Xbra*, did not prevent the activation of these two genes (Fig. 1C; Table 1). *Xwnt-8* is activated in dissociated cells at least 30-fold from stage 1 to stage 10.5, and *goosecoid* is activated more than 5-fold in dissociated cells over the same period. Dissociation leads to a more than 30-fold reduction in the number of *Xbra* transcripts, but causes only an approximately 2-fold reduction in the number of *Xwnt-8* and *goosecoid* transcripts (Table 1 column 5). Conversely, reaggregation of dissociated cells at stage 9, which is sufficient to activate *Xbra* more than 30-fold, does not lead to more than a 2- to 5-fold increase in the number of *Xwnt-8* and *goosecoid* transcripts (Fig. 1B,C). From these results, at least half of the activation of *Xwnt-8* and *goosecoid* appears independent of mesoderm induction.

To rule out the idea that these results were due to an inductive event occurring before the 32-cell stage, we removed the vitelline membrane at the end of the first cell cycle. The blastomeres were immediately separated so that they were only in contact via thin cytoplasmic bridges linking daughter cells. Care was taken to avoid any other form of cell-cell interactions until disappearance of these bridges around the 8- to 16-cell stage. The cells were then kept dispersed as previously. This treatment did not significantly reduce the activation of *Xwnt-8* and *goosecoid* (Table 1, compare columns 5 and 6). We therefore conclude that, in contrast to *Xbra*, *Xwnt-8* and *goosecoid* can be activated in the absence of cell-cell interactions. These data raised the question of whether *goosecoid* and *Xwnt-8* were activated in each dissociated cell or whether their expression was restricted to a subset of these cells. The next two sections deal with this issue.

Dorsoventral expression of *goosecoid* and *Xwnt-8* in dissociated cells

Previous reports have described *goosecoid* expression in the dorsal-most marginal zone of early gastrulae during normal

embryogenesis, whereas *Xwnt-8* is expressed in a complementary pattern (Cho et al., 1991; Christian and Moon, 1993; Christian et al., 1991). We therefore asked if the dissociated cells expressing each of these genes came from the dorsal or ventral side of the embryo. The dorsal- and ventral-most quarters of embryos were labelled with nile blue sulphate and neutral red respectively before the first cleavage (see Fig. 2A and Materials and Methods). Embryos were then transfered to calcium- and magnesium-free medium until the 32-cell stage at which time the vitelline membranes were removed. Blue dorsal cells and red ventral cells were then manually collected, transferred to separate dishes, and cultured in a dispersed state until control embryos reached stage 10.5. The disruption of cell-cell interactions was demonstrated by the absence of detectable *Brachyury* expression in the dissociated samples (data not shown). *goosecoid* mRNA was found predominantly in cells originating from the dorsal quadrant whereas *XWnt-8* was expressed predominantly in cells originating from the ventral-most quadrant (Fig. 2B). Moreover, we found that the ratio of the dorsal and ventral levels of expression of each of these two genes was similar in disaggregated and control embryos (Fig. 2B).

We therefore conclude that, in the absence of mesoderm induction, *Xwnt-8* and *goosecoid* are not activated uniformly in all cells. Their dorsoventral pattern of expression in the dispersed cells reflects the situation found in normal development. Next, we wanted to assess whether, in our assay, *Xwnt-8* and *goosecoid* were activated in presumptive mesoderm cells as they have been described to be in normal development. We have therefore analysed the origin, along the animal-vegetal axis, of the dissociated cells expressing these genes.

goosecoid and *Xwnt-8* expression in dissociated cells in relation to the animal-vegetal axis

In the 32-cell embryo, Dale and Slack (1987a) have determined the contribution of the progeny of the 4 tiers A, B, C and D to the three germ layers. Tiers A and D contribute mainly to ectoderm and endoderm respectively, while the mesoderm originates from the two middle tiers B and C (Fig. 3A). We have therefore studied the expression of *goosecoid* and *Xwnt-8* in dispersed cells descending from tier A, tier D or from a combination of tiers B and C. Fertilised eggs were transferred to calcium- and magnesium-free medium before first cleavage. The vitelline membranes were removed at the 32-cell stage and cells from tier A, tier D or from a combination of tiers B and C of regularly cleaving embryos were gently separated, transferred to different agarose-coated dishes and kept dissociated by frequent agitation as previously until stage 10.5.

Xbra expression was detected in none of the dissociated cell samples under our assay conditions, as is consistent with our previous results demonstrating the effectiveness of dissociation (Fig. 3C). Fig. 3B illustrates that neither *Xwnt-8* nor *goosecoid* were detected in cells derived from the A blastomeres, but both could be detected in cells derived from blastomeres B+C or D. *goosecoid* was activated mainly in cells originating from the B+C tier and to a lesser extent in cells derived from the D tier. This distribution is consistent with an activation of this gene in dispersed cells that would have contributed to the marginal zone in normal development. *Xwnt-8* mRNA was mainly expressed in the dissociated progeny of tier D blastomeres (Fig. 3A and Dale and Slack, 1987a).

From this section, we conclude that activation of *goosecoid* and *Xwnt-8* in dispersed cells is not uniform along the animal-vegetal axis. Consistent with previous studies (Green et al., 1992), neither of these genes is activated in animal cap cells. Moreover, the pattern of expression of *goosecoid* in dispersed cells is consistent with its expression pattern in normal development. However, as *Xwnt-8* has been reported to be expressed mainly in the presumptive mesoderm (Christian and Moon, 1993; Christian et al., 1991; Smith and Harland, 1991), we were surprised to find that, in our assay, it was significantly activated in dispersed future endoderm cells. These results therefore prompted us to reinvestigate the spatial pattern of expression of *Xwnt-8* in normal development.

Comparative study by in situ hybridisation of the expression patterns of *Xbra* and *Xwnt-8* in early gastrulae

Since our results suggested that *Xwnt-8* is mainly expressed in endoderm, we wanted to determine accurately the spatial distribution of the *Xwnt-8* transcripts in normal stage 10.5 embryos. Thus, we compared the expression pattern of *Xwnt-8* and *Xbra* using digoxigenin-labelled probes on adjacent sections of early *Xenopus* gastrulae. We chose to use non-radioactive probes on sectioned *Xenopus* embryos because this method of analysis, which is described here for the first time, alleviates penetration problems found with the whole-mount procedure. In addition it gives a single cell resolution that cannot be attained with conventional radioactive probes.

The spatial expression patterns of *Xbra* and *Xwnt-8* at stage 10.5 are presented in Fig. 4. As noted previously by Harland and coworkers (Smith and Harland, 1991) for other newly activated genes, the hybridization signal detected with digoxigenin probes is mainly found in the nuclei of cells. Superposition of adjacent sections hybridised with *Xbra* or *Xwnt-8* allowed us to position on a single diagram the nuclei positive for each gene on adjacent sections (Fig. 4 panels C,F,I). Since the thickness of the sections used here (20 μm) was greater than the diameter of a single cell, adjacent sections generally contain nuclei from different cells. Still, the diagrammatic representation allows us to compare precisely the regions containing nuclei positive for one or the other gene. We estimate that approximately 70% of the nuclei positive for *Xwnt-8* (represented in red) are in an area that does not express *Xbra* (positive nuclei represented in blue) and which probably corresponds to endoderm. The remaining 30% of the *Xwnt-8*-positive nuclei are in the same region as the *Xbra* RNA-positive nuclei and are therefore located in the mesoderm. In addition, although many endoderm cells are positive for *Xwnt-8* in the dorsolateral areas of the young gastrula, the areas where both genes are expressed tend to be located in the ventral quadrant of the embryo (see Fig. 4C for example).

From this section we conclude that, at stage 10.5, *Xwnt-8* is expressed predominantly in the non-dorsal endoderm underlying the mesoderm with some expression in the ventral mesoderm, a distribution similar to that found in dissociated cells. This distribution is wider than was reported by Smith and Harland (1991) and Christian and Moon (1993) using a whole-mount in situ hybridisation technique. Frank and Harland (1992) indeed suggested that this latter technique may not detect transcripts in the vegetal cells, presumably because of poor penetration of the probe in the yolky endoderm. The

method that we have used here seems to alleviate this problem and has allowed us to detect the transcripts present in the endoderm. Finally, it is important to realise that, since the *Xwnt-8* gene encodes a secreted protein, its pattern of expression does not preclude a role for its product in the patterning of mesoderm.

DISCUSSION

In this article, we have shown that, in dissociated cells, *goosecoid* and *Xwnt-8* can be activated to a nearly normal extent and in a spatial pattern close to what is observed in normal development. Two conclusions can be derived from these results. The first one is that the cell-autonomous activation of *Xwnt-8* and *goosecoid* reveals the presence of cytoplasmic determinants in *Xenopus* eggs. Second, our results, combined with the observation of others that *Xwnt-8* and *goosecoid* act as modifiers of the response to mesoderm induction, suggest a model for the generation of the different mesodermal cell types that significantly differs from the three-signal model proposed by Slack and coworkers (Dale and Slack, 1987b).

Involvement of cytoplasmic determinants in the definition of the dorsoventral axis

The activation of *Xwnt-8* and *goosecoid* in dispersed cells could have several explanations. One possibility is that a mesoderm-inducing factor (MIF) might be released by the dispersed cells in the dissociation medium and this might activate *Xwnt-8* and *goosecoid* but not *Xbra*. We think this is unlikely for two reasons. First, we usually dissociated an embryo in 5-10 cm^3 of calcium-free medium. Since the volume of an embryo is approximately 10^{-3} cm^3, the concentration of the mesoderm-inducing factors released by the cells in the conditioned medium should therefore be at least 5000-fold lower than during normal embryogenesis. We think it unlikely that such a low concentration would be sufficient to account for the activation of *Xwnt-8* and *goosecoid*. Second, even if this very low concentration was sufficient, our results would imply that the activation of *Xwnt-8* and *goosecoid* are more sensitive assays for the presence of MIFs than is the activation of *Xbra*. In fact the contrary seems to hold true. Green and colleagues (1992) have shown that, in animal cap cells, *Xbra* can be activated by a much lower concentration of activin than *goosecoid*. We therefore conclude that the activation of *Xwnt-8* and *goosecoid* in dispersed cells is not due to the presence of MIFs in the dissociation medium.

More generally, since we can dissociate embryos from the 2-cell stage onwards without marked effect on the expression of *goosecoid* and *Xwnt-8*, it appears that these genes can be activated in the absence of cell-cell communication. The dissociation assay therefore provides the first evidence that inheritance of cytoplasmic determinants can lead to the accurate dorsoventral expression of early regional markers. In addition, *Xwnt-8* and *goosecoid* are not only useful markers for ventrolateral and dorsal embryonic cells respectively, but also have important effects on mesoderm differentiation. Dorsal overexpression of *Xwnt-8* from the MBT onwards prevents formation of dorsal-most tissues (Christian and Moon, 1993), while ventral overexpression of *goosecoid* leads to the transformation of ventral mesoderm into more dorsal cell types (Cho et al, 1991; Steinbeisser et al., 1993). Thus, the cell-autonomous activation of these genes probably represents an important step in the formation of a complete dorsoventral axis.

We therefore propose that accurate formation and patterning of mesoderm requires the simultaneous activation of two different pathways (Fig. 5). At the midblastula transition, genes such as *Xbra* are activated throughout the future mesoderm by an inductive pathway. At the same time, genes such as *Xwnt-8* and *goosecoid* are locally activated along the dorsoventral axis by a cell-autonomous pathway. As exemplified by the pattern of expression of *Xwnt-8,* expression of this latter class of genes is not necessarily restricted to the mesoderm. Cooperation between cell-autonomously activated and induction-dependent gene products then initiates a gene regulatory cascade that will give rise to the full spectrum of mesodermal tissues.

Comparison with other works

The importance of cytoplasmic determinants in mesoderm formation has been previously demonstrated by egg ligation experiments (Gurdon et al., 1985b) in which it was shown that subequatorial material isolated at the 1-cell stage could differentiate muscle. This first indication that cytoplasmic determinants positioned along the animal-vegetal axis could play a role in mesoderm differentiation is now confirmed by our finding that the animal-vegetal pattern of expression of *Xwnt-8* and *goosecoid* does not depend on cell interactions. In addition, our results imply that cell-autonomous events also play a role in the definition of the amphibian dorsoventral axis, a situation similar to what is observed in ascidians (reviewed in Davidson, 1990). However, a number of studies suggest that the definition of the frog dorsoventral axis can also be obtained through cell-cell interactions suggesting a possible redundancy of mechanisms.

First, transplantation of a dorsal-vegetal blastomere of a 32-cell embryo into a ventral-vegetal position of a similarly aged host embryo induces the creation of a secondary axis composed of host tissue (Gimlich, 1985; Kageura, 1990). This indicates that at least one dorsal determinant is a signalling molecule, perhaps Vg1 (Thomsen et al., 1993) or a member of the Wnt-family of secreted polypeptides (McMahon and Moon, 1989; Smith and Harland, 1991; Sokol et al., 1991). However, the relevance of this signal for normal development is not known since, by the 32-cell stage, dorsal-equatorial blastomeres, which should be the target of this signal, are already committed to becoming dorsal mesoderm. Second, *Xwnt-8* and *goosecoid* can both be activated in animal cap cells in response to activin (Christian and Moon, 1993; Cho et al., 1991). Since these two genes are not normally expressed in animal cap cells, their activin-dependent activation in these cells may not be relevant for normal development. However, a more attractive possibility is that in vivo both cell-autonomous and induction-dependent mechanisms are required to make sure that the amount of *Xwnt-8* and *goosecoid* proteins are tightly regulated, and to control their precise spatial expression. Finally, Sokol and Melton (1991) have shown that animal caps have a dorsoventral prepattern as early as stage 7.5. It is not known if this prepattern is the result of a cell-autonomous or an inductive event but as neither *Xwnt-8* nor *goosecoid* are expressed at any time in animal caps dissected at this stage,

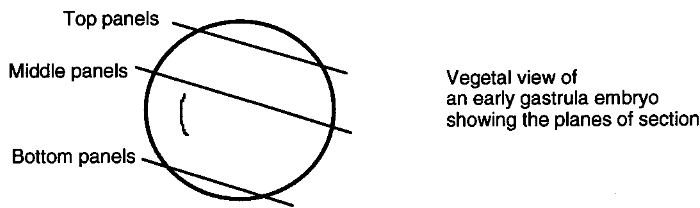

Fig. 4. Comparison of the expression patterns of *XWnt-8* and *Xbra* in normal early gastrulae. Non-radioactive probes for the genes *Xbra* (A,D,G) or *Xwnt-8* (B,E,H) were hybridised to alternate sections of early *Xenopus* gastrulae. (C,F,I) Computer-generated reconstructions of the nuclei staining for either *Xbra* (blue dots) or *Xwnt-8* (red dots) on the neighboring adjacent slides. In all panels, the dorsal side is to the left and the ventral side to the right. Scale bar, 200 μm.

they cannot be the mediators of this prepattern and the dorsal determinants must have additional target genes.

None of these experiments argues against the existence of cytoplasmic determinants. Rather, these data combined with ours suggest that the establishment of the dorsoventral axis is much more complex than previously anticipated, and may involve different, partially redundant, mechanisms. The model presented in Fig. 5 cannot account for all the available data but its value is that its generality can be tested. As more and more immediate early genes are isolated, it will be of great interest to classify them as induction-dependent or -independent genes. Also, the characterisation of the regulatory elements of the *Xwnt-8* and *goosecoid* genes responsible for the differential dorsoventral expression of these two genes should help to determine the relative contribution of cell-autonomous and inductive events in the regulation of these two genes during normal embryogenesis and may lead to the identification of the determinants involved.

We are grateful to J. C. Smith, and E. De Robertis for the gift of plasmids used in the RNase protection assays. We thank Emma Tiller for the gift of the protocol for performing non-radioactive in situ hybridisations on sectioned embryos, Patty Harger for showing how to label the dorsal or ventral sides of embryos and Robert Goldstein for help during the early stages of this work. During the course of this work, we greatly benefited from many enlightening discussions with Kazuto Kato and other members of our laboratory. Finally we want

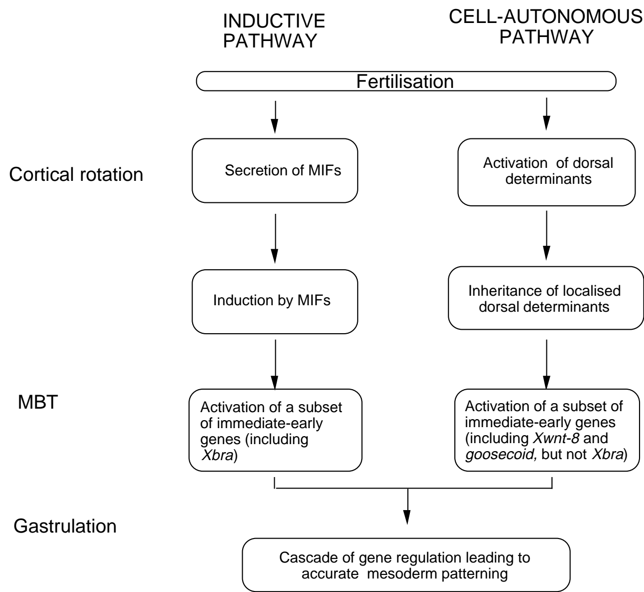

Fig. 5. Model of the early formation and patterning of *Xenopus* mesoderm. See text for details. MIFs, mesoderm inducting factors.

to thank, D. Tannahill, K. Kato and P. Harger for critically reading this manuscript. This work was supported by a CRC grant P2184/0101 to J. B. G. and by an ICI France/Churchill College junior research Fellowship to P. L. who is on leave of absence from the INSERM unit 368, Ecole Normale Supérieure, 46 rue d'Ulm, F-75005 Paris, France and acknowledges the generous support of the French Centre National de la Recherche Scientifique.

REFERENCES

Amaya, E., Musci, T. J. and Kirschner, M. W. (1991). Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in *Xenopus* embryos. *Cell* **66**, 257-269.

Amaya, E., Stein, P. A., Musci, T. J. and Kirschner, M. W. (1993). FGF signalling in the early specification of mesoderm in *Xenopus*. *Development* **118**, 477-487

Cho, K. W. Y., Blumberg, B., Steinbeisser, H. and De Robertis, E. M. (1991). Molecular nature of Spemann's Organizer: The role of the *Xenopus* homeobox gene *goosecoid*. *Cell* **67**, 1111-1120.

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

Christian, J. L. and Moon, R. T. (1993). Interactions between *Xwnt-8* and Spemann organizer signaling pathways generate dorsoventral pattern in the embryonic mesoderm of *Xenopus*. *Genes Dev.* **7**, 13-28

Dale, L. and Slack, J. M. W. (1987a) Fate map of 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.

Davidson, E. H. (1990). How embryos work: a comparative view of diverse modes of cell fate specification. *Development* **108**, 365-389

Frank, D. and Harland, R. M. (1992). Localised expression of a Xenopus POU gene depends on cell-autonomous transcriptional activation and induction-dependent inactivation. *Development* **115**, 439-448.

Gimlich, R. L. (1985) Cytoplasmic localization and chordamesoderm induction in the frog embryo. *J. Embryol. Exp. Morph.* **89**, (suppl) 89-111

Green, J. B. A., New, H. V. and Smith, J. C. (1992). Responses of embryonic *Xenopus* cells to activin and FGF are separated by multiple dose thresholds and correspond to distinct axes of the mesoderm. *Cell* **71**, 731-739.

Gurdon, J. B., Brennan, S., Fairman, S. and Mohun, T. J. (1984). Transcription of muscle-specific actin genes in early *Xenopus* development: nuclear transplantation and cell dissociation. *Cell* **38**, 691-700.

Gurdon, J. B., Fairman, S., Mohun, T. J. and Brennan, S. (1985a). The activation of muscle specific actin genes in *Xenopus* development by an induction between animal and vegetal cells of a blastula. *Cell* **41**, 913-922.

Gurdon, J. B., Mohun, T. J., Fairman, S. and Brennan, S. (1985b) All components required for the eventual activation of muscle specific genes are localized in the subequatorial region of an uncleaved egg. *Proc. Natl. Acad. Sci. USA* **82**, 139-143

Harland, R. M. (1991). In situ hybridisation: an improved whole-mount method for *Xenopus* embryos. *Meth. Cell. Biol.* **36**, 685-695.

Hemmati-Brivanlou, A., Frank, D., Bolce, M. E., Brown, R. D., Sive, H. L. and Harland, R. M. (1990). Localization of specific mRNAs in *Xenopus* embryos by whole-mount *in situ* hybridization. *Development* **110**, 325-330

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

Hopwood, N. D., Pluck, A. and Gurdon, J. B. (1989). *MyoD* expression in the forming somites is an early response to mesoderm induction in *Xenopus* embryos. *EMBO J.* **8**, 3409-3417.

Jones, E. A and Woodland, H. R. (1987). The development of animal caps in *Xenopus*; a measurement of the start of animal competence to form mesoderm. *Development* **101**, 557-563

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

Krieg, P. A. and Melton, D. A. (1985). Developmental regulation of a gastrula-specific gene injected into fertilised Xenopus eggs. *EMBO J.* **4**, 3463-3471.

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

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

Mohun, T. J., Brennan, S., Dathan, N., Fairman, S. and Gurdon, J. B. (1984). Cell type specific activation of actin genes in the early amphibian embryo. *Nature* **311**, 716-721.

Musci, T. J., Amaya, E. and Kirschner, M. W. (1990). Regulation of the fibroblastgrowth factor receptor in early *Xenopus* embryos. *Proc. Natl. Acad. Sci. USA* **87**, 8365-8369.

New, H. V., Howes, G. and Smith, J. C. (1991). Inductive interactions in early embryonic development. *Curr. Opin. Genet. Develop.* **1**, 196-203.

Nieuwkoop, P. D. (1969). The formation of the mesoderm in urodelean amphibians. I. The induction by the endoderm. *Wilhelm Roux Arch. Dev. Biol.* **162**, 341-73.

Nieuwkoop, P. D. and Faber, J. (1967). *Normal Table of Xenopus laevis* (*Daudin*), 2nd ed. Amsterdam: North-Holland.

Rupp, R. A. W. and Weintraub, H. (1991). Ubiquitous *MyoD* transcription at the midblastula transition precedes induction dependent *MyoD* expression in presumptive mesoderm of *X. laevis*. *Cell* **65**, 927-937.

Sargent, T. D., Jamrich, M. and Dawid, I. B. (1986). Cell interactions and the control of gene activity during early development of *Xenopus laevis*. *Dev. Biol.* **114**, 238-246.

Smith, J. C., Price, B. M. J., Green, J. B. A., Weigel, D. and Herrmann, B. G. (1991). Expression of the *Xenopus* homolog of *Brachyury* (T) is an immediate-early response to mesoderm induction. *Cell* **67**, 79-87.

Smith, W. C. and Harland, R. M. (1991). Injected *XWnt-8* RNA acts early in *Xenopus* embryos to promote formation of a vegetal dorsalizing center. *Cell* **67**, 753-765.

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.

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

Steinbeisser, H., De Robertis, E. M., Ku, M., Kessler, D. S. and Melton, D. A. (1993). *Xenopus* axis formation: induction of *goosecoid* by injected *Xwnt-8* and activin mRNAs. *Development* **118**, 499-507.

Symes, K., Yaqoob, M. and Smith, J. C. (1988). Mesoderm induction in *Xenopus laevis*: responding cells must be in contact for mesoderm formation but suppression of epidermal differentiation can occur in single cells. *Development* **104**, 609-618.

Thomsen, G. and Melton, D. A. (1993). Processed Vg1 protein is an axial mesoderm inducer in *Xenopus*. *Cell* **74**, 433-442.

(Accepted 21 January 1994)