Mesoderm-inducing factors and Spemann's organiser phenomenon in amphibian development

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Summary

Certain proteins from 'growth factor' families can initiate mesodermal development in animal cap cells of the amphibian blastula. Cells that are in early stages of their response to one such factor, XTC-MIF (Smith et al. 1988), initiate the formation of a new axial body plan when grafted to the ventral marginal zone of a similarly aged host embryo (Cooke et al. 1987). This replicates the natural control of this phase of development by the dorsal blastoporal lip when similarly grafted; the classical 'organiser' phenomenon. I have explored systematically the effect, upon the outcome of this pattern formation using defined inducing factors, of varying graft size, XTC-MIF concentration to which graft cells were exposed, length of exposure before grafting, and host age. The 'mesodermal organiser' status, evoked by the factor, appears to be stable, and the variables most influencing the degree of completeness and orderliness of second patterns are graft size and factor concentration. Inappropriately large grafts are not effective. A Xenopus basic fibroblast growth factor homologue, present in the embryo and known to be a strong inducer but of mesoderm with a different character from that induced by XTC-MIF, produced no episode of pattern formation at all when tested in the procedure described in this paper. Organiser status of grafts that have been exposed to mixtures of the two factors is set entirely by the supplied XTC-MIF concentration. Lineage labelling of these grafts, and of classical dorsal lip grafts, reveals closely similar though not identical patterns of contribution to the new structure within the host. Implications of the results for the normal mechanism of body pattern formation are discussed.

Key words: organiser, body pattern, XTC-MIF, bFGF, dorsal blastoporal lip, lineage labelling, Xenopus.

Introduction

Spemann and Mangold (1924) first described the graft, from the dorsal blastoporal lip of an early newt gastrula into the presumptive ventral region of a similar host, that led to formation of an extra set of axial body parts centred on the graft region. Donor cells contributed in an integrated way to the anterior and dorsal regions of new mesodermal pattern, while the associated nervous system was very largely host-derived. These conclusions have been extended to other amphibian types, notably the frog Xenopus, and it is understood that the twinning is based not on extra growth with local intercalation of new structure, but upon widespread alteration of the map that describes cells' allocation to particular 'fates' within an embryo of normal tissue extent (Cooke, 1972a,b, 1973a, 1979, 1981; Gimlich and Cooke, 1983; Smith and Slack, 1983; Jacobson, 1984). Since the original work, it has usually been proposed that this process has two separate components; the graft first interacts with its surroundings to complete a new map for dorsal axial mesodermal differentiations, and the resulting midline mesoderm, migrating beneath ectoderm via a blastoporal lip of it own, then delivers signals that elicit a coordinated pattern of neural development there (e.g. Nieuwkoop, 1973; Spemann, 1931). But Spemann remained convinced that he was witnessing a more unified episode of interactions (see 1938 for self-review and Hamburger, 1988 for an intellectual biography).

The classical 'organiser' graft is of tissue whose normal fate in the donor would have been to become those same dorsal–anterior mesodermal structures that it now contributes to the new pattern. At least in Xenopus, however, the most profound and symmetrical twinning results instead from surgical or other manipulations at much earlier stages (Gerhart et al. 1981; Gimlich and Gerhart, 1984; Black and Gerhart, 1986). These cause doubling with respect to a normally single regional specialisation, situated within the egg's yolky hemisphere that contributes the endodermal layer to the body. Mesoderm, and the overall pattern developing within it, result from subsequent inductive interaction. Signals originating in the endodermal regions recruit cells from the overlying equatorial region into mesodermal development during roughly the 10^2- to 10^3-cell, or blastula stages (Nieuwkoop, 1969, 1973; Sudarwati and Nieuwkoop, 1971; Gurdon et al. 1985;
230 J. Cooke

Warner and Gurdon, 1987). Although mesoderm is induced all around the meridians of the normal embryo, these signals vary in character such that a relatively restricted sector has the pattern-organising power at early stages, and transfers this to the mesodermal zone above because it induces mesodermal tissue with a special character. Thus twinning occurs whenever organising potential is made to appear at two positions, either by early endodermal grafting (Gimlich and Gerhart, 1984), later mesodermal grafting (e.g. Cooke, 1972b), or other, mechanical or biochemical manipulations (Black and Gerhart, 1986; Cooke, 1987; Kao et al. 1986).

It has been proposed on independent grounds, both from morphogenetic theory (Dale and Slack, 1987) and from close observation of cellular behaviour during normal gastrulation (Keller and Danilchik, 1988), that only two types of mesoderm are induced at the outset (~ three if prechordal head mesoderm is counted as distinct). Mesodermal body patterns would then form in relation to juxtapositions of these initially induced mesodermal territories, on the basis of gradients in one or more further morphogen signals that could be set up and maintained if the 'dorsal axial' territory were a source and the remaining type of mesoderm a diffusion sink for such molecules. Further evidence for such an arrangement in normal development, i.e. for an early interaction between mesoderms of differing initial specifications rather than direct quantitative or qualitative gradation in one inductive activity, comes from ultraviolet irradiation of eggs before the midpoint of the first cell cycle (e.g. Scharf and Gerhart, 1983; Cooke and Smith, 1987). This prevents the normal creation of what appears to be a quite small special sector within the endodermal source region for inducers, and is followed by development of radially symmetrical bodies possessing essentially normal amounts of mesoderm, but mesoderm only of lateroventral type. Two distinct types of mesoderm-inducing factor (MIFs) that may function in development have now been identified and characterised. On the basis of their effects upon competent Xenopus blastula ectoderm in vitro and, after their microinjection into the blastocoel, in vivo, it has seemed likely that they respectively specify initial mesodermal states corresponding to the two proposed above. XTC-MIF, a protein secreted by a Xenopus cell line, shows evidence of belonging to the transforming growth factor – β family (Smith, 1987; Smith et al. 1988; Rosa et al. 1988). At picomolar concentrations, it induces mesoderm resembling that naturally produced in the dorsal sector of the embryo in the timing and character of its gastrulation movements (Symes and Smith, 1987; Cooke and Smith, 1989) and in its later differentiations as notochord and somite muscle with associated neuralisation of adjacent ectoderm. Like the natural signaling of Xenopus mesoderm formation (Warner and Gurdon, 1987), it respecifies ectoderm only as an extracellular signal via the cell surface (Cooke et al. 1987). MIFs of the other class are members of the heparin-binding fibroblast growth factor (FGF) family (Slack et al. 1987, 1988). They induce mesoderm, which resembles in its mechanical behaviour during gastrulation the natural mesoderm of lateroventral type (Keller and Danilchik, 1988; Cooke and Smith, 1989), and which rarely differentiates as axial formations of notochord and segmenting somite with neural inductive capacity. The gene for a Xenopus homologue of basic FGF, present in eggs and early embryos, has now been cloned (Kimelman and Kirschner, 1987; Kimelman et al. 1988, henceforth in this paper called XbFGF). This is effective in giving mesoderm of lateroventral or 'mesenchymal' differentiation capacities at very low picomolar concentrations in vitro. In response to much higher concentrations some muscle is seen, but it seems unlikely on the basis of at least one estimate of the protein's concentration in vivo, that much muscle would be directly induced (Slack and Isaacs, 1989).

It seemed likely that mesoderm having organiser status might be distinctively specified by an in situ counterpart of XTC-MIF, possibly that molecule itself. A grafting operation has provided striking support for this hypothesis (Cooke et al. 1987). Shortly after injection of the soluble inducer into the blastocoel of a blastula stage donor embryo, a small (approx. 10^5 cell) piece of its blastocoel roof tissue is cut out, washed and then integrated into the ventral subequatorial region (marginal zone) of a host like a classical dorsal blastopore lip 'organiser' graft. Second axial body patterns develop that can be as well organised and complete as are those due to the dorsal lip graft. The scheme of such operations is shown in Fig. 1A. This procedure has been chosen rather than the more obvious in vitro treatment of competent tissue with MIF before grafting, because pilot experiments have revealed that it allows selection of more standardised, homogeneously stimulated graft tissue pieces. Variation in the rate of rounding up of explants in vitro, and perhaps in response rate to the factor, causes great variation in intensity of response in material from different egg batches. It also, we believe, leads in itself to spatial organisation within the newly induced tissue (Cooke et al. 1987). In the present paper I investigate in detail the conditions affecting this formation of 'second body patterns', and compare the natural organiser tissue with MIF – activated grafts of blastocoel roof, henceforth referred to as experimental organiser tissue. I have also assessed the relative role of the FGF class of inducer. Conclusions are based on more than 350 larval patterns resulting from grafts after donor injections with purified XTC-MIF, 200 after injection with cloned purified XbFGF or with XbFGF/XTC-MIF mixtures, and 20 after injection with saline – the control operation. Operations were also made to graft either supra-blastoporal mesodermal pieces or the subjacent endodermal cells, from the natural 'organiser' region of Xenopus early gastrulae, into blastula hosts. 70 of the operations in all were accompanied by lineage labelling of the grafted cells, after initial injection of donor eggs with rhodamine–lyse–dextran (RLDX).

The results of these experiments do furnish strong evidence that a morphogenetic gradient system within
Inducing factors and the organiser

231

Fig. 1. Experimental grafting operation. (A) The scheme of the operations. Major variables are MIF concentrations in blastocoel of blastula donor (injected in 2/3 strength saline, pH 8.0 + 0.5 mg ml⁻¹ BSA), time from donor injection to grafting, size of graft and absolute age of host blastula (usually 1 h younger than donor). Operation in 2/3 saline pH 7.2, lowered to 1/5 before onset of host gastrulation. Lower left shows an enlarged section of the normal mid-dorsal marginal region. This is opposite the experimental graft implantation site in hosts, and is also the origin of ‘natural organiser’ grafts from donors. The site of bottle cell formation separates the suprablastoporal mesodermal region, the most effective organiser when grafted with preservation of animal-vegetal (a–v) orientation, from yolky endoderm cells that are by this stage ineffective as organiser (see Results). Figure B shows views, from the side, of natural organiser (right) and XTC-MIF experimental grafts in position at onset of donor’s gastrulation. Invagination and underlying involution are spatially sequenced in dorsal lip but not in ectopically XTC-MIF-induced tissue.

Mesoderm is the basis of the organiser effect. They cannot yet help us understand the further cellular mechanisms, however, whereby such an initial gradient is utilised in controlling the pattern and proportions for cellular differentiation. This could be by direct (Wolpert, 1971; cp Summerbell, 1979; Tickle et al. 1985), or indirect (Cooke, 1983; Pate, 1984; Smith, 1989) responses to morphogen concentration levels. The relevance of the quantitative observations to the idea of a signal gradient in axial pattern formation is explained in the discussion.

Materials and methods

Synchronously fertilised embryos of Xenopus were obtained by standard procedures. They were dejellied with 2% cysteine HCl at pH 7.9, washed with 3/4 normal amphibian medium (NAM) and placed animal pole up in fitting wells under 1/10 NAM containing 5% Ficoll (SIGMA) at early blastula stages immediately prior to injection of graft donors with factors. Relative stages of donors and hosts for individual versions of the operations were manipulated by culture at temperatures of 15, 18 or 23°C from early cleavage stages.

Donors were injected with a standard 200 (stage 7 and early 8) or 300 (stage late 8 and 9) nannolitres of 3/4 NAM, pH 8.0 + 0.5 mg ml⁻¹ fraction V bovine serum albumin, containing purified factors at concentrations calculated to give those required in the blastocoel, using glass micropipettes and an oil-filled AGLA micrometer system. Injection over a few seconds caused effective subirrigation of the animal cap region and homogeneous exposure of the roof tissue (Cooke and Smith, 1989). Donor injections for the control operation contained no factors.

After the required response periods in donors, donor and host embryos were washed in 3/4 NAM, demembranated using fine forceps and placed on 2% agar beds that had been equilibrated overnight with 2/3 NAM, pH 7.2 for operations. Blastocoel roof grafts, two to four per donor from around the animal pole, were cut out, washed for several seconds by aspiration in a Spemann pipette and transferred to the vicinity.
of host embryos for implantation in their ventral marginal zones as depicted in Fig. 1. Operations were performed in small groups such that grafts remained free in the bathing solution for several minutes between excision and implantation, in addition to the washing and transfer procedures. Ionic strength of the solution was lowered to 1/5 NAM after 30 min, or at least 30 min before the expected onset of gastrulation movements in hosts. Batchs of embryos were selected as hosts in which the pigmentation and cell size gradations predicting the dorsoventral orientation were rather reliably visible during blastula stages, and operations proving to have placed grafts at much less than 180° to the host-controlled dorsal midline (Cooke 1972a, b) were rejected.

Fixation of larval bodies resulting from operations was at mid 30s stages (Nieuwkoop and Faber, 1967). Most second axial patterns were assessed structurally in relation to the full 'host' pattern (see Results) by dissection under the microscope after a few minutes fixation in K$_2$Cr$_2$O$_7$/acetic acid (Cooke and Smith, 1989). This allowed visualisation of each landmark structure of the body plan. Other examples were embedded in 58°C wax and sectioned at 7 μm for staining with Feulgen, light green and orange G (Cooke, 1979, 1981). For lineage labelling of graft contributions, donors were injected at onset of second cleavage with 15 nl of rhodamine-lysinedextran at 30 mg ml$^{-1}$ in water. Fixation of operated embryos was in 4% fresh formalin in 0.08 M-phosphate buffer, overnight at room temperature, followed by graded dehydration in butanol, and embedding in wax at 58°C via a butanol/wax mixture. Sections were cut at 10 μm and examined with appropriate filter sets in the Zeiss epifluorescence microscope.

Results

Classification of individual results

Individual examples of a body plan, while being coherent and well-ordered, can vary in completeness. Incomplete Xenopus plans, possessing only partial sets of mesodermal and induced neural structure, are seen following nonsurgical interventions that diminish the size or intensity of the centre for dorsal–anterior specification (Scharf and Gerhart, 1983; Cooke and Webber, 1985: Cooke, 1985). They form a 'nested' series, in that omission first occurs at one extreme of pattern, i.e. for dorsal and anterior structures directly associated with the organiser region, and then progresses in a posterior and ventral direction. Second initiations of pattern formation, in response to grafts of tissue that has what I here refer to as organiser status, will result in the same series of partial plans as well as the complete one. Total tissue available for morphogenesis is essentially unaltered after the operation (Cooke, 1979), but second patterns can be relatively extensive, embracing almost half the tissue into their structure, or relatively small. Extent and structural completeness tend to correlate in examples, as would if determined by the profiles and spatial extents of gradients, attained on particular occasions by the activity of some morphogen system. This is outlined in Fig. 2, a classification of the patterns seen into a series from 1, the minimal recognisable formation, to 5 the complete plan, in relation to the 'host-centred' plan in twinned larval bodies. Mean scores for second body patterns of twins, attained after particular variations of the operating procedure, thus represent their relative effectiveness in setting up instances of this 'primary' morphogenetic gradient field. The small inset diagrams in Fig. 2, interpreting the normal and examples of twinned body patterns in terms of hypothetical profiles for the primary gradient, will be referred to more in the Discussion section.

XTC-MIF specifies a special cell state

I have conducted experiments with electrophoretically pure XTC-MIF to assess its role in relation to XbFGF, and any possible synergy with that molecule, in inducing organiser status in target cells that are after all competent to respond to both factors. XbFGF is an extremely potent inducer, causing animal cap tissue to behave as mesoderm during gastrulation after exposure to considerably lower concentrations (approx. 0.1 ng ml$^{-1}$) than are required in the case of XTC-MIF (unpublished results). Both Xenopus-derived factors cause stable induction after well under an hour's exposure in vitro, and the altered cellular texture and thickness of blastocoel roof tissue such as is used for grafts, after injection of either factor into donors, already indicates respecification of this tissue as mesoderm before the donor age of onset of gastrulation (see Cooke and Smith, 1989). But in 60 operations using XbFGF, involving groups of 100–400 donor cells from blastocoel roofs 1½–2 h after injection to give exposure at 2–500 ng ml$^{-1}$ (approx. 20–5000 of its 'units' ml$^{-1}$), no host embryo developed even a grade 1 second pattern. This range of conditions embraces those optimal for the twinning result after XTC-MIF injection (see below). Furthermore, as shown in Table 1, XbFGF gives no evidence for synergy with XTC-MIF in inducing organiser status in blastocoel roof, whether present as the major or the minor component in a mixture of the factors at relevant concentrations. The 'intensity' of organiser status in grafts from blastocoel roofs is dictated solely by XTC-MIF concentration previously injected beneath them (Table 1).

The 'organiser graft', found in earlier Xenopus work to give an incidence of large and complete second patterns, contains the presumptive mesoderm (and future archenteron roof) just above the earliest external blastoporal lip at Nieuwkoop and Faber stage 10 (Fig. 1A, lower left), but also an equal mass of the subjacent, larger yolky endoderm cells (~ future archenteron floor). The precursors of these latter cells, at the young blastula stage, are those most effective in inducing new dorsal axial mesoderm when cultured in combination explants (Nieuwkoop, 1973; Dale and Slack, 1987) or grafted into host embryos (Gimlich and Gerhart, 1984). It is of importance to be sure that the cellular state caused by response to XTC-MIF corresponds to that of organiser mesoderm, rather than only its distinctive inducing endoderm. I have accordingly grafted these anatomical components of the natural organiser region separately, from beginning gastrulae into younger hosts. The results confirm that the dorsal endoderm cells of donors are almost past their period of
Fig. 2. The classification of degree of completeness in second body plans. The normal larval body plan (0) and bodies showing second plans of increasing (1–5) completeness and relative extent within their tissue mass. Major body parts represented symbolically, and labelled for the complete 'host' plan, can accordingly be followed as subsets present in second plans of increasing grade. Neural axis is omitted for clarity, except for landmark positions that diagnose pattern grades. ev—ear vesicle; ec, eyecup; fb, forebrain field; cg, cement gland; pc, prechordal mesoderm; nc, notochord; s, somite segments; h, heart; pn, pronephros; g, gill structure; bp, blastopore site. Notochords seldom fuse but tail somites do, with a tendency to terminalise the blastopore. Numbers of segments anterior to the level of fusion are usually equal, counting to landmark positions of pn or ev, in host and second patterns (Smallcombe and Cooke unpublished). Insets below and to right of certain drawings represent profiles proposed for a primary morphogen gradient system, to account for typical lower and higher grade twinned bodies in relation to the normal single one, with the relative extents of sources (i.e. numbers of effectively localised 'organiser' cells, see Discussion) shown as heavy baseline.
Table 1. Effectiveness of tissue grafts as organiser, after exposure to XTC-MIF/XbFGF mixtures and to their XTC-MIF component alone

<table>
<thead>
<tr>
<th>Factor mixture in blastocoel</th>
<th>Response grades (see text and Fig. 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XTC-MIF 900: XbFGF 90</td>
<td>7 9 3 1 2.9</td>
</tr>
<tr>
<td>XTC-MIF 900: XbFGF 180</td>
<td>6 7 6 7 2.9</td>
</tr>
<tr>
<td>XTC-MIF 900 ALONE</td>
<td>6 8 6 3.0</td>
</tr>
<tr>
<td>XTC-MIF 180: XbFGF 900</td>
<td>13 7 1 1.35</td>
</tr>
<tr>
<td>XTC-MIF 90: XbFGF 900</td>
<td>2 14 3 1.05</td>
</tr>
<tr>
<td>XTC-MIF 180 ALONE</td>
<td>12 8 1 1.40</td>
</tr>
<tr>
<td>XTC-MIF 90 ALONE</td>
<td>1 14 5 1.20</td>
</tr>
</tbody>
</table>

Grafts of c. 250 stage 9 donor cells were used, excised from blastocoel roofs 2 h after injection to allow for a possible slower recording of cellular response to FGF. 1 unit of activity ml⁻¹ of factors is the concentration just causing an in vitro inductive response in Xenopus animal cap tissue (Keller and Danilchik, 1988).

inductive signalling by onset of gastrulation (Jones and Woodland, 1987), causing minimal disturbance to pattern when transferred alone to a ventral marginal position even in young blastula hosts (30 operations). The mesoderm component immediately above the lip is at this stage the efficacious organiser. Even when divided longitudinally and used as smaller grafts to two hosts, this tissue initiates patterns that compare in size and completeness with those due to XTC-MIF experimental grafts. Blastocoel roof exposed to appropriate injected concentrations of XTC-MIF begins a subtle change of cellular anatomy that results in close similarity to the natural suprablastoporal organiser by stage 10+ (Cooke and Smith, 1989). Strikingly, both these tissues are most effective when placed in midblastula ventral marginal zones some hours before onset of gastrulation activity in the latter (Table 3, and see Cooke, 1973b), as if specific signalling between ‘organiser’ cells and others can begin before the time of morphogenetic movements with some benefit. It is clearly the status of organiser mesoderm that is specified in competent ectoderm by XTC-MIF. Once evoked, this status is stable.

Optimal conditions for initiation of body pattern

There is a reduced probability of obtaining the anterior headparts that mark the complete (grade 5) body plan in development controlled by the experimental, as opposed to natural organiser, grafts. Experimental graft–organised second axes are more likely than dorsal lip graft–organised ones to run behind the host axis in their schedule of morphogenesis (convergent extension, neural plate and tube formation etc.). This may account for their tendency to gain a smaller ultimate share in the host tissue. Dorsal lip tissue must be in homopolar orientation with its host, as regards the animal-to-vegetal dimension (presumptive head–tail, see Fig. 1), in order to set up second patterns (Cooke, 1972c; Keller, 1986). Reversed implantation leads to mechanical disturbance to gastrulation but not to any new axial structure. This can be related to a quite fine-grained and durable organisation among the cells of this region even before gastrulation, that actively controls their temporal order of involution and is often maintained even if the grafted tissue is reversed relative to its new surroundings. The graft’s movements then create a barrier that leads to mechanical conflict. But appropriately orientated dorsal lip mesoderm grafts recreate the lip-like invagination overlying a site of involution at their lower edge. This new centre for gastrulation movements comes to involve considerable host tissue, and a second pattern develops with a morphogenetic schedule nearly up to that in the ‘host’ axis. By contrast, brick-shaped pieces of the experimental organiser tissue have no ascertainable axes other than ‘inside–outside’ polarity, and when implanted tend to produce a more radially symmetrical, placode-like formation in the host’s marginal zone when their donor reaches stage 10+. For such ‘MIF’ grafts that are of near-optimal size, however, shape in relation to the host structure is relevant. A ‘brick’ whose width, subtending about 40° around the annular marginal zone of the host, is twice its height tends to give the best second patterns. These comparative features of natural and experimental organiser tissue are represented in Fig. 1B.

The variables most dramatically influencing mean size and completeness scores of second patterns are the original concentration of XTC-MIF in donor blastocoel and graft size. 200 ng (approx. 10⁻⁸ units) ml⁻¹ XTC-MIF has been, overall, the optimal blastocoel concentration for graft donors. The optimum graft is then a tissue piece as in Fig. 1B, and corresponding to some 250 cells of a stage 9 (approx. 4×10⁹ cell) donor. Cell number progresses very rapidly in the cleaving but nongrowing blastula, but it is graft size that matters. Second patterns after optimal versions of the experimental operation are most commonly of grades 3 and 4, while after comparable natural organiser and mesoderm grafts, grade 4 is the commonest result. Grade 5 (i.e. complete) second patterns, a minor class in Xenopus even after natural organiser grafts of these sizes, nevertheless occur three times as often as with the experimental grafts. As few as 50 grafted stage 9 cells, after exposure to 20 ng ml⁻¹ XTC-MIF, can lead to a grade 1 or 2 axial formation.

The effects of these variables, though strong, are hard to tabulate. There is inevitably a unique response level to the whole procedure among the embryos of different ovolutions, and only a limited sample of individual operations can be done on each experimental occasion. Table 2 nevertheless shows pooled means of second pattern scores in 3 experiments that have used varied MIF concentrations and graft sizes. Other variables were kept optimal at 1–2 h from factor injection to the grafting of early stage 9 cell groups into stage 8 hosts (see below). An optimal combination of factor concentration and graft size emerges. Appropriately small grafts that have responded to saturating concentrations of the factor, placed exactly midventrally in hosts, cause all the grade 4 and the occasional grade 5 results (Fig. 3A, B). Results from smaller dose×size products
Table 2. Effectiveness of blastocoel roof tissue as organiser, related to graft size and to concentration of XTC-MIF experienced

<table>
<thead>
<tr>
<th>XTC-MIF conc. (units ml⁻¹ in donor blastocoel)</th>
<th>Graft size (st. 9 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>500+</td>
</tr>
<tr>
<td>3000</td>
<td>1.7</td>
</tr>
<tr>
<td>1000</td>
<td>2.1</td>
</tr>
<tr>
<td>300</td>
<td>1.1</td>
</tr>
<tr>
<td>200</td>
<td>N.D.</td>
</tr>
<tr>
<td>100</td>
<td>N.D.</td>
</tr>
<tr>
<td>50</td>
<td>N.D.</td>
</tr>
<tr>
<td>Dorsal lip</td>
<td>4.4</td>
</tr>
</tbody>
</table>

Entries are mean pattern grades for pooled samples of operations totalling around 25 per entry. Thus, after middle-sized grafts and 1000 units ml⁻¹, 12 grade 3, 12 grade 4 and 1 grade 5 pattern resulted. After middle-sized grafts and 100 units ml⁻¹, 4 zero responses, 12 grade 1 and 10 grade 2 patterns resulted. Other variables, notably those of timing (see Table 3 and text) were near optimal in the present experiments.

...tend to be small and of lower score though internally well-ordered, while those from much larger than optimal grafts, regardless of MIF concentration, are usually large but ill-organised, poorly differentiated and low-scoring disturbances in the host body plan (see Fig. 4D). Natural organiser tissue grafted with proper orientation shows a contrasting property, in that the larger the piece that is implanted, the larger and more vigorous is the new set of cellular movements and ultimately the more complete (frequently grade 5) is the second body pattern formed. This contrast in behaviour between dorsal lip and ectopically MIF-induced tissue constitutes striking evidence that further spatial organisation must be an early part of natural 'dorsal axial' induction.

Donor tissue reaches the full potential as organiser that is to be evoked by any particular concentration of the factor 40 min to 1 h after injection. No appreciable change then occurs in this potential even if grafting is delayed for the further 3 h that may elapse before it displays new cellular behaviour resembling that in the natural stage 10 organiser (Table 3). Advancing host age at grafting, between large-celled blastula (st.7) and onset of gastrulation, exerts only a mild downgrading effect (Table 3), suggesting that the relevant signalling interactions occur largely during the gastrula period, though they may begin beforehand. Ambient temperature between healing-in of grafts and the end of gastrulation is kept near 18°C, when *Xenopus* develops at near half its 'optimal' rate (~ mid 20°C). It has been the author’s overall experience that such cool ambients during gastrulation optimise extent and completeness of second patterns after organiser grafting, and this remains true of XTC-MIF experimental grafting.

Cross-species recognition of the inductive function of XTC-MIF

I have asked whether *Ambystoma* (the axolotl), which may be distant from the frog by something closer to class than to subclass status taxonomically (Nieuwkoop...
Table 3. Time and stage related variables and the effectiveness of dorsal lip and of XTC-MIF experimental tissue grafts

<table>
<thead>
<tr>
<th>Time to host gastrulation (h)</th>
<th>Time and stage related variables and the effectiveness of dorsal lip and of XTC-MIF experimental tissue grafts</th>
<th>Time and stage related variables and the effectiveness of dorsal lip and of XTC-MIF experimental tissue grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5-4.0 (st. 7-8 host)</td>
<td>3.9  N.D.  3.6</td>
<td>3.9  N.D.  3.6</td>
</tr>
<tr>
<td>Around 1.5 (early st. 9 host)</td>
<td>3.8  3.0</td>
<td>3.8  3.0</td>
</tr>
<tr>
<td>&lt;0.5 (donor gastrulation)</td>
<td>2.9  2.8  N.D.</td>
<td>2.9  2.8  N.D.</td>
</tr>
<tr>
<td>ca. 0.75</td>
<td>3.5  3.3  3.1</td>
<td>3.5  3.3  3.1</td>
</tr>
<tr>
<td>ca. 1.0</td>
<td>3.4  3.3  3.1</td>
<td>3.4  3.3  3.1</td>
</tr>
<tr>
<td>ca. 1.75</td>
<td>3.8  3.1  3.0</td>
<td>3.8  3.1  3.0</td>
</tr>
<tr>
<td>2.5-3.5</td>
<td>3.1  3.0</td>
<td>3.1  3.0</td>
</tr>
</tbody>
</table>

Entries are mean pattern grades (see Table 1 last column and Fig. 2) for second axes of 15–20 operations, pooled from 3–5 experimental occasions (i.e. outgrowths of eggs) in which operations were performed with the stated combination of variables. Graft sizes was always near optimum. Dorsal lip grafts were similar-sized to control grafts in their contribution on an individual basis (18 experimental and 20 control-grafted larvae analysed). More descendents may tend to populate midtrunk levels and lateral somite while fewer remain in mesoderm near blastopore and tailbud, but like control cells they never enter anterior structure, notochord or parachordal parts of the somite cross-section of the normal, singly organised host pattern. In situ inductive signals at the graft site therefore seem able largely to substitute for XbFGF experienced beforehand by implanted cells, in controlling their long-term fate. Their 'preinduction' within the donor embryo may nevertheless ensure that experimental graft cells are among earlier-involuting, thus somewhat more anteriorly fated, mesoderm.

Descendents of 18 XTC-MIF experimental and of 18 natural mesodermal organiser grafts were in all cases centred upon the midlines of substantial second axial mesodermal patterns. They by no means constitute these mesodermal axes, however. They are typically intermixed with host-derived mesoderm in the anterior regions, and also form the roof of a new (endodermal) archenteron or else give rise to an integral strip of the endoderm beneath the new mesodermal dorsal midline. They give a progressively more scattered minor contribution to somite, or are absent, as progressively more posterior levels of the somite segment series are examined in the new axis that has been initiated by their implantation. The anteriorly confined distribution is particularly seen for experimental graft-produced patterns of low grade, after grafting both small numbers and suboptimally large numbers of mesodermised cells. Notochord, in patterns that include it, is the only structure essentially made of graft cells even at posterior axial levels, and also the only occasion when an anatomical and a graft–host lineage boundary coincide over any distance in pattern. A case from an XTC-MIF graft is shown in Fig. 4F. The compact contribution of labelled cells to notochord is also seen in the more frequent occurrences of this structure after grafting dorsal lip tissue. Whether dorsal lip grafts cause notochord formation or not, their contribution tends to be more compact and dorsally centred in the cross-section of second patterns, as well as more completely rostro-caudally distributed, than is that of experimental MIF grafts.

XTC-MIF grafts leave a substantial contribution in the non-neural ectoderm overlying the anterior end of the mesodermal pattern they have organised, suggesting that they contain a variable proportion of cells that have not been diverted from their epidermal pre specification at time of implantation. Grafted dorsal lips in the present series contributed only occasionally and to a small extent to the ectodermal pattern of hosts. This was entirely in ventral spinocaudal parts of second nervous systems. Such a contribution might be explicable in terms of our conventional view of neural induction, according to which this occurs in response to newly involuting dorsal axial mesoderm. Alternatively, it might indicate that dorsal lip grafts contain cells that were already part of a pattern of biass or 'induction' towards membership of the CNS at the onset of gastrulation in the donor (see Keller, 1986; Sharpe et al.)
Fig. 4. Structure and graft contributions in second axes. (A–C) Feulgen/light green/Orange G-stained wax histology, 7 μm transverse sections. (A, B) Eye/diencephalic and pronephric levels, respectively, of the second axial pattern in the *Ambystoma* embryo of Fig. 3D. (C) Trunk level section showing equal partitioning of mesoderm and sizes of nervous systems in XTC-MIF graft-organised twin body of *Xenopus*. (D–L) Rhodamine–lysine–dextran lineage-labelled grafts in *Xenopus*. Buffered paraformaldehyde fixation and wax histology, 10 μm sections transverse to single bodies and of equal oblique angles to T.S. for axes of twins. ‘Host’ dorsal midline (off-frame except in C and K) is towards top for D–K and right for L. (D) Neighbouring anterior levels populated by suboptimally large XTC-MIF graft from which little organisation resulted. Note extent and solid distribution of labelled tissue. (E, F) Ear vesicle/hindbrain and pronephric levels, respectively, of grade 4 XTC-MIF graft-organised second pattern. Extensive head mesenchyme, the notochord and a mid-dorsal endodermal strip populated, and parachordal somite contributed to, by graft. (G–J) Ear vesicle/hindbrain, pronephric and successive trunk levels, respectively, of grade 3 pattern after XTC-MIF grafting. Disorganised head mesoderm and the anterior somite axis largely populated by graft, but contribution rapidly decays posteriorly to give well-organised but unlabelled somite. (K, L) Unilateral tailbud somite and symmetrical midventral mesodermal contribution to a single normal axis after XbFGF grafting. This contribution, according with normal fate of graft surroundings, is not distinguishable from that of untreated blastocoel roof grafts. Scale bar=100 μm approx. Labelling as Fig. 2.

1987; Spemann, as related in Hamburger, 1988). The difference in character of ectodermal contribution as between experimental and natural organiser tissue tends to support the latter idea, and to suggest once again that the normal dorsal marginal zone achieves more early spatial organisation into subdomains than can be achieved by the homogeneously stimulated experimental tissue.
Fig. 5. Sequence of intercellular signalling proposed in the '3 signal' class of model. Pregastrula stage represented in lateral view, dorsal at right. In the downward-hanging vegetal hemisphere, a sector emitting inductive signal for 'dorsal axial' mesoderm abuts against or is superimposed on the more widely distributed source of 'ventrolateral' vegetal inductive activity (signals 1 and 2). The minor and major mesodermal sectors of different character which are thus induced in the marginal zone act respectively as source and sink for subsequent intramesodermal morphogen gradients (signals 3). Although shown on the same diagram, this intramesodermal signalling may occur largely during gastrulation. Dorsal axial sector is shown stippled with density decreasing from vegetal border (first - gastrulating, future anterior) into animal cap (later - gastrulating, more posterior). a, animal pole; v, vegetal pole. Shorter arrowheads=initial inductive signals, longer arrowheads=gradient fields within mesoderm occurring before and during gastrulation, either in signals 3 or by self-organising interactions within dorsal axial sector (see Discussion).

Discussion

First steps in regionalisation of mesoderm

Ectopic mesoderm in the blastocoel roof of XTC-MIF-injected embryos resembles, in the timing within gastrulation of its 'involution' behaviour and in the mode of intercellular adhesion it adopts, the mesoderm beneath the early dorsal lip. The differences in character between it and experimentally XbFGF-specified mesoderm are independent of the concentrations and prior time of application of the inducers. The hypothesis represented in Fig. 5 is supported by these and the other findings reviewed in the Introduction and by those reported in this paper. It is a version of the '3-signal model' first proposed by Slack and explicitly laid out by Dale and Slack (1987), and follows the general notion that pattern is realised in response to a morphogen gradient system. Initial inductive signals from the endodermal region of two types (i.e. signals 1 and 2) are proposed, with their effective sources so distributed as to give rise to a restricted sector of 'dorsal axial' type marginal zone and a larger remaining sector of the lateroventral type. Intramesodermal gradients in a further morphogen or morphogens (i.e. 'signals 3') then arise because the dorsal axial sector is a source for the molecules involved, and the remaining tissue acts as a sink by actively sequestering/destroying, and/or passively providing diffusion space. This model, and all the present work, relate only to proposed mechanisms underlying the reliability of normal development. It is now clear (e.g. Kao et al. 1986; Slack et al. 1988) that distortion of intracellular mechanisms of signal transduction, as presumably with the Lithium ion, can erode the normal distinction between cell states achieved via the heparin-binding and the expected TGF-β-like classes of factor and their distinctive cellular receptors. This piece of cell biology will only be unravelled with further understanding of the mechanisms of the signal transductions themselves.

The present work strengthens belief in some version of the overall model depicted in Fig. 5, for the initial organisation of pattern in the mesoderm. XTC-MIF and XbFGF might be, or might closely resemble, the initial signals 1 and 2 that set up source and sink states for the signals 3. The evidence for an actual, corresponding in situ role for XbFGF is currently somewhat stronger than is that for XTC-MIF, since it is present in the egg and blastula although its distribution is not yet known to be appropriately localised (Kimelman and Kirschner, 1987; Kimelman et al. 1988; Slack and Isaacs, 1989). The vegetal region of the Xenopus egg and blastula appears to contain RNAs for several TGF-β relatives, of which the best characterised, Veg. 1 (Weeks and Melton, 1987), does not code for XTC-MIF. Furthermore, various TGF-β family members are ineffective as mesoderm inducers of animal cap tissue on their own but can potentiate the action of factors of the FGF family so that mesoderm of dorsal character is produced (e.g. Kimelman and Kirschner, 1987). In the present experiments, all groups of animal cap cells probably receive a certain intensity of natural ventrolateral inductive signal after their grafting to the host ventral margin, and this could conceivably replicate an additional and necessary signal found earlier on in the dorsal marginal zone where the natural organiser originates. The mechanical behaviour and differentiations induced in animal cap tissue in vitro by pure XTC-MIF, however (Smith et al. 1988; Rosa et al. 1988; Symes and Smith, 1987), indicate that this particular signal molecule, whether or not it is the relevant in vivo one, induces the mesodermal organiser status in cells in the schema of Fig. 5 when acting alone. Work of the present type aims not so much to add to the evidences about which particular molecules are the in vivo components, but to help in understanding of the logical sequence of signals and cell states whereby pattern is built up. A distinctive experimental prediction of the 3 signal class of model, with the presently postulated
candidates for signals 1 and 2, concerns expected results of experiments where pieces of animal cap tissue with differing histories of exposure to inducers in vitro are joined together (Cooke et al. 1987). Such combining of pieces that have recently received XTC-MIF and XbFGF should result in significantly greater spatial organisation of mesodermal structure than occurs in either 'homotypic' combination, or in combinations where one component piece had not previously received any mesoderm-inducing signal. This prediction is under test.

Alternative mechanisms to the above for the primary arrangement of the body pattern would be (a) direct use of a gradient in the XTC-MIF signal, from its restricted source, to order pattern, (b) direct responses to different 'source intensities' of a mesodermal inductive stimulus, graded around the endodermal zone (Cooke and Webber, 1985), or (c) a variety of schemes whereby both receptor and potential FGF class of inductor were ubiquitously present in competent tissue, but this 'autocrine' mechanism of mesoderm formation was only made active, in a graded way, by encounter with an XTC/TGF-β class signal. But against (a) and (b), there is evidence that the XTC-MIF molecule's concentration and time of delivery does not in itself set 'position value' of mesoderm in the whole embryo (Cooke and Smith, 1989), and that any spread of signal from new dorsal axial mesoderm to adjacent but as yet not mesodermally induced tissue is a slow and thus local process (Cooke et al. 1987). Whereas it is straightforward in principle to see, from our understanding of transcriptional control processes, how quantitative gradation in an intracellular trans-acting factor might determine more than one switch (thus, two states) within previously equivalent cells (e.g. Driever and Nüsslein-Volhard, 1988), a receptor-binding intercellular signal protein does not appear apt for such direct control of several cell states. Alternative (c) above has difficulty in accounting for the occurrence of extensive mesoderms of radially symmetrical 'lateroventral' character, in embryos which have been prevented from forming any dorsal-axial-type inductive sector (Scharf and Gerhart, 1983; Cooke and Smith, 1987).

The 3 signal class of model tallies best with the present results, particularly in accounting for the stable development of incomplete (Fig. 2, 1–4) versions of the body plan. But it can only do so if it explicitly proposes that the cell states for the two initial mesodermal sectors, directly induced by signals 1 and 2, do not lead directly and uniquely to any particular differentiations. Instead, by their interaction, they define the polarity and extent of a morphogen landscape that will more directly order such differentiations. Thus XTC-MIF may specify a single new state – the 'organiser' or signal 3 source state, in an all-or-nothing manner at the individual cell level. Maximum signal value attained in the signal 3 gradients, thus the 'completeness score' (1–5) of patterns, should then be conditioned by the product of graft size and original XTC-MIF concentration. These will have set the effective number of signal 3 source cells present (cf. Summerbell, 1979; Tickle et al. 1985). The influence of the effective extent of source territory in relation to surroundings, in setting a gradient profile from source tissue of constant properties, is represented in the baselines below left-hand parts of the profiles sketched in Fig. 2. These profiles each correspond to single or twinned body patterns that are illustrated above and to their left. The source area must nevertheless be suitably small within the surrounding 'sink' mesoderm to ensure a coherent gradient; hence the low grade and disorganised patterns after over-large grafts (see Fig. 4D). Naturally originating body pattern is assumed to be complete and well proportioned insofar as earlier events ensure rightly proportioned source and sink sectors.

It cannot be discounted that some of the effect of grafting both natural and experimental organiser tissue comes from passive transfer, into the new surrounding intercellular spaces, of unbound factor of the same functional class as that which has induced dorsal specification in the grafted tissue. This would correspond to operation of a version of mechanism (a) above for the normal organisation of pattern within the dorsal axial sector, which is re-enacted after grafting. There is no reason to propose different classes of event, in this regard, after experimental as opposed to natural organiser tissue grafts, and in the future only experiments involving antibody reagents unique for the fully characterised factors may be able rigorously to distinguish between the classes of mechanism. It is already known that ventral implantation of a small, non-living, initially high-density source of XTC-MIF, but one from which the molecule will rapidly diffuse (agarose or sephadex), does not simulate the organiser phenomenon. Such implantation merely causes the blastocoel-wide ectopic mesoderm formation seen when a comparable, low total dose of factor is injected free as in the present procedure for creating donors (Cooke and Smith, 1989; J. Cooke and Emma J. Smith, unpublished work).

Spatial organisation within the natural organiser region

Ectopically XTC-MIF-induced animal cap tissue clearly corresponds, in general, to dorsal lip mesoderm. Why then should it be appreciably less effective, mass for mass, as organiser, and tend to make somewhat more rostrocaudally restricted and yet less compactly distributed lineage contributions to second patterns that it organises as a graft? Future head-to-tail and dorsoventral coordinates of the body plan are evident within normal mesoderm by gastrulation itself, as sequences of local time schedules of active involution. These gastrular gradations of mesodermal state are so laid out that they must be conditioned by relative distances of cells in the marginal zone from the endodermal sources of the inducers, as well as by spatial segregation of those sources around the marginal zone. The endogenous MIF source is assumed to lie at the vegetal edge of the induced dorsal axial tissue which is thereby able to acquire graded properties for a rostrocaudal sequence of axial pattern which ectopic, homogeneously induced tissue lacks (Cooke and Smith, 1989). This further
refinement of spatial organisation must occur soon in the normal development, because the dorsal lip tissue reveals itself in grafting experiments to be finely and importantly polarised (Cooke, 1972c; Keller, 1986, see also present Fig. 1B). Signal circuitry having the formal properties of local autocatalysis and then long-range inhibition, following initiation at one edge of a field, could mediate such an organisation (Gierer and Meinhardt, 1972; Meinhardt, 1982; Cooke, 1989), and TGF-β biosynthesis shows the first of these dynamics (Van-Obbergen-Schilling et al. 1988). Properly orientated grafts of dorsal lip may retain their graded range of preliminary specifications. Hence their tendency for compact but quite extended cellular contributions to axes, with relatively little developmental delay. Newly and homogeneously MIF-stimulated experimental grafts, by contrast, must start the new gradient system ab initio by selforganisation, as well as by being source for signals 3. The success of the outcome, within the time available, may depend upon the variable initial conditions as observed in this work. Hence also the more radially symmetrical arrangement of mechanical activity after implantation (Fig. 1B), and the anteriorly confined lineage contribution with more posterior axial pattern organised, but not populated, by the graft.

The normal initiation of induction from one (vegetal) edge of the competent region, that is not properly simulated by injection of soluble XTC-MIF beneath a cell sheet, may also be required for reliable attainment of the most ‘anterior’ specification within the general dorsal axial region. See Meinhardt (1982) for full theoretical discussion of symmetry-breaking and spontaneous pattern formation in morphogen systems. This most anterior tissue, of ‘prechordal’ specificity, is required for development of the complete plan in both mesoderm and induced neural system (Nieuwkoop, 1973), and in normal development it shows different cell-mechanical behaviour from, and involutes sooner than, dorsal axial tissue. But observation of even the occasional complete (grade 5) axes that are seen in response to XTC-MIF-stimulated implants does obviate the need to postulate a third, as yet undiscovered type of initial inducer, for dorsal prechordal mesoderm.

The completeness, extent and integration in the second organisations in Ambystoma are significant, confirming the folklore existing among experimental embryologists that the pattern of differentiation in urodele embryo tissue is ‘less determined’ than is that of anurans at each blastula and gastrula stage (Holtfreter and Hamburger, 1955). In Xenopus the negative effects upon outcome of the operations of advancing host age at grafting, and of increased ambient temperature, may indicate that the necessary interactions across tissue can be overtaken and limited by the onset of cell determination. The inherently slower pace at which loss of competence of tissue to be diverted in fate is occurring in Ambystoma would then allow superior results from both ‘natural’ and MIF-stimulated organiser grafting, and diminish the advantage of implanted but naturally preorganised dorsal lip mesoderm over a homogeneously induced cell group.

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