Properties of the dorsalizing signal in gastrulae of *Xenopus laevis*

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SUMMARY

According to the ‘three signal model’, the regional specification of tissue type within the mesoderm of *Xenopus laevis* occurs in a process called ‘dorsalization’. We have studied the timing and transmission characteristics of this signal, and assessed the dorsalizing activity of the lithium ion and a panel of cytokines.

The marginal zone has been fate mapped during gastrulation by colloidal gold labelling and it is shown that the ventral tissue undergoes substantial circumferential expansion. The fate map information is used to provide tissues of constant cellular composition for experiments conducted at different stages.

The stage at which dorsalization can occur has been investigated by means of heterochronic dorsal-ventral combinations. The results indicate that the interaction occurs during gastrulation, with a decline in both signal strength and competence of the ventral marginal zone to respond as gastrulation proceeds.

The signal is capable of passing through arrangements of membranes that exclude the possibility of cytoplasmic contact, implying that it can be carried by a diffusible morphogen.

The effect on the ventral marginal zone of lithium and a number of cytokines has also been studied. While none appears to function as a dorsalizing signal, lithium acts during blastula stages to alter the response to the mesoderm-inducing signal such that the inductions are of a more dorsal character.

These data confirm that the dorsalizing signal is independent of and operates later than the signal(s) from the vegetal hemisphere that induce mesoderm during the blastula stages.

Key words: *Xenopus*, gastrulation, organizer, dorsalization, mesoderm induction, lithium, transfilter induction

INTRODUCTION

The formation of the body plan of the amphibian embryo is thought to be the result of a series of inductive interactions, the first being the induction of mesoderm round the equator of the embryo. This was first demonstrated by Nieuwkoop (1969) who combined isolated explants from animal and vegetal pole regions. Alone, neither forms mesoderm and yet in combination several mesodermal tissue types are formed. In particular, depending on the vegetal portion used, two distinct types of mesoderm are formed (Boterenbrood and Nieuwkoop, 1973). The dorsal vegetal region induces notochord and muscle, while the ventral vegetal region induces blood, mesenchyme and mesothelium.

These two classes of mesoderm are revealed in the specification map which summarizes the self-differentiation behaviour of small explants from different regions of the embryo (Dale and Slack, 1987b; Slack, 1991b). Until the beginning of gastrulation, all but the most dorsal 90° of the marginal zone is ventral in character. While large blocks of muscle are characteristic of inductions using dorsal vegetal tissue, fate mapping studies show that most of the muscle in the embryo actually comes from the ventral half of the embryo (Dale and Slack, 1987a; Moody, 1987a,b). This apparent contradiction has been rationalized by the ‘three signal model’ (Smith and Slack, 1983; Slack et al., 1984; Smith et al., 1985; Dale and Slack, 1987b) in which the first two signals induce dorsal and ventral type mesodermal territories in the blastula and a third signal from the dorsal mesoderm, or ‘organizer’ brings about a regionalization of the ventral type mesoderm.

Although the early events in the blastula had not yet been elucidated, this third signal was apparent in the original organizer grafts of Spemann and Mangold (Spemann and Mangold, 1924), where a dorsal lip was grafted into the ventral marginal zone of a host embryo. This led to a second axis being induced. The most obvious consequence is the induction of a second nervous system, but a contribution of host tissues to the mesoderm was also noticed. When these experiments were repeated using horseradish peroxidase-labelled grafts (Smith and Slack, 1983), it was shown that the notochord of the secondary embryo was completely labelled but that its somites were substantially derived from the host ventral marginal zone. As the ventral marginal zone normally contributes very few cells to the somites, this is evidence for the existence of a dorsalizing signal emitted by the organizer and acting on the surrounding mesoderm. Further evidence for the existence of the signal was obtained by making direct combinations of explanted dorsal and ventral marginal tissue. In these combinations, the fate of the dorsal tissue remains unchanged, but the ventral
tissue becomes dorsalized (Slack and Forman, 1980; Dale and Slack, 1987b).

In recent years, it has proved possible to begin to identify candidate molecules for the early signals. The signal from the vegetal hemisphere responsible for the induction of ventral type mesoderm is believed to be a member of the fibroblast growth factor family (Kimelman and Kirschner, 1987; Slack et al., 1987; Amaya et al., 1991; Isaac et al., 1992). In the dorsal vegetal sector, this factor may be acting in conjunction with a maternal member of the wnt gene family (Smith and Harland, 1991; Sokol et al., 1991; Christian et al., 1992) to give rise to dorsal or organizer mesoderm. It is probable that the cytokine activin, which is a potent inducer of axial type mesoderm, is also involved in the process (Asashima et al., 1990; Smith et al., 1990; Thomsen et al., 1990). The newly induced mesoderm itself expresses a number of genes, among them are, goosecoid (Cho et al., 1991) in the organizer region, XWnt-8 (Christian et al., 1991) in the ventral region and brachyury (Smith et al., 1991) and snail (Sargent and Bennett, 1990) throughout the whole marginal zone. Before the importance of these, or other genes can be assessed fully and their interactions understood, more information is needed about the characteristics of the dorsalizing signal. The present study was undertaken in order to establish the time of dorsalization, whether it was carried by a diffusible substance and if it could be mimicked by known cytokines.

MATERIALS AND METHODS

Labelling of embryos

Embryos were obtained and handled as previously described (Godsave et al., 1988) and staged as in Nieuwkoop and Faber (1967). For uniform labelling, embryos were placed in full-strength Normal amphibian medium (NAM) (Slack and Forman, 1980) containing 5% Ficoll and label injected with a microinjection pump (Carnegie Medicin). Both fluorescein lysine dextran (Molecular Probes; called FDA after Gimlich and Braun, 1985), at a concentration of 100 mg/ml, and 0.5% colloidal gold (Poly-science no. 09285) were used. 20 nl of FDA was injected into the animal hemisphere upon first cleavage. The colloidal gold (CG) was prepared as in Niehrs and De Robertis (1991); as its diffusion rate, whether it was carried by a diffusible substance and if it could be mimicked by known cytokines.

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Fate mapping

A 60° wedge of either the dorsal marginal zone (DMZ) or ventral marginal zone (VMZ) was excised from an appropriately CG-labelled embryo at stage 10 and grafted orthotopically into an unlabelled, synchronous host. (The pieces of tissue grafted are illustrated in Fig. 1.) Typically, the graft healed within 30 minutes and the embryo continued to gastrulate normally to the desired stage. The embryos were fixed in 4% formaldehyde for 2 hours and the CG visualised by whole-mount silver enhancement (Niehrs and De Robertis, 1991).

Dorsoventral combinations

A ventral marginal zone explant was excised from FDA-labelled gastrulae at an appropriate stage and combined directly with a dorsal marginal zone explant from an unlabelled sibling. In order to ensure that explants from different stages were of similar cellular composition, the size and shape of the tissues excised correspond to the results of the fate mapping experiments. After 3 days in culture, the embryos were fixed in 4% formaldehyde for 2 hours, embedded in paraffin wax and sectioned at 6 µm. Specimens were rehydrated and stained in the nuclear stain DAPI (1 µg/ml) for 10 minutes, washed and mounted in Gelvatol.

In most cases, the presence of muscle was scored by its characteristic appearance: cells were large, elongated and had prominent lipid-filled vacuoles. Several specimens were stained with the anti-muscle monoclonal antibody, 12-101 (Kintner and Brockes, 1985), using a goat anti-mouse secondary coupled to tetramethylrhodamine isothiocyanate (TRITC). To reduce background, the sections were blocked with 5% bovine serum albumin (BSA) and 10% normal goat serum and 5% BSA was used in all antibody solutions.

In order to verify that dorsalization is indeed distinct from mesoderm induction, a set of combinations was made between stage 8 vegetal cores and FDA-labelled tissues: stage 8 caps to verify that the core were indeed capable of inducing mesoderm; stage 11 animal caps to ensure that by this stage the cap tissue is no longer responsive to mesoderm induction; stage 11 VMZs to investigate the dorsalizing properties of the signals emanating from the vegetal cells. All these were cultured for three days, embedded, sectioned and stained with DAPI as above.

Transfilter experiments

The apparatus used has been described previously (Slack, 1991a). Control combinations have VMZs on both sides of the filter while experimental ones are composed of a VMZ juxtaposed to a DMZ. Nucleopore filters of pore sizes 0.4 µm, 3 µm and 12 µm were used and in all cases, combinations were set up at stage 10 and disassembled some time later. The VMZs were then cultured in isolation until control embryos had reached stage 44 when they were processed for histology (Godsave et al., 1988).

In studies using Nucleopore filters, it is important to note that the total cross-sectional area of pore is much less for the small pore types. For example, the flow rate through 0.4 µm filters at 10 psi is only 70 ml/minute per cm² compared with 3000 ml/minute per cm² through a 12 µm filter. Passive diffusion is proportional to cross-sectional area, so we should expect the 12 µm filter to be 43 times better at passing the signal than the 0.4 µm, even in the absence of cytoplasmic contact. Hence, although 0.4 µm pore filters have been shown to prevent cytoplasmic contact (Grunz and Tacke, 1986), while the larger pores do not, a final set of transfilter combinations was made with the tissues separated by two 12 µm pore filters with a piece of nylon mesh sandwiched between them. This guarantees separation of the tissues since there is a gap of 100 µm between the two Nucle-
 pores, while still allowing a high cross-sectional area for communication.

**Lithium**

Embryos used for blastula-stage explants had their dorsal sides marked with Nile Blue at the 4-cell stage where there is a clear dorsal/ventral pigment difference. The VMZ was excised at stages 7, 9, 10, 11, 12 and 12+ i.e from the period of mesoderm induction through to the end of gastrulation. These were incubated in 0.1 M LiCl for 1 hour, rinsed in 1/3 strength NAM and then cultured for 3 days at 24°C, until control embryos had reached stage 44. They were then fixed, embedded and sectioned as described in Godsave et al. (1988).

**Cytokine treatment**

When the factor under investigation showed mesoderm-inducing properties, three columns of six serial, 1:3 dilutions of the factor were made in a Terasaki plate (as in the MIF assay of Godsave et al., 1988). Animal caps from stage 8 and stage 12 embryos were placed in two of the columns and stage 12 VMZs transferred into the third. Two further columns were filled with untreated stage 8 caps and VMZs. Treatment of stage 8 caps demonstrates the biological activity of the MIF, while the stage 12 caps act as a control for de novo mesoderm production, by demonstrating that ectodermal competence to respond has ended by this stage. The caps were scored visually from the plate, the induced ones form large vesicles, while the rest remain as small, undifferentiated spheres. The VMZs were processed for histology. Where the factor had no known MIF activity, the dilutions were applied only to stage 12 VMZs.

**Determination of the quantity of muscle**

Most specimens were classified as ventral or dorsalized by visual inspection of the amount of muscle. But it was felt important to have an objective measure of muscle content in order to assess borderline cases.

A number of control and dorsalized explants were examined and tracings of every tenth section made on graph paper using a camera lucida. Once these squares had been counted, the size of muscle blocks was then calculated. This volume is considered to be similar to the size of tissue that was dorsalized initially, as there is very little volume change from gastrula-stage cells to differentiated muscle cells.

The volumes of muscle observed in the lithium experiment are shown in the histogram in Fig. 2. A cut off for the amount of muscle permissible in control explants was selected at less than 3.5×10⁶ µm³, the same as was used by Dale and Slack (1987b). This figure is somewhat arbitrary but was chosen to be well above the highest observed muscle content of an untreated explant. It may slightly underestimate the number of cases affected, but does ensure that all the explants scored as positive are indeed dorsalized.

**RESULTS**

**Behaviour of marginal zone tissues during gastrulation**

During gastrulation, cell relationships alter and tissues change shape as different cohorts of cell invaginate at the blastopore lip, to be replaced by cells from the animal hemisphere as it undergoes epiboly (Keller et al., 1985). This means that if any tissue is to be excised at different stages during gastrulation, then it is essential that changes in its shape are mapped to ensure the use of equivalent cell cohorts at each stage.

From the colloidal gold grafts, it could be seen that the dorsal wedge did not expand ventrally in the course of gastrulation, but remained at a constant 60° throughout. When the embryo was cut in half along the dorsal and ventral midlines and the interior examined, labelled cells were observed along the entire anteroposterior extent of the invaginated marginal zone.

The labelled cells from a ventral marginal zone invaginated very little, but expanded dorsally around the circumference of the blastopore. This means that a wedge which measured 60° when it was grafted, had expanded to 90° by stage 11 and to 130° by stage 12. By the end of gastrulation, one half of the circumference of the blastopore is surrounded by labelled tissue. The ventral expansion is summarized in Fig. 3.

**Heterochronic dorso-ventral combinations**

Combinations of dorsal and ventral marginal zone tissue from gastrula stages were examined. Explants were scored as positive if they contained blocks of fluorescent muscle in excess of 3.5×10⁶ µm³. Most of the positive cases also contained pronephric tubules, which are not seen in isolated VMZ explants. In a few cases, lengths of fluorescent notochord were also observed. The results are summarized in Table 1. The first figures for each set of combinations show the number of positive explants as a fraction of the total number of that stage combinations examined.

There is a decline in both the strength of signal and in the competence of the ventral marginal zone to respond to it as gastrulation proceeds. All ventral explants combined at stage 10 became dorsalized, with a reduction in this proportion as both tissues got progressively older e.g. a stage...
Fig. 3. Summary of ventral marginal zone expansion during gastrulation. (A) A ventral graft (hatched) when it is first made at stage 10. This 60° area expands during gastrulation, so that, by stage 11 (B), it measures 90° and, by stage 12 (C), it has expanded to 130°. By the end of gastrulation (D), one half of the circumference of the blastopore is surrounded by labelled cells.

10 DMZ gave only 83% dorsalization in combination with a stage 12 VMZ or a stage 10 VMZ was only dorsalized in 71% of cases when combined with a stage 12 DMZ. By looking along the top row of Table 1, we can follow the competence of the VMZ. This drops sharply only between stages 12 to 12½, suggesting that competence is maintained throughout most of gastrulation. Looking down the first column of the Table, we see what happens to the signalling capacity of the DMZ. This seems to fall gradually as gastrulation proceeds but is still present at stage 12½. The other entries in the Table are consistent with these interpretations.

The decline in the strength of the signal is reflected in the fact that in 10 out of the 11 cases where fluorescent notochord, (i.e. notochord derived from the VMZ), is observed, it is in combinations with a stage 10 DMZ.

When the final position of the tissues was examined in detail, it could be seen that there was much cell movement in the combinations, especially on the part of the ventral explant, with fluorescent cells frequently entirely surrounding the dorsal explant. This results in labelled muscle being intermingled with unlabelled muscle cells surrounding an unlabelled notochord. (See Fig. 4). The fact that only those parts of the ventral explant that were closest to the DMZ became dorsalized, may suggest that the signal has a short range. However, it is conceivable that dorsalized cells from the ventral explant collect together into blocks before differentiating and the final distribution of tissues merely reflects these cell movements.

The results of the combinations involving blastula tissues were as expected. When a stage 8 core was combined with a stage 8 cap, mesoderm was induced (Nieuwkoop, 1969), but when the cap was taken from a stage 11 embryo, it differentiated as epidermis (Jones and Woodland, 1987). In all cases (n=12) where combinations were made between an entire stage 8 core and a stage 11 VMZ, the VMZ fails to be dorsalized and differentiates into a vesicle containing the typically ventral mesodermal tissues, mesenchyme, mesothelium and blood. This confirms that the signal(s) that induce mesoderm in the blastula are distinct from the dorsalizing signal in the gastrula.

Transfilter experiments

From the heterochronic combinations, it was evident that stage 10 was to be the optimal stage, for both dorsal and ventral marginal zones, for dorsalization to occur. Hence, all the transfilter experiments were assembled with tissues from this stage. As in the heterochronic combinations, pronephric tubules were found in most of the explants that were scored as dorsalized on the basis of their muscle content. Notochord was never observed in ventral explants from transfilter combinations. The results of the combinations are summarized in Table 2 and typical explants are illustrated in Fig. 5.

It can be seen that while the dorsalizing signal is capable of crossing a Nuclepore filter of pore size 0.4 μm, it does so only rarely. The apparent weakness of the signal may be due to the very low cross-sectional area of pore in 0.4 μm filter. Since 0.4 μm pores have been shown to be sufficiently small to exclude the possibility of cytoplasmic contact (Grunz and Tacke, 1986) even the small number of cases is suggestive of a signal carried by an extracellular factor.

The cross-sectional area increases with pore size in the ratio 1:2:1:43 for 0.4 μm, 3 μm and 12 μm filters respectively. This obviously serves to allow the signal better access to the ventral explant and so may explain the increase in percentage of explants dorsalized between 0.4 μm and 12 μm filters, in contact for the same length of time. This argument assumes that binding of the factor to the cell surface occurs at a rate comparable to or faster than that of diffusion, so that the diffusion is rate limiting.

Table 1. Results of heterochronic dorsoventral combinations

<table>
<thead>
<tr>
<th>Dorsal MZ Stage</th>
<th>Ventral MZ Stage</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>12%</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td></td>
<td>22</td>
<td>26</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>15</td>
<td>12</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>12</td>
<td>11</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>12½</td>
<td></td>
<td>6</td>
<td>2</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

The figures under the dorsal and ventral marginal zone labels represent the stages of each tissue when the combinations were made.

The denominator of the fraction is the total number of each combination examined and the numerator is the number which were scored as positive.

*marks those combinations in which fluorescent notochord has been observed - in one of the the 11/12 combinations, two of the 10/10 and the 10/12 and in six of the 10/11 combinations.
The combinations made with the nylon mesh sandwich arrangement gave percentages of explants dorsalized that are comparable with the single 12 µm filter (59% and 53% respectively). As the explants with the sandwich are separated by in excess of 100 µm, we do not believe there is any cytoplasmic contact and hence the signal must be carried by an extracellular factor. However, even with optimum arrangements only 50-60% of dorsalized explants are obtained in comparison with the 100% for direct combinations. This might be due to the even greater area available for diffusion, or to additional effects of contact, or to some role of the extracellular matrix.

The effect of cytokines and the lithium ion on the VMZ

The panel of cytokines with known MIF activity that have been tested for dorsalizing capacity are acidic and basic fibroblast growth factor (FGF), XeFGF, kFGF, TGF-β2, BMP-4 and XTC-medium (Xenopus activin A). The other factors studied were heparin, nerve growth factor, insulin-like growth factor 2, mammary derived growth inhibitor, granulocyte macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), NN′-Hexamethylene-bis-acetamide (HMBA) and the murine interferon α (IFN). In all cases, the concentration of factor applied ranged from very high through to physiological levels. The period of treatment lasted from stage 10, when the explants were excised, to the end of the three day culture period.

On examination of the sections from the VMZs, all explants were found to contain the ventral tissues, blood, mesenchyme and mesothelium. Where muscle was observed it was in small wisps, smaller than 3.5×10^6 µm^3, comparable to the controls. In all cases, the treated explants were indistinguishable from untreated and hence none of these substances has dorsalizing activity. Where the factor was known to have mesoderm-inducing activity, parallel titrations were also done with stage 8 and stage 12 ectoderms. In all cases, the stage 8 ectoderms were induced and the stage 12s were not.

In light of the negative results obtained with the VMZs, we were concerned about the accessibility of the internal

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**Table 2. Results of transfilter experiments**

<table>
<thead>
<tr>
<th>Filter pore size</th>
<th>Dis-assembled</th>
<th>Ventrail</th>
<th>Dorsalized</th>
<th>No. of explants</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4 µm</td>
<td>St 13</td>
<td>38</td>
<td>100%</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>St 30</td>
<td>36</td>
<td>84%</td>
<td>43</td>
</tr>
<tr>
<td>3 µm</td>
<td>St 13</td>
<td>3</td>
<td>43%</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>St 30</td>
<td>6</td>
<td>46%</td>
<td>7</td>
</tr>
<tr>
<td>12 µm</td>
<td>St 13</td>
<td>9</td>
<td>57%</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>St 30</td>
<td>9</td>
<td>47%</td>
<td>19</td>
</tr>
<tr>
<td>12 µm Sandwich</td>
<td>St 30</td>
<td>7</td>
<td>41%</td>
<td>17</td>
</tr>
</tbody>
</table>

76 explants from control combinations were examined, all of which were ventral in character. Dorsalized explants contain in excess of 3.5×10^6 µm^3 of muscle.

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**Fig. 4.** Dorsalization of ventral tissue. A section of a stage 10 VMZ was labelled with FDA was cultured in combination with an unlabelled stage 12 DMZ. A shows nuclei in the entire combination stained with DAPI. B shows those areas that contain FDA. The epidermis, some mesenchyme and a substantial amount of muscle are labelled, surrounding an unlabelled notochord. C illustrates staining with the anti-muscle antibody, 12-101. The large muscle block encompasses not only those cells FDA labelled, but also those cells immediately adjacent to the notochord. Bar: 10 µm.
surface of the explant to the factors, so a number of stage 10 explants were cultured in a 1:20 dilution of fluorescein-coupled wheat germ agglutinin. These were fixed at stage 13, the end of competence of the VMZ to respond to the dorsalizing signal, embedded and sectioned. In all cases, a substantial amount of fluorescence was observed on the cell surface of the marginal zone and endoderm cells (data not shown). This indicates that the VMZ explants do not round up immediately on excision and the blastocoel-facing surface is accessible to factor treatment.

The results of lithium treatments are summarized in Table 3, with typical explants shown in Fig. 6. It can be seen from these that explants that are treated with lithium at stage 7 form large blocks of muscle, in excess of $3.5 \times 10^6 \mu m^3$, and also notochord, while those treated at stage 9 will still give rise to a lot of muscle, but are never observed to contain notochord. When the treatment is conducted later than this, i.e. at gastrula stages, there is no discernible effect, with explants giving rise to ventral tissues such as mesenchyme, mesothelium, blood and, occasionally, wisps of muscle smaller than $3.5 \times 10^6 \mu m^3$. Pronephric tubules are never observed in lithium-treated explants.

These results show that lithium has a much stronger dorsalizing effect when applied to blastula stages than at any point during gastrulation. Hence, it is not mimicking the in vivo dorsalizing signal but rather is altering the embryo’s response to the earlier ventral mesoderm-inducing signal.

**DISCUSSION**

The establishment of the dorsal-ventral axis in *Xenopus* can be divided into a number of discrete steps. Soon after fertilization, the egg cytoplasm undergoes a rotation relative to the cortex (Gerhart et al., 1989), imparting a bilateral symmetry to the egg. The midline where the core moves vegetally relative to the cortex becomes the dorsal side. By blastula stages, the dorsal-vegetal portion has acquired a dorsal type mesoderm-inducing activity (Elinson and Kao, 1989; Gerhart et al., 1989; Kageura, 1990), which by gastrulation has resulted in the formation of the Spemann organizer (Gerhart et al., 1989).

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**Table 3. Results of lithium treatments**

<table>
<thead>
<tr>
<th>Explanted</th>
<th>Control ventral</th>
<th>Control +muscle</th>
<th>Treated ventral</th>
<th>Treated +muscle</th>
<th>Treated +notoch.</th>
</tr>
</thead>
<tbody>
<tr>
<td>St 7</td>
<td>15 100%</td>
<td></td>
<td>27 8%</td>
<td>13 48%</td>
<td>12 44%</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td></td>
<td>27</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>St 9</td>
<td>15 100%</td>
<td></td>
<td>9 56%</td>
<td>7 44%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td></td>
<td>16</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>St 10</td>
<td>19 100%</td>
<td></td>
<td>30 91%</td>
<td>6 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19</td>
<td></td>
<td>33</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>St 11</td>
<td>22 96%</td>
<td>1 4%</td>
<td>27 100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>23</td>
<td></td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>St 12</td>
<td>7 88%</td>
<td>1 8 12%</td>
<td>11 92%</td>
<td>1 8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>8</td>
<td>12</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>St 12½</td>
<td>6 100%</td>
<td></td>
<td>4 100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td></td>
<td>4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

‘Treated’ explants were soaked in 0.1 M LiCl solution for 1 hour after explantation. They were then cultured for 3 days and scored. Explants which are classed as ‘ventral’ contained less than $3.5 \times 10^6 \mu m^3$ muscle. Those included in the ‘+muscle’ column contained more than this.

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**Fig. 5.** Explants from transfilter experiments. (A) An explant from a control transfilter combination. Typical mesenchyme (ms), mesothelium (mt), blood (bl) and endoderm (en) are clearly visible. (B) Section of a ventral explant cultured in combination with a dorsal marginal zone. The explants were separated by a 3 µm Nuclepore filter until stage 13, when the apparatus was dismantled. The influence of the dorsalizing signal is seen by the presence of organized blocks of muscle (mu) among the more ventral tissues. Bar: 10 µm.
Dorsalization in *Xenopus* gastrulae

From the fate maps for 16/32 cell *Xenopus* embryos (Dale and Slack, 1987a; Moody, 1987a,b), it can be seen that much of the somites arise from the ventral half of the body. However, the specification map (Dale and Slack, 1987b; Slack, 1991b) illustrates that muscle arises only from the dorsal quadrant. This led to the postulate of another interaction from the organizer responsible for patterning of the mesoderm.

This postulate has been supported directly and indirectly by results from several other workers. The isolation studies of Kageura and Yamana (1983), on very early stages, confirmed that isolated ventral halves form vesicles lacking in axial structures (Spemann’s belly piece (1938)). Keller and Danilchik (1988) showed, from the study of explanted pieces of the marginal zone, that the extension leading to elongation of the axis is confined to the dorsal quadrant while the convergence movements leading to closure of the blastopore occurred equally around the marginal circumference. Sater and Jacobson (1990) showed that the dorsal 60° sector is required for the formation of the heart from more lateral marginal zone tissue, suggesting that the heart as well as the somites and kidney needed the dorsalization signal for its specification.

Although the ventral half of the embryo is usually perceived as the passive partner in these interactions, the present study shows that it is undergoing a substantial amount of cell movement, which cannot be explained merely as a consequence of the convergent extension of the dorsal marginal zone (Keller et al., 1985; Keller and Danilchik, 1988; Keller and Tibbetts, 1989). Keller et al. (1992) may begin to explain at least part of the late ventral expansion when he states that, after stage 11.5, the notochord begins to elongate and pushes the blastopore region ventrally (Keller and Danilchik, 1988). This results in the ventral marginal zone being seen ‘streaming round the sides of the blastopore’ (Wilson et al., 1989; Keller et al., 1989). However, as Nieuwkoop and Faber (1967) describe first indications of notochord formation at stage 12.5, about the same time as the embryo begins to elongate visibly, it would seem that any extension prior to this time would be so slight as to explain very little of the ventral expansion at all.

This expansion of the ventral marginal zone does mean that the dorsalizing signal need only have a short range because cells of ventral origin are continuously migrating into the dorsal half. Also compatible with a short-range signal would be the final position of cells in the heterochronic combinations, where it appears that only the ventral cells immediately in contact with the dorsal explant are affected. This may however reflect dorsalized cells collecting into groups before differentiating.

The heterochronic combinations indicate that the competence of the ventral marginal zone declines between stages 12 and 12½ (looking at the stage 10 DMZ row in Table 1, the only difference between the last 2 columns is 1 hour in development, yet there is a marked decline in the percentage of explants dorsalized). Taking the stage 10 VMZ column, there is less of a difference between the stage 12 and 12½ DMZ, indicating that the signal may persist until somewhat later. The decline in the strength of the dorsalizing signal is also detected in the ability of the DMZ to dorsalize as far as notochord. 10 out of the 11 cases, where fluorescent notochord was observed, were in combinations with a stage 10 DMZ explant. This ability to obtain notochord from lateral mesoderm has been observed by Stewart and Gerhart (1991) who made combinations between normal and U.V. embryos at the late blastula stage. Notochord was shown to be induced and by comparison with fate mapping experiments they concluded that this may happen in normal development. Other workers have suggested that lateral mesoderm cannot be dorsalized as far as notochord (Smith, 1985; Gimlich and Gerhart, 1984). In this study, we have seen only a limited number of cases and conclude that the contribution to the notochord in vivo from dorsalization is probably small.

In a similar series of tissue combination experiments,

Fig. 6. Effects of lithium on ventral marginal zone. The control untreated explant (A) contains the ventral tissues blood, mesenchyme (ms) and mesothelium (mt), while those explants treated at stage 7 (B), around the time of mesoderm induction, have large blocks of muscle (mu) and notochord (no). Explants treated at stage 9 (C) show large blocks of muscle (mu) but are never found to contain notochord, while explants treated from stage 10 (D) through to the end of gastrulation are indistinguishable from the controls. Bar: 10 μm.
Jones and Woodland (1987) investigated the competence of animal cap cells to respond to mesoderm induction and the ability of vegetal cells to induce mesoderm. They concluded that animal caps become responsive at stage 6½ and lose their responsiveness at stage 10½. The signalling ability of the vegetal region is in place by stage 6 and is lost between stages 10½ and 11. The different intervals of competence and signalling in their series and ours suggests that the dorsalizing signal is distinct from and operates later than the signals that initially induce the mesoderm. This was confirmed by the combinations reported here between an entire stage 8 vegetal core, capable of inducing mesoderm from a stage 8 cap, and a mid gastrula VMZ. In these, the VMZ was not dorsalized.

The results of the transfilter experiments conducted with 0.4 µm Nucleopore filters show that in 16% of cases ventral explants become dorsalized. While a low proportion, this is at least suggestive of a signal carried by an extracellular factor. This is confirmed in the 12 µm sandwich arrangement, where explants are separated by approximately 100 µm, excluding the possibility of cytoplasmic contact but still allowing a large cross-sectional area for communication. In these almost 60% are dorsalized. It must be remembered that the ability to cross this liquid gap by passive diffusion says nothing about the range of the signal in vivo and should not be taken as evidence against the possibility that the signal has a short range within the embryo, only that it can be secreted.

Even with this optimum arrangement, the percentages obtained are lower than in the direct combinations, suggesting that cellular contact can greatly enhance the effect. Comparable transfilter experiments on mesoderm induction can give 100% positives (Slack, 1991a). Even in those experiments, there is a substantial reduction in signal strength, with the inductions being smaller and more ventral in character than those obtained in direct combinations. The weakening of the signal observed by Gurdon (1989) was even greater. He found that with a 0.1 µm pore filter, only 50% of his blastula animal cap/vegetal core combinations were induced and in those that were positive, the response was weakened by 3-10 times.

Of the factor treatments, lithium obviously shows biological activity, but seems to be altering the response to mesoderm induction (also shown by Cooke and Smith, 1988; Kao and Elinson, 1989; Slack et al., 1988) rather than mimicking dorsalization. In the light of the findings of Yamaguchi and Shinagawa (1989), what is rather unexpected is that lithium does still seem to have a dorsalizing effect at stage 9. However, given that the stage of the mid-blastula transition, with respect to Nieuwkoop and Faber stages, varies between batches of embryos, if our stage 9 embryos were only slightly early, it is possible that they were pre-MBT and hence in accordance with the published observations. It is surprising, however, that none of the other factors investigated are capable of acting as a dorsalizing agent, despite in many cases being able to induce dorsal type mesoderm from competent animal caps. Frank and Harland (1992) recently reported that activin can induce muscle differentiation in isolated ventral marginal zone explants, as assayed by MyoD expression. The treatment in their experiments was done between stage 8 and stage 11 and hence actually reflects the ability of activin to induce dorsal mesoderm, not to dorsalize ventral mesoderm.

When explants from the ventral marginal zone are cultured as has been described, very little muscle is formed, but when these cells are dispersed and grown as monoclonal cultures, many more clones give rise to muscle than would be predicted (Godsave and Slack, 1991). A possible explanation for this is that the ventral half of the embryo contains an inhibitory factor that represses myogenesis. The dorsalizing signal might then represent the destruction or removal of the inhibition, and this would be compatible with the failure so far to find a substance that has dorsalizing activity. If there is a gradient of inhibitor superimposed upon a constant myogenic capacity around the entire marginal zone, we would expect to see some myogenic factors present in the lateroventral marginal zone. One such is MyoD, for which the message and protein is initially found in stage 10½ and stage 11 as an incomplete ring around the closing blastopore (Frank and Harland, 1991; Hopwood et al., 1992). The gap in the staining of the marginal zone has been shown to be in the most dorsal region, the prospective notochord.

A number of genes are now being discovered that are specifically expressed in the organizer (Cho et al., 1991; Dirksen and Jamrich, 1992; Taira et al., 1992). We feel that the present study should be helpful when assessing their role, and that of other genes, in the function of the organizer. We have shown that the dorsalizing signal acts during gastrulation and can diffuse but is more effective when there is a large area of contact. Components that have these properties would be good candidates for the molecular basis of dorsalization.

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