The organization of mesodermal pattern in *Xenopus laevis*: experiments using a *Xenopus* mesoderm-inducing factor

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Summary

In this paper, we study the mechanism by which a *Xenopus* cell line-derived mesoderm-inducing factor (MIF) might establish the spatial pattern of cellular differentiation in the mesoderm. The effects of the factor on competent animal pole tissue are consistent with it being identical to the natural mesoderm-inducing factor. The signal can only act on those membrane domains of the animal pole that face the blastocoel, but it can be stably recorded there, such that axial mesoderm is formed, after 15 min exposure or less. This exposure can end some hours, or several cell cycles, before the onset of RNA synthesis yet nevertheless be fully effective, although competence to respond also extends well after the onset of transcription.

Exposure of the entire blastocoel lining of intact embryos to MIF causes a synchronous and sudden transformation of the behaviour and adhesive properties of all inner animal cap cells. This transformation mimics and is contemporaneous with the involution behaviour of normal mesoderm in the early gastrula marginal zone. Although high concentrations of MIF totally disorganize gastrulation, lower concentrations permit gastrulation to proceed. However, the pattern of mesoderm in these embryos is disrupted and ectopic mesoderm is formed around the blastocoel remnant.

When MIF is injected directly into blastomeres, rather than into the blastocoel, it has no effect. This suggests that the molecule is secreted from source cells and affects target cells through an extracellular receptor.

Finally, we show that small pieces of animal pole tissue recently exposed to MIF go on to produce morphogenetic signals perhaps distinct from MIF. We discuss the role of these signals in establishing and modifying the spatial pattern of cellular differentiation in the mesoderm of *Xenopus*.

Key words: *Xenopus*, amphibia, mesoderm induction, gastrulation, mesoderm inducing factor, pattern formation, intracellular injection, homeogenetic induction.

Introduction

Gurdon (1987), reviewing the role of inductive interactions in early development, distinguishes between heterogenous (foreign) and homogenous sources of inducing factors. A factor that induces mesoderm from competent amphibian ectoderm has recently been described, which, while not strictly homogenous in coming from the natural anatomical locality and time in the life cycle for this signal, is at least a gene product of the organism in which it is being studied (Smith, 1987a). The hope that this factor is identical, or is closely related, to a natural agent of mesoderm induction is based on two alternative ideas. First, culture-adapted cells derived from late stages of development might express embryo-specific proteins as accidental 'breakthrough' products. Alternatively, they may synthesize substances having normal roles in the adult body that were also used at earlier stages of development as intercellular signals (see Slack, Darlington, Heath & Godsave, 1987).

The present mesoderm-inducing factor (MIF) is secreted by the *Xenopus* XTC cell line (Pudney, Varma & Leake, 1973). It leads to differentiation of mesodermal structures characterizing 'dorsal' or axial parts of the normal body (Smith, 1987a). It does this
via a pathway that leads responding tissue to participate in just those sequences of mechanical and adhesive activities that distinguish the precursors of these mesodermal regions in vivo (Symes & Smith, 1987). There is currently no reason, however, to think of the factor as a direct stimulus to the determination of any one mesodermal cell type. There is indeed no reason to think of the natural process that specifies tissue as mesodermal (Nieuwkoop, 1969) as simultaneously causing responding cells to differentiate as any of the cell types ultimately produced. Elaboration of pattern within the responding tissue probably requires further sequences of signalling interactions coming under the general concept of homeogenetic induction. If, as suggested for some cases studied in amphibia (Kaneda, 1981; Kurihara & Sasaki, 1981; Kaneda & Suzuki, 1983), the propagation of an induced state is accompanied by alteration in the cell types specified, such a process could be part of the mechanism for establishing positional information (Wolpert, 1969). The most elegant and incisive recent experiments on the conditions and time course required for primary mesodermal induction have, nevertheless, utilized the onset of muscle-specific transcription as a molecular marker for successful interaction (Gurdon, Fairman, Mohun & Brennan, 1985; Gurdon & Fairman, 1986; Warner & Gurdon, 1987).

Here, we report the results of several different kinds of experiment, on whole embryos and on explants, using partially purified preparations of MIF, and also poly (A)* RNA from MIF-producing cells (see also Woodland & Jones, 1987). Together with the studies of cell movement and cell cycle kinetics in explanted responding tissue (Symes & Smith, 1987), our results make it seem increasingly likely that the XTC cell MIF is, or is closely related to, a natural initiator of mesoderm formation. They are also helping us to begin the difficult task of understanding the process whereby the natural mesoderm becomes organized or patterned, at or soon after its inception.

Materials and methods

Embryos
Embryos of Xenopus laevis were obtained by artificial fertilization. They were chemically dejellied, washed and transferred to 10% normal amphibian medium (NAM: Slack, 1984) until required. They were staged according to Nieuwkoop & Faber (1967).

Partially purified mesoderm-inducing factor
Serum-free conditioned medium from the XTC cell line was obtained as described by Smith (1987a), except that the cells were grown on glass roller bottles. The medium was heated to 100°C (Smith, 1987a), concentrated tenfold by ultrafiltration and exchanged into 20 mM-Tris pH 8.0. Mesoderm-inducing activity was partially purified by ion exchange chromatography on DEAE-Sepharose, where activity eluted at approximately 0.5 M-NaCl, followed by hydrophobic interaction chromatography on phenyl-Sepharose, where activity eluted with approximately 30-40% ethylene glycol (Smith & Yaqoob, unpublished data). This material was active at concentrations of 0.1-0.2% in an assay in which internal cells of the blastocoel roof are exposed to mesoderm-inducing activity at stage 8 and subsequently tested for mesodermal differentiation (Smith, 1987a). We define one unit of mesoderm-inducing activity as the minimum quantity which must be present in 1 ml medium for induction to occur. Our partially purified preparation of MIF therefore contained 500-1000 units ml⁻¹, representing about 7000 units mg⁻¹ protein.

0.5 ml of this partially purified preparation was dialysed for 3 h against 67% NAM adjusted to pH 8.0, after addition of bovine serum albumin (BSA) to 0.5 mg ml⁻¹ to reduce nonspecific loss of protein. The final dialysate was active at 0.3-0.5% (250 units ml⁻¹) in the above assay. Control dialysates were made of the dialysis medium and of 30 times concentrated XL-conditioned medium, previously adjusted to 30% ethylene glycol and 0.5 mg ml⁻¹ BSA. XL cells (Anizet, Hawe, Pays & Picard, 1981) produce little mesoderm-inducing activity (Smith, 1987a). Dialysates were microfuged and stored at −70°C as 50 μl aliquots. Each experiment was carried out with dialysate that had been kept for not more than 72 h at 4°C after thawing. Intracellular and blastocoelic injections were performed with micropipettes of appropriate tip size, using pulses of air at 250 g cm⁻² or by means of a completely oil-filled micrometer-syringe apparatus. Injection was performed under 10% NAM containing 5% Ficoll (Sigma) to clamp the vitelline membrane to the cell surface, and injected embryos were kept in Ficoll for 30 min before being transferred to 10% NAM.

RNA isolation
RNA was extracted from XTC or XL cells as described by Krumlauf, Holland, McVey & Hogan (1987). Poly (A)* RNA was isolated by oligo(dT)-cellulose chromatography as described by Maniatis, Fritsch & Sambrook (1982) and quantified according to its A260. Proteins of up to 100 000 molecular mass were seen after in vitro translation of our RNA preparations (data not shown).

For microinjection, poly (A)* RNA was dissolved in sterile distilled water at 1 mg ml⁻¹ and micropipettes were heated overnight at 180°C to destroy RNase activity.

Blastula animal cap explants and combinations
Animal cap regions were excised in 67% NAM containing 5% Ficoll (Sigma) to clamp the vitelline membrane to the cell surface, and injected embryos were kept in Ficoll for 30 min before being transferred to 10% NAM.

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Blastula animal cap explants and combinations
Animal cap regions were excised in 67% NAM, pH 7.3. According to the experiment, they were then cultured in the same medium, or in heated XTC-conditioned medium diluted with 67% NAM to concentrations known to produce strong 'dorsal' mesodermal induction in stage-8 animal caps (see Smith, 1987a). In different experiments, animal caps were excised between the 128 to 256-cell stage and late stage 8. It was found that a larger cell sheet than represented by the '60° solid angle around the animal pole'
of Dale, Smith & Slack (1985) can be cultured without spontaneous production of mesoderm, if care is taken to exclude the thickened zone of white opalescent material appearing at the margin from stage 8. In some experiments, freshly excised animal caps were fused with others, or with matched pieces of stage-11 gastrula ectoderm, in ‘sandwich’ combinations that were sealed by the weight of one donor embryo in a shallow agar well for 10 min. These then rounded up to become a sealed, hamburger-shaped wall of tissue. In most culture experiments the ionic strength was lowered to 20% NAM about 1 h after excision, as this prevented excessive contraction of unsealed explants with consequent extrusion of inner tissue, perhaps the equivalent of exogastrulation in whole embryos.

**Lineage labelling**

Fertilized eggs were injected with 20–30 nl of 100 mg ml⁻¹ rhodamine-lysine-dextran (RLDx: see Gimlich & Cooke, 1983; Gimlich & Braun, 1985). The resulting embryos were uniformly labelled with the lineage marker (see Smith & Slack, 1983; Dale et al. 1985).

**Indirect immunofluorescence for Xenopus muscle**

Muscle differentiation was detected on PEDS wax-embedded sections by indirect immunofluorescence with the monoclonal antibody 12/101 (Kintner & Brockes, 1984). The technique was identical to that of Dale et al. (1985) and Smith (1987a) except that material was fixed in 4% fresh formaldehyde in PBS to preserve RLDx lineage labelling.

**Preparation of stained sectioned material**

Gastrulae were fixed overnight in 2% paraformaldehyde, 2% glutaraldehyde in 0.08 M cacodylate buffer, pH 7.3, leaving the vitelline membrane intact. They were then rinsed in buffer alone for 2 h, and slowly dehydrated to 95% ethanol over 3 or 4 h after puncture of a small hole with a tungsten needle to minimize distortions during dehydration. The embryos were embedded in Historesin (LKB) via a preinfiltration in resin:alcohol (50:50). 2 μm sagittal sections were dried onto clean glass slides, stained with 0.1% toluidine blue in 1% borax at 70°C for about 2 min, rinsed and dried for mounting.

Embryos and explants at axial larval stages were fixed and embedded in 58°C m.p. wax for Feulgen staining with light green/orange G counterstain (Cooke, 1979, 1981).

**Examination of stage-9 to -12 embryos by fixation-dissection**

Embryos to be examined by dissection were demembranated and a small hole was punctured in the blastocoel roof. They were then placed in a fixative of 1% K₂Cr₂O₇, 2% glacial acetic acid in water and examined against black wax in oblique lighting under a Wild dissecting stereomicroscope. After 5–10 min, the embryos could be cracked in desired planes by sharpened tungsten needles, and the presence of tissue layers and cell social behaviour could be examined on a gross scale. Ectoderm, postinvoluted mesoderm with the inner ‘blastoporal lip’ (Keller, 1975, 1976) and endoderm could easily be identified under these conditions.

**Results**

The effect of MIF is stably recorded within minutes of exposure at the ‘inner’ surface of blastulae cells

Freshly excised blastula animal caps were exposed to diluted XTC-conditioned medium followed after various times by thorough washing and culture in 67% NAM. In most experiments, the Ca²⁺ and Mg²⁺ concentrations in the initial inducing medium were one third the normal, to delay contraction and adhesion of the explant cells so that washing would be effective. Explants exposed to MIF for more than 10 min showed by their convergent extension movements and subsequent differentiation that mesoderm had developed, as described by Smith (1987a) and Symes & Smith (1987). In three such experiments, the test animal caps were excised at stage 7– (128-cell stage). Both faces of their single layer of cells were fully exposed to the wash that followed 20 min of culture in MIF. 17 out of 21 cases showed a strongly induced appearance, and histology confirmed that notochord and massive, organized somite tissue had resulted (Fig. 1A,B). This suggests that MIF is stably bound at the cell surface, or causes the activation of a stable second messenger, within minutes. This initial phase of recording the signal is unlikely to depend upon RNA synthesis since transcription is barely detectable before the mid-blastula transition (Newport & Kirschner, 1982), which occurs about 2 h after this short exposure to MIF.

The single layer of cells in the stage 7– animal cap is polarized, with the outer surface being derived from the egg membrane and the lateral and basal membranes being newly assembled (see Gerhart, 1980). We investigated which surface(s) could respond to MIF by fusing two animal pole regions such that after 30 min healing they formed a sphere, of which the external surface was composed entirely of the outer domain. On electron microscopical and electrophysical grounds (Kalt, 1971; Slack & Warner, 1973; Regen & Steinhardt, 1986) the inner cell membrane domains should be inaccessible to macromolecules introduced into the culture medium.

Ten double explants of this type were transferred to XTC-conditioned medium when healing was complete. None formed mesoderm, all differentiating as ‘atypical epidermis’. As a control, at the same time the fused explants were transferred to MIF, nine additional double explants were constructed from sibling embryos in the presence of MIF. All formed mesoderm. We conclude that only the inner membrane domain is capable of mediating the response to MIF.

This observation makes it possible to expose different proportions of cells in an explant to MIF. When animal pole regions are dissected from embryos, over
Fig. 1. Differentiation in explanted animal caps after various conditions of early exposure to MIF. (A,B) Sections transverse and longitudinal to axial structure in explants exposed to MIF for 20 min immediately after excision from the 128-cell-stage embryo. All explants were extensively washed after exposure and cultured in 67% NAM until controls reached stage 35. (C) Differentiation indistinguishable from that of controls (67% NAM only) and (D) differentiation corresponding to the 'weakest' grade of mesoderm induction, as observed after culture in limiting dilutions of MIF. These appearances follow continuous culture in the same MIF preparation that gives rise to A and B but beginning 30 min after dissection from the donor, when little or no original 'inner' blastomere membrane was exposed to the medium. nc, notochord; s, somite; ab, abnormal, wrinkled epidermis; ep, properly stretched, bilayered epidermis over a fluid-inflated cavity, not seen in complete absence of induction. Scale bar, 300 μm.

the next hour or so they tend to curl into a solid ball, in which the original outer surface encloses those parts of the cells which would have faced the blastocoel (see Symes & Smith, 1987). In three experiments, therefore, stage 7+ explants were prepared in 67% NAM and allowed to heal progressively into spheres. At intervals, samples of five to seven of these were transferred to highly inducing concentrations of XTC-conditioned medium and allowed to develop to control stage 40. In all 38 cases, explants first experiencing MIF when considerable 'inner' cell surface was still visible went on to show convergent extension movements and the appearance of induction. However, as healing time increased, the intensity of response decreased. When only a small focus of inner cell membrane was apparent, at 30–40 min after excision, only 3 out of 18 explants showed any kind of induction (Fig. 1C,D). This was a 'weak' or 'ventral-type' induction, resembling those obtained from newly dissected animal pole explants in response to fibroblast growth factor (Slack et al. 1987) or low concentrations of XTC-conditioned medium (Symes & Smith, unpublished observations). In each experiment, a control group of stage-9 explants, made from synchronous embryos of the same egg batch, was exposed to MIF at the same time as the final healed explants. All 15 were strongly induced, confirming that the cells were still competent to respond to MIF.

Injection of MIF into the Xenopus blastocoel disorganizes gastrulation and alters the subsequent body pattern

In the remaining experiments (other than those involving poly(A)+ RNA), partially purified MIF has been microinjected into the blastocoels of embryos, either to study its effect on pattern formation in the mesoderm or to provide a source of animal pole tissue which, unable to heal into a sphere, has received homogenous exposure to MIF. Control dialysates consisting of saline containing only 0·5 mg ml⁻¹ BSA,
or of concentrated XL-conditioned medium, are without effect when used at a volume of 200 nl that will effectively irrigate the cavity and assume a uniform concentration to the lining cells. The effective concentration of MIF has thus been varied by delivering this constant volume diluted as necessary with XL-derived dialysate, to give doses of 0.003 to 0.1 units per embryo (see Materials and methods for details and origin of units).

Irrespective of dose or stage of injection (from stage 7 to 9+; 5 h to 30 min before gastrulation) MIF has the same striking effect on cell behaviour. The primary response occurs at stage 10 and is complete within 10 min; this is after the onset of normal mesoderm involution but several hours before its completion. The change in cell behaviour is consistent with the entire blastocoel roof being induced to form mesoderm. This results in changes in cell shape, locomotion and adhesion that correspond to those occurring in a spatially and temporally organized fashion around the marginal zone (Keller, 1976, 1986; Keller & Schoenwolf, 1977).

We have studied these early events and their effects on development by fix-dissection and histology of MIF-injected and control embryos from stage 9 onwards. The observations are summarized in Figs 2 and 3. These illustrate how cells of the inner layer of the blastocoel roof initially become less adhesive and more motile. Soon afterwards, this inner layer contracts due to deepening of cell shapes and maximization of cell contact area and a prominent interface separates it from the outer layer of cells. Rather than migrate over this substrate, the newly involuted normal mesoderm fuses with the inner blastocoel wall, while peripheral endodermal cells migrate to make contact with the entire blastocoel lining. The volume of the embryo is preserved by the appearance of a new cavity within the endoderm in the vegetal region.

Although the initial appearance of embryos injected with MIF does not depend upon the amount received, their further development does, for there is a dose-dependent 'recovery' of normal gastrulation. We have no explanation of how this occurs but it is clearly of great interest. Embryos injected with the lowest doses of MIF (0.003 units) recover to form an embryo whose only external abnormality is a reduced head. However, somite morphogenesis is significantly disorganized in such embryos (Fig. 2G) and no blood is formed. As the dose of MIF is increased, the embryos recommence normal gastrulation activity at successively later stages and form only posterior or tailbud structures. Heart and then pronephros development are removed from the body pattern. 47 embryos have been examined in this series of experiments, 35 using conventional histology and 12 by immunofluorescence using an antibody to *Xenopus* globins (Cooke & Smith, 1987).

Embyros are unable to recommence normal gastrulation after being injected with more than 0.02 units MIF. Real embryonic mesodermal pattern is not detectable and the abnormal single-layered epidermal remnant is sloughed off. However, ectopic mesoderm is invariably present at the site of the obliterated blastocoel, as it is in embryos injected with lower doses of MIF which have managed to recover normal gastrulation movements (Fig. 2H).

At the highest doses of MIF this ectopic mesoderm includes muscle, but never notochord. Interestingly, when isolated animal pole regions are exposed to MIF there is a dose–response relationship such that concentrations greater than 10–20 units ml⁻¹ frequently result in notochord formation (Symes & Smith, unpublished observations; see Smith, 1987a; Symes & Smith, 1987). The MIF concentration range in the blastocoel in these experiments is about 15–500 units ml⁻¹ and thus the required dose range in intact embryos is elevated with respect to that in isolated animal pole tissue. This suggests that the normal mechanisms of mesoderm formation and patterning include a widely disseminated inhibitor, which down-regulates the threshold of response in embryos, but which is absent in animal pole explants (see also Smith, 1987a). We shall present further evidence for this elsewhere.

**MIF behaves like a secreted protein that acts via cell surface receptors and a second messenger system**

Equal volumes of MIF or of control dialysates were injected into five separate batches of sibling embryos, either intracellularly, into the animal hemisphere at the 1–4-cell stage, or into the blastocoel at the 128-cell stage. More than 150 embryos were involved, with five groups of 15–25 receiving intracellular injection but smaller groups receiving intrablastocoelic injection to confirm the familiar positive response (see above). Ectopic mesoderm is formed after intrablastocoelic injection of approximately 0.003 units MIF. However, this dose can be injected into the animal hemisphere of the precleavage zygote or the 2- to 4-cell-stage embryo without visible effect on development. Even doses of 0.005 unit into the single cell are without effect, though the control (XL-derived) dialysate is slightly toxic, producing some misalignment and pregastrula arrest at the required injection volume.

It is possible that proteins with special function early in development are particularly rapidly degraded, so that intracellularly injected MIF would be destroyed before the onset of mesodermal competence at the 32- to 64-cell stage (Jones & Woodland, personal communication). Accordingly, in
three of the experiments, three or four animal blastomeres at the 16- to 32-cell stage were given an aggregate dose of 0.003 units MIF from a very fine (and thus slowly delivering) micropipette. Embryos showing delayed cleavage after this treatment were discarded. Subsequently MIF was injected into the blastocoel of stage 7- embryos from the same egg batch under identical conditions but with only 75% of the total aggregate delivery time per embryo. A striking MIF effect (see Figs 2, 3) was observed in all ten of the latter embryos, but 21 out of 25 intracellularly injected embryos were normal. The four abnormal embryos each had large single patches of cytolysis, possibly resulting from original overfill of one blastomere.

We conclude that microinjected MIF protein in an undamaged cell is ineffective, and is not secreted from the cell to act via extracellular receptors.

In contrast, like Woodland & Jones (1987), we find that intracellular injection of poly(A) + RNA from XTC cells, but not XL cells, is effective. Approximately 10 nl of 1 mg/ml RNA, or water, was injected into the animal hemisphere of embryos at the 2- to 4-cell stage. These embryos were allowed to reach the mid-blastula stage (stage 8), when the animal pole regions were dissected out and allowed to develop until control stage 38 in 67% NAM. Nine explants from embryos injected with water were sectioned and examined by immunofluorescence with an anti-muscle antibody. All formed atypical epidermis. Seven explants were taken from embryos injected with XL poly(A) + RNA, of which six formed atypical epidermis (Fig. 5A,B) and one formed some mesenchyme and mesothelium. Of the 14 explants from embryos injected with XTC poly(A) + RNA, five formed atypical epidermis, and one mesenchyme and mesothelium but eight formed large masses of muscle (Fig. 5C,D). One even formed notochord.

These results suggest that newly synthesized MIF is capable of being secreted from blastomeres and is effective once outside the cell.

**Blastocoel roof tissue exposed to MIF acts as 'organizer' mesoderm when grafted to normal host ventral marginal zones**

Animal pole ectoderm was dissected from stage-9 *Xenopus* embryos that had received, at earlier blastula stages, intrablastocoelic injections of MIF. These pieces of tissue were washed and grafted to the ventral marginal zones of stage-9 embryos and the embryos were allowed to develop. In 65 out of 80 cases, from five egg batches, where donor tissue had received at least 0.025 units of MIF between 30 and 90 min beforehand, the grafted embryos developed secondary axes with spinocaudal to hindbrain levels of neuraxis (Fig. 4), or else complete rostrocaudal pattern. Similar grafts from normal embryos or from embryos injected with control solutions are without effect (see control embryo, Fig. 4A); the grafted tissue integrates into the posterior trunk or tail region, visible as an abnormally pigmented patch of epidermis.

These experiments suggest that MIF-treated animal pole ectoderm acts as 'organizer' tissue, capable both of respecifying ventral mesoderm and of causing neural induction (Spemann & Mangold, 1924; Gimlich & Cooke, 1983; Smith & Slack, 1983; Jacobson, 1984). It is unlikely that the secondary axes are caused by passive release of MIF from the treated explants because in seven cases when MIF was injected into the blastocoels of stage-11 gastrulae and one hour later a larger panel of the thinner stage-11-5 blastocoel roof was used as a graft, no secondary axes were produced. Stage-11 tissue is no longer competent to respond to MIF (Symes & Smith, unpublished observations), and this suggests that induced
stage-9 animal pole tissue causes pattern respecification by producing further inductive signals.

Further evidence that induced animal pole ectoderm acts as organizer tissue comes from its cellular behaviour. At the time of grafting the donor tissue is of quite normal appearance for its tissue of origin (see above). About 1 h after grafting, however, when gastrulation would be commencing in the donor, active surface convergence and invagination begin at the graft site, as occurs with grafted dorsal lip tissue (Cooke, 1972). Furthermore, it is possible to count the number of induced animal pole cells in the graft, and as few as 100 are required for successful double axis formation. This indicates that there is a large host contribution to the secondary axes, and we are currently investigating this by cell lineage labelling, as well as examining the effects of varying graft size and dose of MIF on the patterns produced.

Homeogenetic induction

We have investigated whether animal pole ectoderm exposed to MIF can itself induce mesoderm by combining stage-8 MIF-treated ectoderm with RLDx-labelled uninduced ectoderm (Fig. 6). Twelve combinations were prepared in which one component was derived from an embryo which had received an intrablastocoelic injection of MIF one hour previously, at stage 7. The dissected animal cap region was washed before combining it with an RLDx-labelled uninduced explant at the same stage.

These combinations formed elongated structures surrounded by epidermis derived from both components. A cement gland was frequently present at one end, and this was invariably labelled with RLDx and so derived from the uninduced animal pole ectoderm. In sections of the explants, large masses of muscle were identified by indirect immunofluorescence with a fluorescein-conjugated second antibody (Fig. 6B,C,E,G). The muscle was sometimes accompanied by notochord, and at least 90% of this axial mesoderm was derived from the unlabelled MIF-treated ectoderm. Lineage-labelled tissue, derived from the uninduced ectoderm, usually abutted the muscle cells (Fig. 6B–D). Significant numbers of lineage-labelled cells, both as individuals and as small groups, had contributed to peripheral parts of the muscle mass (Fig. 6E–H), but most of the lineage-labelled cells differentiated as mesenchyme, resembling the tissue seen in ‘weak’ or ‘ventral-type’ inductions (see above). It is probable that this tissue induces the cement gland from overlying ectoderm; in our hands, cement gland never forms spontaneously from uninduced animal pole ectoderm.

The mesenchymal tissue formed by lineage-labelled animal pole ectoderm must have arisen through a form of induction because fused pairs of uninduced animal cap regions never form mesoderm. The inducing signal is unlikely to be MIF diffusing from the induced animal pole tissue because in 12 experiments when RLDx-labelled blastula animal pole ectoderm was combined with ectoderm that had been exposed to MIF at stage 11 (Fig. 6A), the labelled tissue formed atypical epidermis. Stage-11 ectoderm is no longer competent to respond to MIF, and in 11 cases the unlabelled cells themselves formed epidermis and mixed with the labelled cells that had not been exposed to MIF. There was one unusual case where a little muscle was formed from the MIF-treated gastrula ectoderm.

These results suggest that passive transfer of MIF cannot account for the formation of muscle and mesenchyme by the uninduced component of experimental combinations. It is probable that these mesodermal cell types arise through a form of homeogenetic induction, in which newly induced ectoderm itself produces a mesoderm-induction signal. This is discussed below.
Discussion

The discovery of soluble factors capable of inducing isolated animal pole ectoderm to form mesoderm (Smith, 1987a; Slack et al. 1987) has made possible a series of novel and informative experiments on mesodermal induction. In a previous paper, for example, we have shown that an early response to induction is the onset of gastrulation movements (Symes & Smith, 1987). The results described in the present paper emphasize some of the differences between induction and other cell signalling systems (see Gurdon, 1987) but also indicate some similarities. In addition, our data pose interesting questions about the specification of mesoderm and the initiation of pattern formation within it.

Mesoderm induction and other cell signalling systems

If poly(A)^+ RNA from the XTC cell line is microinjected into the animal pole region of early *Xenopus* embryos, the prospective ectoderm of the blastula becomes capable of differentiating as mesoderm (Woodland & Jones, 1987; see our Fig. 5). By contrast, when partially purified MIF is microinjected into the same cells it is without effect, although it will induce mesoderm if applied extracellularly, by injection into the blastocoel. This suggests that the activity of microinjected poly(A)^+ RNA is due to secretion of MIF and that MIF acts via extracellular receptors. In this respect, the mesoderm-induction signal resembles, for example, the polypeptide growth factors like epidermal growth factor (EGF) (Carpenter, 1985) rather than steroid hormones (reviewed by Tata, 1984).

In two respects, however, mesoderm induction clearly differs from growth factor action. The first is in the timing of the response. Gurdon et al. (1985) and Symes & Smith (1987) have previously demonstrated that the time of muscle-specific gene activation and the onset of gastrulation movements do not depend upon the time of mesoderm induction. Here we show that the change in structure of the blastocoel roof after MIF injection begins at the same stage irrespective of the time of injection or the concentration of MIF. This change appears to be the abnormal, deepening of the archenteron, and the advance of the blastopore over the yolk plug, all driven by mesoderm involution (Keller, 1986), are halted. Meanwhile the anterior edge of the endodermal mass alters its normal sharp outline, and its cells rapidly and abnormally achieve contact with the entire lining of the former blastocoel. The volume of the embryo is preserved by the appearance of an abnormal cavity within the endoderm in the vegetal region. After lower MIF doses (about 0.006 units), dorsal lip activity reappears and a pre- and post-involution interface forms by stage 11-12 (arrowheads), whereas with doses above 0.02 units this never occurs, and the ectopic mesoderm may reveal its ‘dorsal’ status by its semiorganized convergent behaviour. (B) Camera lucida drawings of normal and a range of MIF-injected embryos at stage 35. The least abnormal case has all major head parts and all germ layers present, but heart and blood do not form. Increasing doses reduce the anterior axial pattern, the pronephros disappears and somite segmentation is disrupted. A significant accessory trunk-tail formation is often present, centred on the ectopic mesoderm region but running into what remains of the embryo’s ‘own’ axis anteriorly where there is no brain. At intermediate doses where gastrulation has only just recovered, the tailbud may be the only externally recognizable structure, although massive, normally positioned notochord and somite tissue are found internally. The ectoderm and nervous system have few cells and often disintegrate. Above this dose, gastrulation never occurs and a thick pad of disorganized mesoderm lies between the thin shell of abnormal ciliated epidermis (stippled) and the enenclosed endodermal mass. e, eyecup; ev, ear vesicle; g, gills and pharynx; pn, pronephros; s, somite segments; fb, forebrain above cement gland; bp, blastopore. Scale bars, 1 mm.
ectopic counterpart of those changes normally causing involution and then convergent extension of mesoderm. The time of response is therefore set by the age of the responding tissue rather than by the time of induction. This is a phenomenon unique to inductive interactions (Gurdon, 1987).

The effect of MIF can be 'remembered' by responding cells within 10 min. This swift response is not unprecedented. Some growth factors, like EGF, need to be present in the culture medium continuously to exert their effect (Shechter, Hernaez & Cuatracasas, 1978) but others, like platelet-derived growth factor (PDGF), rapidly induce a long-term cellular memory which is retained in the absence of the factor (Pledger, Stiles, Antoniades & Scher, 1977; Singh et al. 1983). Acquisition of this 'memory' requires RNA synthesis (Smith & Stiles, 1981) and may depend upon the transcription of oncogenes such as \( c\text{-}myc \) and \( c\text{-}fos \) as well as other genes (Cochran, Reffel & Stiles, 1983; Armelin et al. 1984; Greenberg & Ziff, 1984). What is unusual about the rapid response to MIF is that it can occur in the virtual absence of transcription; that is, before the mid-blastula transition (Newport & Kirschner, 1982). This may indicate that the signal is remembered in some other way, perhaps by binding of the factor to the cell surface. Alternatively, the extremely low level of transcription that can be detected before the MBT (Kimelman, Kirschner & Scherson, 1987) may be sufficient to record the effect of MIF.
Fig. 5. Injection of poly(A)$^+$ RNA from XTC, but not XL, cells into animal pole blastomeres causes mesoderm induction. Poly(A)$^+$ RNA from XL cells (A, B) or XTC cells (C, D) was injected into the animal hemisphere of Xenopus embryos at the 2- to 4-cell stage. At the mid-blastula stage (stage 8) the animal pole regions of these embryos were dissected out and allowed to develop in 67% NAM. (A) DAPI staining. (B) The same section stained with 12/101; no muscle is formed. (C) DAPI staining. (D) The same section stained with 12/101; large amounts of muscle are formed in response to XTC poly(A)$^+$ RNA. Scale bar in (D) is 200 μm and applies to all frames.

Fig. 4. The effect of blastocoelic MIF injection on morphogenesis of embryos, and on properties of grafted blastocoel roof tissue. (A) A group of one normal and five abnormal siblings at stage 37. The normal individual is second from bottom. The upper group show external appearances after low (top) and moderate (2nd and 3rd) doses of MIF. Even individuals such as that at top have no heart or blood and little pronephric development, while somite disorganization (see Fig. 2G) and reduced epidermal and CNS cell populations are evident. As the syndrome becomes more severe, only posterior axial structure and notochord is recognizable, while unorganized muscle or significant accessory tail and CNS formation may occur anteroventrally. Higher MIF doses abort gastrulation.

Above and below the control embryo are two individuals with highly organized but anteriorly incomplete second axial patterns, following the grafting operation described in B. (B) An operation in which a piece of blastocoel roof tissue from a donor blastula, previously injected with MIF or a control solution, is grafted into the ventral marginal zone of a late blastula host. Grafts (100–300 cells) from control injected donors or from those receiving less than about 0.06 units MIF, integrate into host posteroventral regions with no disturbance to morphogenesis (see lower larval sketch). Grafts treated with higher doses of MIF, even after careful washing before implantation, act like dorsal blastoporal lip tissue and organize the development of second axial patterns (see upper larval sketch and A, C). The host blastula is represented in sagittal section with its own prospective dorsal lip site to left. (C) Anterior tissue structure in horizontal sections from the two secondary axes following grafting shown in (A). The level of anterior structure reached in these depends upon graft size and original donor MIF dosage. Both those shown are incomplete, one reaching pronephric levels but original donor MIF dosage. Both those shown are incomplete, one reaching pronephric levels but showing a small notochord piece in its otherwise fused somite axis, the other reaching ear vesicle and hind brain induction levels, without notochord. Note highly organized somite segmentation, which does not occur anywhere within donor embryos after the doses of MIF used in these experiments (see Fig. 2G). cg, cement gland; nc, notochord tissue; ev, ear vesicle; pn, pronephros; s, somites; tb, tailbud. Scale bar: 1 mm for drawings of B.
Mesodermal specification and pattern formation

The establishment of the spatial pattern of cellular differentiation within the mesoderm of *Xenopus* depends upon signals received before and during gastrulation as well as on the gastrulation movements themselves (Smith, Dale & Slack, 1985; Smith, 1987b; Keller, 1986; Cooke & Smith, 1987). The work described in this paper uses partially purified MIF to investigate these signals. The experiments fall into two classes, those on intact embryos and those on explants.

Injection of MIF into the blastocoel of intact embryos

Microinjection of partially purified MIF into the blastocoel of intact embryos causes all the cells lining the blastocoel to experience a homogenous concentration of inducing factor. As a result the cells undergo changes in cell shape, locomotion and adhesion which mimic those occurring during gastrulation in the marginal zone (see also Symes & Smith, 1987).

These changes in cell behaviour begin shortly after the onset of normal mesoderm involution. However, a large body of evidence (reviewed by Cooke, 1985; see also Cooke, 1987) suggests that the time at which the movements of involution occur in different regions of the normal mesoderm is characteristic of each region, and reflects its preliminary specification for body position and mode of differentiation. On this hypothesis, the time of change of behaviour of ectopic mesoderm might equally reflect its positional character. This time may be estimated very precisely by frequent inspection of synchronous sibling embryos, and it is thus possible to ask whether it depends upon the time of receipt, or concentration, of an inducing signal. Preliminary results, based on several hundred injected embryos, show that the greatest time difference that can be observed is less than 30 min. This is short compared with the hours-long progression of normal mesoderm involution (Keller, 1976; Gerhart & Keller, 1986). Thus differences in timing and intensity of a single endogenous mesoderm-inducing factor may be insufficient to establish the time course of gastrulation and the spatial pattern of cellular differentiation.

One consequence of the change in behaviour of blastocoel roof tissue in response to MIF is the inhibition of gastrulation. However, although the timing of the change and the initial appearance of the cells do not depend upon the dose of MIF, there does occur a dose-dependent recovery of normal gastrulation movements, giving rise to an organized axis and induced nervous system (Cooke & Smith, unpublished data; see Fig. 3).

There are two effects of injected MIF on cell differentiation. Consistent with our observation that higher doses of MIF tend to induce dorsal structures and lower doses ventral (Symes & Smith, unpublished observations; see Grunz, 1983), injection of MIF suppresses blood and pronephros differentiation; it is as if the concentration of MIF within the embryo is nowhere low enough to permit such cell types to form. On the other hand, even when high doses of MIF are injected into the blastocoel, the blastocoel roof cells form muscle but never notochord. At equivalent concentrations of MIF isolated animal pole regions frequently form notochord, and this suggests that the embryo contains an inhibitor of mesoderm formation as well as an activator.

Explant experiments

One conclusion from the experiments in which MIF is injected into whole embryos is that the factor is capable of modifying pattern within the mesoderm but is not a molecule which determines particular pathways of cell differentiation; it is not, for example, the notochord-inducing factor, or the muscle-inducing factor.

Further evidence for this view comes from experiments using animal pole explants. A significant observation is that the proportion of cells in an explant that are exposed to MIF determines its subsequent appearance and cellular constitution.

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*Fig. 6.* Explants combining MIF-treated and lineage-labelled animal cap tissue. (A) Preparation of explant combinations. The left-hand side shows how one donor embryo is lineage labelled with RLDx and allowed to develop to stage 8 (about 7 h). The centre column shows how competent animal cap tissue at stage 7 is exposed to MIF and excited with careful washing after about 1 h at stage 8+. The right-hand side shows how the same tissue, but past its competent period at stage 11+, is similarly exposed to MIF. Experimental combinations are prepared from left and centre components, control combinations from left and right. (B) Upper (muscle immunofluorescence) and lower (lineage label) images from a sectioned combination at control larval stages. The bulk of muscle develops from the directly MIF-exposed component, but morphogenesis frequently results in such muscle being overlain by lineage-labelled epidermis. In C–H, muscle immunofluorescent images from sections are on the left, and their paired lineage label images on the right. (C,D) Even within the mesodermal tissue that fills the explant combinations, nonmuscle regions including lineage-labelled cells frequently abut onto muscle. Freely diffusing transfer of the original MIF does not seem to cause systematic muscle induction adjacent to treated tissue. (E,F and G,H) Two examples of the incorporation of lineage-labelled cells into the periphery of a region of muscle development. Arrowheads indicate cells labelled with RLDx that are also stained with anti-muscle antibody. Scale bar: 1 mm in drawings of A, and 65 μm on photomicrographs B–H.
Further patterning of the mesoderm of *Xenopus* is believed to arise through ‘dorsalization’, an interaction distinct from both mesoderm induction and homeogenetic induction, in which dorsal ‘organizer’ mesoderm induces ‘ventral’ mesoderm to adopt an intermediate level, such as muscle (see Dale & Slack, 1987b). Dorsalization is one of the interactions revealed by the ‘organizer graft’ (Smith & Slack, 1983) and our results (Fig. 4) show that animal pole ectoderm exposed to MIF is also active in this assay. The other interaction demonstrated by the organizer graft, neural induction (Gimlich & Cooke, 1983; Smith & Slack, 1983; Jacobson, 1984), also occurs.

The impression from these results is that pattern formation within the mesoderm of *Xenopus* is not a single-step process. It may be initiated by an inducing factor (for which the present partially purified molecule is a good candidate) and polarity of the pattern may be set by different concentrations, or by the localized origin, of that factor in vivo (e.g. Gimlich & Gerhart, 1984). Further building up and refinement of the pattern could result from subsequent homeogenetic induction and dorsalization, probably involving the serial production of, and response to, additional signalling molecules. These may act to ‘fine-tune’ the early specification, thus ensuring the remarkable constancy of pattern seen in the mesoderm of *Xenopus* (Cooke, 1981; Cooke & Smith, 1987).

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