Regional specification within the mesoderm of early embryos of
Xenopus laevis

L. DALE and J. M. W. SLACK

Imperial Cancer Research Fund, Developmental Biology Unit, University of Oxford, Zoology Building, South Parks Road, Oxford, OX1 3PS, UK

Summary

We have further analysed the roles of mesoderm induction and dorsalization in the formation of a regionally specified mesoderm in early embryos of Xenopus laevis. First, we have examined the regional specificity of mesoderm induction by isolating single blastomeres from the vegetalmost tier of the 32-cell embryo and combining each with a lineage-labelled (FDA) animal blastomere tier. Whereas dorsovegetal (D1) blastomeres induce 'dorsal-type' mesoderm (notochord and muscle), lateroventral and ventrovegetal blastomeres (D2–4) induce either 'intermediate-type' (muscle, mesothelium, mesenchyme and blood) or 'ventral-type' (mesothelium, mesenchyme and blood) mesoderm. No significant difference in inductive specificity between blastomeres D2, 3 and 4 could be detected. We also show that lateroventral and ventrovegetal blastomeres from early cleavage stages can have a dorsal inductive potency partially activated by operative procedures, resulting in the induction of intermediate-type mesoderm.

Second, we have determined the state of specification of ventral blastomeres by isolating and culturing them in vitro between the 4-cell stage and the early gastrula stage. The majority of isolates from the ventral half of the embryo gave extreme ventral types of differentiation at all stages tested. Although a minority of cases formed intermediate-type and dorsal-type mesoderm we believe these to result from either errors in our assessment of the prospective DV axis or from an enhancement, provoked by microsurgery, of some dorsal inductive specificity. The results of induction and isolation experiments suggest that only two states of specification exist in the mesoderm of the pregastrula embryo, a dorsal type and a ventral type.

Finally, we have made a comprehensive series of combinations between different regions of the marginal zone using FDA to distinguish the components. We show that, in combination with dorsal-type mesoderm, ventral-type mesoderm becomes dorsalized to the level of intermediate-type mesoderm. Dorsal-type mesoderm is not ventralized in these combinations. Dorsalizing activity is confined to a restricted sector of the dorsal marginal zone, it is wider than the prospective notochord and seems to be graded from a high point at the dorsal midline.

The results of these experiments strengthen the case for the three-signal model proposed previously, i.e. dorsal and ventral mesoderm inductions followed by dorsalization, as the simplest explanation capable of accounting for regional specification within the mesoderm of early Xenopus embryos.

Key words: mesoderm, Xenopus laevis, regional specificity, dorsalization, induction, blastomere, specification, fluorescein–dextran–amine.

Introduction

The formation of the basic body plan during early amphibian development is believed to result from a sequence of inductive interactions between different regions of the embryo (Slack, 1983; Nieuwkoop, 1985; Smith, Dale & Slack, 1985). The first of these is mesoderm induction, through which the equatorial marginal zone of the blastula acquires the capacity to differentiate into mesodermal tissue types (Nakamura, 1978). This interaction can be demonstrated by combining isolated explants from animal and vegetal pole regions; alone neither piece forms any mesoderm whereas in combinations the animal explant produces several mesodermal tissue types (Nieuwkoop, 1969; Nakamura, Takasaki & Ishihara, 1970;
Sudarwati & Nieuwkoop, 1971; Nieuwkoop & Ubbels, 1972; Dale, Smith & Slack, 1985; Gurdon, Fairman, Mohun & Brennan, 1985). Two types of mesoderm can be induced in these experiments: dorsovegetal cells induce ‘dorsal’-type mesoderm characterized by notochord and muscle, while ventro-vegetal cells induce ‘ventral’-type mesoderm characterized by mesothelium and blood (Boterenbrood & Nieuwkoop, 1973; Dale et al., 1985; Maufroid & Capuron, 1985). This suggests that the vegetal hemisphere of the amphibian blastula is subdivided into two or more differently specified regions and that this division is transmitted to the animal hemisphere as a result of mesoderm induction.

Although large muscle blocks are characteristic of dorsal inductions, the fate map of the early *Xenopus* embryo shows that more than half of the somite tissue comes from the ventral half in normal development (Cooke & Webber, 1985; Dale & Slack, 1987). This is why we believe that a second inductive interaction, dorsalization, is responsible for further subdivisions of the mesoderm (Slack, Dale & Smith, 1984; Smith et al., 1985). This interaction can be shown directly in combinations of dorsal and ventral marginal zones from gastrulae. In such combinations the fate of the dorsal tissue remains unchanged but large muscle masses are formed by the ventral component. Ventral marginal tissue in isolation differentiates mainly into mesenchyme, mesothelium and blood (Slack & Forman, 1980). Dorsalization is also demonstrated by the organizer graft. When a lineage-labelled dorsal marginal zone fragment is grafted into the ventral marginal zone a secondary dorsal axis is formed from the host ventral marginal zone (Spemann & Mangold, 1924; Smith & Slack, 1983). Furthermore ventral marginal zone fragments grafted into the dorsal marginal zone become dorsalized and differentiate into large blocks of muscle (Smith & Slack, 1983). These experiments suggest that the formation of a normal set of axial mesodermal structures requires a dorsalizing signal from a quite restricted sector of the blastula/gastrula dorsal marginal zone. In this paper, as in previous works, we have used the term organizer for this region since we regard dorsalization of the mesoderm as the inductive activity which is primarily involved in the formation of the double embryo following an organizer graft.

In the absence of this region we might expect isolated fragments to form extreme ventral-type mesodermal tissues, lacking dorsal axial structures such as somites. However, many cases of ventral isolates from early cleavage stages have been reported to develop mesodermal part patterns, including somites, that in many ways resemble those formed by these blastomeres during normal development (Kageura & Yamana, 1983, 1984; Cooke & Webber, 1985; Render & Elinson, 1986). Several of these authors have suggested that dorsoventral pattern formation is a function of the mesoderm-inducing signal(s) and this would imply that what we call dorsalization does not occur in normal development.

To test this view against our own we have performed a number of new experiments. (1) We have examined further the regional specificity of mesoderm induction by dividing the vegetal tier of the 32-cell *Xenopus* embryo into four regions along the D/V axis and combining single blastomeres from these regions with a lineage-labelled animal tier of blastomeres. (2) We have determined the state of specification of ventral blastomeres by isolating and culturing them *in vitro* at all stages between the 4-cell and the early gastrula stages. (3) We have made a comprehensive series of combinations between different regions of the marginal zone using a lineage label to distinguish the components.

### Materials and methods

Fertilized embryos were obtained using methods described by Dale & Slack (1987) and were staged according to Nieuwkoop & Faber (1967). Embryos requiring lineage label were injected within 1 h of fertilization with 15 nl of fluorescein–dextran–amine (FDA: Gimlich & Braun, 1985) at 100 mg ml⁻¹ in water, using techniques described previously (Dale et al., 1985; Dale & Slack, 1987).

The location of the future dorsal centre was ascertained at the 8-cell stage when dorsal micromeres are more lightly pigmented than ventral micromeres (Nieuwkoop & Faber, 1967). The dorsal equatorial zone was then marked with a crystal of alkaline-precipitated Nile blue (Kirschner & Hara, 1980). The accuracy of this method was checked on a number of egg batches by measuring the angle between the stain and the dorsal blastopore at the early gastrula stage. In 83 % of cases (n = 70) they were coincident and in 95 % of cases the stain was within 60° of the dorsal blastopore lip.

Operations performed at stage 6 (32-cell embryo) utilized embryos in which the blastomeres were regularly arranged in four tiers of eight cells (Fig. 1A). Blastomeres were isolated by sacrificing all unwanted blastomeres using ground forceps and a tungsten needle. For the single-cell induction experiments the vegetalmost tier (tier D) was isolated and then divided into individual blastomeres, one from each column, by sacrificing half the cells. Blastomeres from the same embryo were noted and then each combined with the eight cells of the most animal tier (tier A) of an FDA-labelled embryo. All operations were performed in 50 % NAM (normal amphibian medium: Slack & Forman, 1980). Combinations were cultured in 50 % NAM until control embryos had reached stage 40–41 when they were fixed in 4 % paraformaldehyde (PF).
Similar procedures were used to isolate ventral-half embryos at the 4- to 32-cell stages. All yolky remains were gradually removed and the media replaced by fresh half-strength NAM. At stages 7, 8, 9 and 10 demembranated embryos were cut into dorsal and ventral halves using tungsten needles and both halves were retained. All half embryos were cultured in half-strength NAM until controls had reached stage 40-41, they were then fixed in Romeis fixative (25 ml saturated HgCl, 20 ml 5% TCA, 15 ml 37% formaldehyde).

Dorsal, dorsolateral, ventrolateral and ventral fragments of both early gastrula (st10) and midblastula (st8) marginal zones (DMZ, DLMZ, VLMZ and VMZ respectively, see Fig. 7) were isolated and cultured until stage 40—41 when they were fixed in Romeis fixative. For the dorsalization experiments stage-10 ventral fragments were combined with either dorsal or dorsolateral fragments from the same stage and cultured until stage 40—41 when they were fixed in 4% PF. One of the two fragments was taken from an FDA-labelled embryo, the other from an unlabelled embryo.

The methods used for histologically processing and scoring fluorescent specimens have been described by Dale & Slack (1987). Similar procedures were used for nonfluorescent specimens except they were stained overnight in borax carmine (saturated in 35% alcohol) and then transferred to 70% alcohol/1% HCl for differentiation. After sectioning they were counterstained with 0.1% naphthalene black in saturated aqueous picric acid.

The results of each experiment is presented in two forms: first, we present the frequency of each tissue in each class of experiments and, second, we classify each case into one of three groups based on the mesodermal tissues formed. Dorsal mesoderm is defined as containing notochord which is always accompanied by muscle and neural tube. Intermediate mesoderm is defined as containing large, contiguous blocks of muscle, usually mesenchyme and mesothelium and less-frequently blood cells or pronephros. The muscle volume represents more than 10% of that formed by the appropriate region of the embryo during normal development (Dale & Slack, 1987) and this was measured by dividing the field of view (×250) into squares of side 9-25 μm using an eyepiece graticule and in every tenth section counting those squares in which at least 50% of the area contained FDA-labelled muscle. Ventral mesoderm is defined as containing mesenchyme, mesothelium, blood cells and in some cases small wisps of muscle representing less than 10% of that formed by the appropriate region of the embryo during normal development. Although the 10% figure may seem arbitrary, in fact most cases of either isolates or inductions contain either much more than this if they are intermediate or much less if they are ventral. It should be noted that the intermediate class is equivalent to mesoderm levels 2 and 3 of Slack & Forman (1980).

Results

D tier blastomeres exhibit two inductive specificities

Regional differences in the inductive specificity of the vegetal hemisphere were analysed at the 32-cell stage by combining single blastomeres from each column of tier D with eight blastomeres from the A tier of an FDA-labelled embryo (Fig. 2). The results of this experiment reveal an important regional difference between the type of mesoderm induced by blastomere D1 and those induced by blastomeres D2—4 (Table 1). Whereas blastomere D1 induces dorsal types (Fig. 3A) in all but a few cases (17/22), blastomeres D2—4 induce a mixture of intermediate (Fig. 3B) and ventral (Fig. 3C) mesoderms. The mesoderms induced by sets of blastomeres taken from the same embryo are particularly revealing. In five cases where all four combinations survived and/or formed mesoderm, only blastomere D1 induced notochord. This strongly suggests that the ability to induce notochord is restricted to a very small region
of the D tier, that region vegetal to the prospective organizer. Although the mesoderm induced by the remaining blastomeres of the D tier are very similar blastomere D2 is a slightly more potent inducer of muscle than either D3 or D4. Not only do more cases contain muscle but the mean volume of muscle is approximately twice that induced by these latter blastomeres.

Since all of the cases described above included an A tier isolated from an embryo labelled with FDA, the provenance of the induced tissues could be ascertained. In all cases most of the induced mesoderm was clearly derived from the A tier (Fig. 3) and it is the labelled tissues that were scored and are presented in the tables. However, in many cases a small but significant proportion of the induced mesoderm was unlabelled and therefore clearly derived from D tier blastomeres. Unlabelled cells were found in all mesodermal tissue types, in all types of combination.

A comparison between these results and what might have been expected from the fate map (Dale & Slack, 1987; Fig. 1B) indicate some differences as far as the ventrolateral blastomeres are concerned. Both blood cells and pronephric tubules are induced by blastomeres D2, 3 and 4, a result contrasting with their restricted location in the fate map (blood – C4, pronephros – C3). Furthermore, dorsal blastomeres (D1 and D2) induce twice the volume of muscle induced by ventral blastomeres, whereas in the fate map 60% of the muscle is derived from ventral blastomeres. Only notochord is induced in a manner consistent with the fate map. The results suggest that regional differences in the inductive potency of vegetal blastomeres are too few to form a complete explanation of regional specification within the mesoderm during normal development.

Table 1. Frequency of occurrence of tissues in stage-6 combinations of single blastomeres of tier D and the eight blastomeres of tier A

<table>
<thead>
<tr>
<th>Blastomere</th>
<th>n</th>
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<th>NT</th>
<th>Me</th>
<th>No</th>
<th>Mu</th>
<th>Pr</th>
<th>Mt</th>
<th>Bl</th>
<th>YM</th>
<th>Dor</th>
<th>Int</th>
<th>Ven</th>
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<tbody>
<tr>
<td>D1</td>
<td>22</td>
<td>100</td>
<td>73</td>
<td>64</td>
<td>77</td>
<td>100</td>
<td>0</td>
<td>18</td>
<td>9</td>
<td>0</td>
<td>77</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>D2</td>
<td>18</td>
<td>100</td>
<td>22</td>
<td>94</td>
<td>11</td>
<td>83</td>
<td>11</td>
<td>30</td>
<td>33</td>
<td>11</td>
<td>11</td>
<td>61</td>
<td>28</td>
</tr>
<tr>
<td>D3</td>
<td>22</td>
<td>100</td>
<td>14</td>
<td>82</td>
<td>5</td>
<td>77</td>
<td>5</td>
<td>68</td>
<td>50</td>
<td>14</td>
<td>5</td>
<td>45</td>
<td>50</td>
</tr>
<tr>
<td>D4</td>
<td>19</td>
<td>100</td>
<td>16</td>
<td>68</td>
<td>16</td>
<td>74</td>
<td>11</td>
<td>63</td>
<td>58</td>
<td>5</td>
<td>16</td>
<td>42</td>
<td>42</td>
</tr>
</tbody>
</table>

N.B. Nomenclature for this and subsequent tables. n, number of cases; Epi, epidermis; NT, neural tube; Me, mesenchyme; No, notochord; Mu, muscle; Pr, pronephros; Mt, mesothelium; Bl, blood; YM, undifferentiated yolk mass; Dor, dorsal mesoderm; Int, intermediate mesoderm; Ven, ventral mesoderm.
Early ventrovegetal explants induce excess intermediate forms

In a previous report (Dale et al. 1985) we have demonstrated that in combinations between animal and vegetal pole material from stage-7½ (about 256-cell) embryos, ventrovegetal material induces predominantly ventral types containing very little muscle. However, in the stage-6 cases described above muscle was found frequently in combinations including ventrovegetal blastomeres, often as a substantial proportion of the induced mesoderm. To facilitate a more accurate comparison between these stages, combinations were made at stage 6 between the four blastomeres of the ventral half of the D tier (D3 and D4) and the eight blastomeres of the A tier. In a majority of cases (15/26) these combinations formed intermediate-type mesoderms (Table 2). The main difference between these and single-cell inductions concerns the volume of mesoderm. Combinations including all four ventrovegetal blastomeres contain, on average, four times as much muscle as combinations including only a single ventrovegetal blastomere (data not shown). In comparison with combinations made at stage 7½, both types of stage-6 combination contain muscle both more frequently and in greater volume ($P<0.01$). This is reflected in the greater proportion of stage-6 cases that have been classified as having intermediate mesoderms. It should however be emphasized that a large minority of both single cell (D2, 3, 4) and 4-cell (D3+4) inductions comprise the extreme ventral types. We have also performed a number of combinations between single vegetal pole blastomeres and animal pole pieces from stage-7½ embryos (data not shown). In all cases ($n=23$) blastomeres from lateral and ventrovegetal locations induced small volumes of ventral mesoderm while only dorsovegetal blastomeres induced notochord. Taken together these experiments demonstrate a clear difference between the mesoderms induced by ventrovegetal material at stages 6 and 7½.

This change in inductive specificity from intermediate to ventral type seems paradoxical because it is a change away from the fate map, and while we are accustomed to regulation preceding mosaicism there are few, if any, examples of mosaicism preceding regulation. We therefore wondered whether this change was perhaps not a reflection of real events in the embryo but arose from our own interventions. The critical test is to find whether change can occur autonomously in isolated ventrovegetal blastomeres. Accordingly groups of four ventrovegetal blastomeres (D3–4) were isolated at stage 6, aged until stage 7½ (2h), and then combined with a stage-7½ animal pole piece. From the results presented in Table 2, it is clear that no change has occurred in the inductive potency of these blastomeres as a result of ageing in isolation. Blastomeres isolated at stage 6 and aged until stage 7½ before combining with animal pole tissue induced the same range of mesodermal tissues, and at a similar frequency, as blastomeres.
Table 2. Frequency of occurrence of tissues in combinations of ventrovegetal and animal pole isolates

<table>
<thead>
<tr>
<th>Specimen</th>
<th>n</th>
<th>Epi</th>
<th>NT</th>
<th>Me</th>
<th>No</th>
<th>Mu</th>
<th>Pr</th>
<th>Mt</th>
<th>Bl</th>
<th>YM</th>
<th>Dor</th>
<th>Int</th>
<th>Ven</th>
</tr>
</thead>
<tbody>
<tr>
<td>St6</td>
<td>26</td>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>65</td>
<td>0</td>
<td>81</td>
<td></td>
<td></td>
<td>12</td>
<td>65</td>
<td>54</td>
</tr>
<tr>
<td>St7</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td>70</td>
<td>11</td>
<td>26</td>
<td></td>
<td></td>
<td>4</td>
<td>70</td>
<td>81</td>
</tr>
<tr>
<td>St6 (aged)</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td>100</td>
<td>13</td>
<td>93</td>
<td></td>
<td></td>
<td>27</td>
<td>87</td>
<td>80</td>
</tr>
</tbody>
</table>

The ventral half of the embryo is in an extreme ventral state of specification

An independent method of investigating the correctness of our contention that there are two distinct mesoderm-inducing signals is to analyse the tissues differentiated in isolates containing different portions of the prospective mesoderm. If the mesoderm-inducing signal is complex, consisting of several distinct components, then a complex pattern of differentiations comparable to the fate map should be observed in isolates. Conversely if the signal is simple, consisting of as little as two components, then a simple pattern of differentiations should be observed. We have analysed two types of isolate: first, in dorsal and ventral half embryos and, second, in small isolates from four locations around the dorsoventral axis of the marginal zone at midblastula and early gastrula stages.

Dorsal-half embryos from stage 7–10 develop into tissues expected both from fate maps and the induction experiments at all four stages tested. Most of these specimens (97/100) developed into dorsal-type mesoderm, including pronephros in about half the cases. Although the culture conditions used throughout these experiments did not allow normal gastrulation, many of the cases developed into embryos that resembled, both externally and internally, those previously described for dorsal blastomeres isolated at the 4-cell stage (Kageura & Yamana, 1983; Cooke & Webber, 1985). Three exceptions were found (5% of cases at stage 7–9), all formed mesoderms characteristic of ventral isolates and we believe these to be the result of mismatches between early pigmentation and the prospective dorsal side.

In a majority (157/215) of cases ventral-half isolates from all eight stages studied develop into radially symmetric embryos whose mesoderms consist only of mesenchyme, mesothelium and blood (Table 3; Fig. 4), the classical belly piece (‘Bauchstücke’) of Spemann (1938). Although muscle is often found in these isolates, it is usually unsegmented and in very small quantities. Of the remaining cases most (43 cases) developed into intermediate-type mesoderms and the remainder (15 cases), dorsal mesoderm. We believe that the dorsals arise from cases in which there was a mismatch between pigmentation and prospective dorsal side. Such mismatches are expected to have a more severe effect on ventral than dorsal halves because a 45° mismatch would include some D1-type cytoplasm in a ventral half while a 135° mismatch is required to exclude D1 cytoplasm from a dorsal half. The muscle content of these specimens is extremely variable, ranging from no muscle at all through to the full amount expected from the fate map. However, the figure of 10% of fate map muscle content was selected as the cutoff between ‘ventral’ and ‘intermediate’ types because there are almost no cases in the range 3–15% (Fig. 5). The mean muscle content of those mesoderms classified as ventral is 0–7% and those classified as intermediate is 45% of that expected from the stage-6 fate map. It is clear that most ventral-half isolates develop in a manner...
Regional specification within mesoderm

Table 3. Frequency of occurrence of tissues in ventral half isolates

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Tissue types (percentage of cases)</th>
<th>Mesoderm level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Epi</td>
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<tr>
<td>Stage 3</td>
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<td>Stage 10</td>
<td>33</td>
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<tr>
<td>Totals</td>
<td>215</td>
<td>100</td>
</tr>
</tbody>
</table>

Much more ventral than that expected from stage-6 and early gastrula fate maps (Keller, 1976; Dale & Slack, 1987).

Intermediate forms arise from mechanically activated early ventral halves

Although the mesoderms formed by ventral halves at the different stages are similar, a few differences are also apparent. This particularly concerns isolates from the 4-cell stage in which the mesoderms are often more complete, frequently containing pronephric tubules. Apart from stage 10, pronephric tubules are found very rarely in ventral halves from other stages and then usually (12/14 cases) in cases classified as dorsal. These results are similar to those obtained elsewhere for ventral blastomeres isolated at the 4-cell stage (Kageura & Yamana, 1983; Cooke & Webber, 1985).

Of particular interest are the mesoderms that develop in ventral halves isolated at stages 6 and 7, for they can be compared with the induction experiments we have already described. Whereas the mesoderms formed by stage-7 ventral halves are predictable from the induction experiments, both being predominantly ventral, those formed at stage 6...
are not since the inductions include many intermediates. We tested the possibility that the difference was caused by microsurgical 'activation' of blastomeres by the following experiment. Following isolation of the 16 ventral blastomeres from a stage-6 embryo, the vegetal 4 blastomeres (tier D) were first isolated from and then recombined with the remaining 12 blastomeres (tiers A, B & C). The result is a stage-6 ventral-half embryo in which the vegetal tier has undergone the operative procedures used in combination experiments (Fig. 6). In a majority of cases (19/29) these half embryos produce intermediate-type mesoderms containing substantial quantities of muscle (mean = 48% of fate map) and in many cases pronephros (14/29), mesothelium and blood (Table 4). It is clear that these embryos differentiate more in accord with stage-6 combinations than stage-6 isolates, suggesting that the procedures used to isolate ventrovegetal blastomeres artificially enhance their inductive potencies. Of course this result could explain the differentiation of intermediate mesoderms in ventral-half embryos, both those described by us and other workers, the less extreme procedures enhancing fewer cases. The similarity between the mesoderms containing substantial quantities of muscle (mean = 48% of fate map) and in many cases pronephros (14/29), mesothelium and blood (Table 4). It is clear that these embryos differentiate more in accord with stage-6 combinations than stage-6 isolates, suggesting that the procedures used to isolate ventrovegetal blastomeres artificially enhance their inductive potencies. Of course this result could explain the differentiation of intermediate mesoderms in ventral-half embryos, both those described by us and other workers, the less extreme procedures enhancing fewer cases. The similarity between the mesoderms

Table 4. Frequency of occurrence of tissues in stage-6 ventral half isolates in which the vegetal tier was first isolated and then recombined

<table>
<thead>
<tr>
<th>Tissue types (percentage of cases)</th>
<th>Mesoderm level</th>
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<tr>
<td>n</td>
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<td>29</td>
<td>100</td>
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Table 5. Frequency of occurrence of tissues in marginal zone isolates

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<td>DLMZ (St10)</td>
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<td>94</td>
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<td>VLMZ (St10)</td>
<td>17</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>18</td>
<td>6</td>
<td>94</td>
<td>94</td>
<td>100</td>
<td>0</td>
<td>12</td>
<td>88</td>
</tr>
<tr>
<td>VMZ (St8)</td>
<td>13</td>
<td>100</td>
<td>92</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>92</td>
<td>77</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>VMZ (St10)</td>
<td>19</td>
<td>100</td>
<td>0</td>
<td>95</td>
<td>0</td>
<td>26</td>
<td>0</td>
<td>100</td>
<td>95</td>
<td>100</td>
<td>0</td>
<td>5</td>
<td>95</td>
</tr>
</tbody>
</table>

* For the origin and nomenclature of fragments see Fig. 7.
differentiated by isolates and combinations at stage 7 and later suggest that by this stage vegetal blastomeres are less sensitive to the operative procedures, presumably because of their smaller size.

Most of the marginal zone exhibits extreme ventral specification

The patterns of mesoderm differentiation in small marginal zone isolates (Fig. 7) from the midblastula (stage 8) and early gastrula (stage 10), are on the whole similar to those developed by the much larger dorsal and ventral halves (Table 5). Ventral (VMZ & VLMZ) and dorsomedial (DMZ) fragments form ventral and dorsal mesoderms, respectively, and dorsolateral (DLMZ) fragments may form all three types of mesoderm (Fig. 8). Indeed at both stages 50% of DLMZ fragments form ventral mesoderm, containing few muscle cells (Fig. 8C).

A comparison of these results with their fate in orthotopic grafts (Dale & Slack, 1987) demonstrates that, whereas isolates of the DMZ consistently develop according to fate, most isolates of the DLMZ and all isolates of the VLMZ and VMZ do not. In all of these cases isolates develop into more ventral tissue types and less dorsal tissue types than expected from the fate map. It is worth emphasizing that about half of DLMZ isolates and all VLMZ isolates contain substantial numbers of blood cells, which is not expected from the fate map. These cases and those of VMZ isolates cannot be distinguished.

The ventral marginal zone can be dorsalized by the organizer

If there are only two states of specification in the marginal zone of the early Xenopus embryo, how might intermediate states be generated? We have previously suggested that dorsalization may account for these intermediate states (Slack et al. 1984; Smith et al. 1985) and this mechanism is further investigated in the experiments described below.

Combinations were performed at stage 10 between ventral fragments and either dorsal or dorsolateral fragments of the marginal zone (Fig. 9). In all combinations one of the fragments was isolated from an embryo uniformly labelled with FDA. Wound healing occurred rapidly and the two fragments remained in close contact throughout the culture period. When combinations are examined in sections, following culture, labelled cells remain as coherent blocks and there is little mingling with unlabelled cells (Fig. 10).

Fig. 7. Stage-10 marginal zone fragments. (A) Viewed from the vegetal pole. (B) Viewed in median section. DMZ, dorsal marginal zone; DLMZ, dorsolateral marginal zone; VLMZ, ventrolateral marginal zone; VMZ, ventral marginal zone; AP, animal pole; VP, vegetal pole.

Fig. 8. Stage-10 DLMZ fragments: examples of actual cases after culture. (A) Dorsal-type mesoderm. (B) Intermediate-type mesoderm. (C) Ventral-type mesoderm. For abbreviations see Table 1. Bars, 100 µm.
Fig. 9. Dorsalization: experimental design. Stage-10 DMZ or DLMZ fragments are isolated and combined with a stage-10 VMZ fragment. One of the fragments is always lineage labelled.

Fig. 10. Dorsalization: actual cases after culture. All photographs show bright fluorescence of labelled cells and faint autofluorescence of unlabelled cells. (A) In combination with an unlabelled DMZ fragment a labelled VMZ fragment is dorsalized and forms a large block of muscle. (B) In the complementary experiment a labelled DMZ fragment is not ventralized by combination with an unlabelled VMZ fragment, it still forms notochord. (C) In combination with an unlabelled DLMZ fragment a labelled VMZ fragment is dorsalized. Here the DLMZ fragment has formed intermediate mesodermal tissues including muscle but not notochord. (D) When an unlabelled DLMZ fragment forms ventral mesodermal tissues a combined labelled VMZ fragment is not dorsalized, it also forms ventral mesoderm. For abbreviations see Table 1. Bars, 100μm.
When DMZ and VMZ fragments are combined the VMZ is invariably dorsalized containing a large mass of muscle and failing to form either mesothelium or blood (Table 6; Fig. 10A). However, in only one case \((n = 40)\) did the VMZ fragment produce any notochord and then only a few cells (<10) were involved. So dorsalization is not complete, rather it involves promotion of ventral tissue to intermediate status, corresponding to mesoderm from the second or third column in the fate map. The fate of the DMZ is not changed by these combinations, in all cases notochord and muscle were the mesodermal tissues formed by these fragments (Fig. 10B). In agreement with previous results (Slack & Forman, 1980; Smith & Slack, 1983) this suggests that while ventral tissue may be dorsalized, dorsal tissue may not be ‘ventralized’.

The organizer occupies about 90° of marginal zone circumference

To determine the extent of dorsalizing activity in the marginal zone, combinations were made between DLMZ and VMZ fragments (Fig. 9). In Table 6 the results of this experiment have been divided into three categories, based on the tissue types formed by the DLMZ fragment (see above). DLMZs forming dorsal mesoderm invariably dorsalize the combined VMZ fragment, a result expected from those presented above. DLMZs forming intermediate mesoderm also dorsalized the combined VMZ in a majority of cases (73%; Fig. 10C), demonstrating that notochord development and dorsalization are not intimately linked. However, DLMZs forming ventral mesoderm failed to dorsalize the combined VMZ fragment at all; in these cases both fragments formed mesothelium and blood (Fig. 10D). This suggests that dorsalizing activity is localized to the dorsomedial sector of the marginal zone and occupies a region of perhaps 90° or less of circumference.

Not all regions of the organizer are equally effective at dorsalizing VMZ fragments, lateral explants being weaker than more medial ones. This is clearly illustrated by Fig. 11, where we compare the volume of muscle differentiated by VMZ fragments under different conditions. VMZs in combination with a DMZ fragment contain a greater volume of muscle than those in combination with the more lateral

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**Table 6. Frequency of occurrence of tissues in both components of recombined stage-10 marginal zone fragments**

<table>
<thead>
<tr>
<th>Fragments</th>
<th>n</th>
<th>Epi</th>
<th>NT</th>
<th>Me</th>
<th>No</th>
<th>Mu</th>
<th>Pr</th>
<th>Mt</th>
<th>Bl</th>
<th>YM</th>
<th>Dor</th>
<th>Int</th>
<th>Ven</th>
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<tr>
<td>DMZ</td>
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<td>100</td>
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<td>0</td>
<td>60</td>
<td>100</td>
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<td>82</td>
<td>35</td>
<td>100</td>
<td>3</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>3</td>
<td>97</td>
<td>0</td>
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<tr>
<td>DLMZ(D)*</td>
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<td>100</td>
<td>42</td>
<td>100</td>
<td>100</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0</td>
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<tr>
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<td>42</td>
<td>58</td>
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<td>100</td>
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<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
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<td>DLMZ(I)</td>
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</tr>
<tr>
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<td>100</td>
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<td>84</td>
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<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>VMZ</td>
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<td>0</td>
<td>98</td>
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<td>2</td>
<td>4</td>
<td>98</td>
<td>93</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
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<td>DMZ</td>
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<td>82</td>
<td>23</td>
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<td>86</td>
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<td>Ventral half</td>
<td>22</td>
<td>100</td>
<td>86</td>
<td>82</td>
<td>0</td>
<td>95</td>
<td>91</td>
<td>77</td>
<td>41</td>
<td>100</td>
<td>0</td>
<td>86</td>
<td>14</td>
</tr>
</tbody>
</table>

*The letters in brackets indicate the type of mesoderm differentiated by DLMZ fragments in these combinations. (D) dorsal type, (I) intermediate type and (V) ventral type.*

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**Fig. 11. Dorsalization: attenuation of the organizer.** The histogram shows the volume of muscle formed by VMZ fragments, under the various experimental conditions described in this paper, as a fraction of the muscle formed following orthotopic grafts (Dale & Slack, 1987). DMZ, in combination with DMZ fragments; DLMZ(D), in combination with DLMZ fragments that form dorsal mesodermal tissues; Graft, in orthotopic grafts; DLMZ(I), in combination with DLMZ fragments that form intermediate mesodermal tissues; DLMZ(V), in combination with DLMZ fragments that form ventral mesodermal tissues; I, in isolation.
Fig. 12. (A) Fate map of the stage-6 embryo, based on Dale & Slack (1987). (B) Specification map of the pregastrula embryo. (C) Schematic diagram of the three-signal model. As a result of early cytoplasmic rearrangements two inductive centres, of dorsal and ventral specificity, are established in the vegetal hemisphere. During mesoderm induction the dorsovegetal region (DV) induces the organizer (O) and the ventrovegetal region (VV) induces ventrally specified mesoderm (M3) in the marginal zone. Lateral mesodermal regions (M1 & M2) are formed as a result of the influence of the organizer, a process we call dorsalization.

DLMZ fragments. Whereas in the strongest cases all or most of the prospective mesoderm is present as muscle, in the weakest cases pronephros, mesothelium and blood cells are formed as well. Of course we do not know whether this represents a gradient in dorsalizing activity in all the cells or results from the inclusion of fewer dorsalizing cells in more lateral fragments. Our own model would favour the latter alternative.

The development of pronephros in combinations with lateral fragments demonstrates that dorsalization is not merely the induction of muscle. This is also shown in combinations between a stage-10 DMZ fragment and a ventral-half embryo of the same stage. Although the ventral half contains a large volume of muscle (mean = 53% of fate map), it also forms substantial amounts of mesothelium, blood and pronephric tubules (Table 6). So it is clear that dorsalization could act as a gradient-like mechanism for generating all the regional differences in the prospective mesoderm of the ventral and lateral marginal zone.

Discussion

The three-signal model

In previous publications (Slack & Forman, 1980; Smith & Slack, 1983; Slack et al. 1984; Dale et al. 1985; Smith et al. 1985) we have proposed that pattern formation in the early amphibian embryo depends on three inductive interactions: a dorsal- and a ventral-type mesoderm induction followed by a regionalization of the ventral mesoderm under the influence of a signal from the dorsal mesoderm, otherwise known as the organizer (Fig. 12C). This model has been contested explicitly or implicitly by various alternatives among which we may mention three in particular.

First, there is the model by which mesoderm arises because of cytoplasmic determinants localized in the egg and partitioned passively to blastomeres during cleavage. This is based on the ability of C tier blastomeres to form muscle in isolation or of nucleated egg fragments to do so when they contain some cytoplasm from this region (Gurdon, Mohun, Fairman & Brennan, 1985). However, recent fate
mapping studies on the 32-cell embryo (Cooke & Webber, 1985; Dale & Slack, 1987) show that a substantial amount of muscle normally arises from the B tier which does not form muscle in isolation. So at least part of the mesoderm must arise by induction in normal development. Indeed it remains possible that all of it arises by induction since C tier blastomeres normally contribute to both the mesoderm and the endoderm and will form substantial explants containing hundreds of cells by the onset of gastrulation. This makes it quite possible that interactions occur between inducing and competent cell populations well after the time of explantation.

Second, there is a model in which the mesoderm is considered only to consist of the axial structures (Gerhart, Vincent, Scharf, Black, Gimlich & Danilchik, 1984). This involves a dorsal mesoderm induction which results in the formation of the organizer, but considers the organizer only as an agent of neural induction and does not account for the formation or regionalization of the rest of the mesodermal mantle. Pure ventral inductions have been known since the study of Boterenbrood & Nieuwkoop (1973) and have been obtained recently by Dale et al. (1985) and Maufroid & Capuron (1985), so this model must be regarded as incomplete, although as far as it goes it is in accordance with our own.

Third, there is the view, most clearly articulated by Cooke (1985a,b), that the entire body plan, comprising not only different tissue types but also postgastrulation anterior–posterior ‘position values’, is set up as a system of cytoplasmic determinants in the vegetal hemisphere following fertilization. This is based on the near-mosaic behaviour of a proportion of isolated ventral halves at the 4-cell stage (Cooke & Webber, 1985). It implies a gradient of mesoderm-inducing signal each concentration capable of inducing a particular level in the anterior–posterior body plan. Although the reality of dorsalization in DMZ/VMZ combinations is not denied, their role in normal development is.

Finally there is a computer model of mesoderm induction by Weyer, Nieuwkoop & Lindenmayer (1978) but this addresses only the shape of the prospective mesoderm territory and not the different specifications within it.

The experiments in the present paper were designed to test further the three-signal model against these alternatives. First, we tried to find how many mesoderm-inducing signals there are by using as inductors single cells from the D tier of a 32-cell stage. This showed two things quite clearly: that the procedures used to isolate single cells do not destroy inductive ability and that only two inductive specificities could be distinguished. Of course, the actual induction probably occurs much later than the operation and by this time the D blastomere will have divided many times so we still cannot say that an isolated cell can signal, but simply that its capacity for later signalling is not impaired. The inductive specificities clearly differ between D1 (dorsal) and D2–4 (ventral or intermediate) but no clear difference was apparent between D2, 3 and 4. The inductions in this experiment were all rather small and we associate this, as have other workers, with the small volume of inducing tissue used. When four ventral blastomeres are used the size of the inductions produced is about four times greater than inductions produced by one blastomere. Significantly however, the increased size of such inductions is not associated with a shift towards a more dorsal character and this is in sharp contrast to the behaviour of the organizer (see discussion of ‘attenuation’ below).

Second, we attempted to find out the state of specification of the nonorganizer parts of the marginal zone by making various kinds of isolate. The overwhelming majority of isolates from the ventral half, whether half embryos or small pieces, gave extreme ventral types of differentiation up to and including stage 10. It was even found that a significant minority of the DLMZ class, taken from the dorsolateral region, gave extreme ventral types. Once again the ventral induction responsible for this specification may have occurred before, during or after the operation, the important point is that the specification of a large sector of the marginal zone is extreme ventral right up to the onset of gastrulation. It is thus much more ventral than the fate map (Fig. 12A,B) and it follows that information from the organizer must be required to promote this tissue to the level of intermediate structures such as pronephros or large muscle masses. This is clear evidence for the importance of dorsalization in normal development.

Third, we have mapped the dorsalizing activity of the organizer directly by making combinations of explants from different levels of the marginal zone. The previous experiments of this type were done before the technique of lineage label and relied on interspecies differences for cell identification (Slack & Forman, 1980). Now, however, we have been able to do this with *Xenopus* tissue alone and the results are very clear: dorsalizing activity is confined to a restricted region of the dorsal marginal zone, it is wider than the prospective notochord although seems to be graded from a high point at the dorsal midline.

We feel that the results of these experiments strengthen the three-signal model as the simplest explanation capable of accounting for regional specification in the embryo up to the time of gastrulation. There are clear operational differences between the
Table 7. Characteristics of the three inducing signals

<table>
<thead>
<tr>
<th>Location (in terms of cell positions at 32 cell stage)</th>
<th>DV inductor</th>
<th>VV inductor</th>
<th>Organizer</th>
</tr>
</thead>
<tbody>
<tr>
<td>DV induction W inductor Organizer</td>
<td>C1, D1</td>
<td>D2, D3, D4</td>
<td>B1, C1</td>
</tr>
<tr>
<td>Mode of formation</td>
<td>Determinant arising from postfertilization cytoplasmic shifts</td>
<td>Determinant arising during oogenesis?</td>
<td>Induction from ectoderm by DV inductor</td>
</tr>
<tr>
<td>Biological activities</td>
<td>Induction of dorsal type mesoderm. Rescue UV5 embryos</td>
<td>Induction of ventral type mesoderm</td>
<td>Induction of intermediate structures from ventral type mesoderm. Induction of neuroepithel from ectoderm</td>
</tr>
<tr>
<td>Time of action</td>
<td>Blastula</td>
<td>Blastula</td>
<td>Gastrula</td>
</tr>
<tr>
<td>Biochemical nature</td>
<td>XTC-MIF (Smith, 1987)?</td>
<td>Fibroblast Growth Factor (FGF)?</td>
<td>Heparin?</td>
</tr>
</tbody>
</table>

three processes as summarized in Table 7 and we feel that these must be taken seriously. In particular it should be noted that each process is mimicked by a different molecule. A factor from a Xenopus cell line induces notochord and muscle from ectoderm (Smith, 1987). Fibroblast growth factor induces extreme ventral structures from ectoderm (Slack, Darlington, Heath & Godsave, 1987). Heparin or heparan sulphate is preferentially synthesized in the marginal zone and has been shown to have dorsalizing effects on ventral mesoderm (Flickenger, 1980). These molecules may not be the true endogenous inducers but their effects do indicate that there are three distinct and separable classes of response.

Activation of early ventral blastomeres

Although the majority of ventral half embryos develop into extreme ventral types, there is a significant minority that form dorsal or intermediate types. Some of these probably arise from mismatches between pigmentation and the prospective dorsal side but the total number is probably too great to be accounted for in this way. In particular intermediates arising at the 4-cell stage make up over one third of the total and tend to contain pronephros and more highly organized somites than intermediates from later stages. Similar results but with even more intermediates were obtained at the 4- and 8-cell stages by Cooke & Webber (1985) and Kageura & Yamada (1983, 1984). Ventral isolates made prior to first cleavage form almost exclusively intermediate types (Render & Elinson, 1986). It is important to note that, although these authors tend to stress the near-mosaic character of these results, the cases actually form a continuum from the extreme ventral type up to those containing neural tubes, which must be induced by organizer-type mesoderm not normally found in the ventral half. So, early ventral halves can fall below, approximately equal, or even exceed the range of structures formed in normal development.

Significantly, Gimlich (1986) found that by the 16-cell stage ablation only of blastomeres D1 and C1 lead to 60% of extreme ventral forms.

This suggests that there may be important differences in the mesoderm formed by large ventral isolates at early and late cleavage stages and is supported by our own induction and isolation experiments. In ventrovegetal—animal pole combinations more muscle is induced at stage 6 than at stage 71. Similarly, whereas isolated lateral and ventral marginal zone fragments from midblastula embryos are invariably ventral in character containing very little muscle, many of those from stage-6 embryos develop into what we would call intermediate mesoderms (Nakamura, 1978; Dale & Slack, data not shown). We have presented experiments to show that this is an artefact of microsurgery on early stages and have done this by focusing on the apparent stabilization of ventral character in the ventral blastomeres between stage 6 (32 cells) and stage 71 (256 cells). First, we showed that stage-6 ventrovegetal blastomeres aged for three cell cycles in vitro have an inducing specificity like stage 6 rather than stage 71, i.e. more intermediates and fewer ventrals. Second, we showed that stage-6 ventrovegetal blastomeres aged for three cell cycles in vitro have an inducing specificity like stage 6 rather than stage 71, i.e. more intermediates and fewer ventrals. Second, we showed that stage-6 ventral halves reconstituted from isolated D34 and ABC34 fragments developed into more intermediate than ventral types, behaving more like the single-cell inductions than like ventral halves prepared with a single cut of the tungsten needle.

From this it appears that ventrovegetal blastomeres from early cleavage stages can have an intermediate-type inductive potency activated by operative procedures. Clearly such activation could also explain the development of intermediate mesoderms in ventral isolates, particularly at early stages, and the variability between studies would result from the different operative procedures between laboratories.
Association of inducing activities with other characters

UV5 rescue

It has been shown that embryos rendered radially symmetrical and extreme ventral by u.v. irradiation can be rescued towards a normal pattern by implantation of D1 or C1 blastomeres (Gimlich & Gerhart, 1984; Gimlich, 1986). D1 cells can also produce twins if grafted to the ventral side in place of D4 cells. It has been shown by lineage labelling that the D1 graft cells do not enter the axial structures, which must, therefore, be formed by induction. This phenomenon is obviously very similar to what we call dorsal mesoderm induction and it seems probable that the same signal is involved. In both cases the D2, 3 and 4 blastomeres are not active.

Neural induction

Although the original organizer graft (Spemann & Mangold, 1924) did reveal the dorsalizing property of the DMZ, attention quickly became focused on the later property of neural induction which occurs as this tissue becomes stretched out to form the archenteron roof. The designation of neural induction as primary embryonic induction in numerous textbooks probably did more than anything else in the long period between 1924 and 1969 to obscure the importance of interactions occurring before gastrulation. Recent work on the organizer graft (Smith & Slack, 1983) has shown that dorsalization of ventral mesoderm and neuralization of ectoderm are two distinct properties of the DMZ region. The spatial extent of the neuralizing tissue has not been mapped in *Xenopus* and, in fact, we are not aware of any study since that of Bautzmann (1926) on Triturus. This showed a zone around the dorsal lip extending perhaps 45° in each direction and this does seem remarkably similar to the extent of the dorsalizing region inferred from our present study. On the other hand, there is some evidence implicating heparin as a dorsalizing factor (Flickenger, 1980) but heparin has no neuralizing effect on isolated ectoderm, so even if the same region is responsible this does not necessarily mean that the two signals are chemically the same.

It is important to note that neither dorsalizing nor neuralizing activities are confined to the prospective notochord, but extend also into the prospective somite region. Thus it is possible to obtain, among DLMZ isolates, some cases containing muscle blocks and neuroepithelium but no notochord. Similarly, some of the activated ventral half embryos from the 4-cell stage contain neural tube but not notochord and Youn & Malacinski (1981) have reported u.v.-irradiated embryos with neural tubes but no notochord.

Attenuation of the organizer

The results from the marginal zone combinations show that different degrees of dorsalization are possible. DMZ–VMZ combinations form more muscle than DLMZ(dorsal)–VMZ and these form more muscle than DLMZ(intermediate)–VMZ. The latter class however contains more pronephros than either the strongly dorsalized or the control ventral cases. This suggests a continuum of outcomes related to the position of the inducer relative to the dorsal pole. Likewise, it is well known that u.v. irradiation yields a range of defects, from slight anterior reductions (UV1) to radially symmetrical extreme ventral types (UV5), which suggests a progressive weakening of the organizer secondary to a reduced formation of the DV inductor (Malacinski, Brothers & Chung, 1977; Scharf & Gerhart, 1980).

What does it mean if the organizer is weakened in this way? An analogy is the well-studied case of the polarizing region in the chick limb bud. Here a strong signal gives duplications up to the level of digit IV while weaker ones may achieve only digit III or II. Experiments in which the cell number in the graft is progressively reduced show that there is a critical cell number required for the attainment of each level (Tickle, 1981) although this number is small. Similarly the activity of an intact graft can be progressively reduced by graded doses of γ irradiation (Smith, Tickle & Wolpert, 1978) also consistent with a reduction of effective cell number. If we apply this idea to the organizer it would follow that u.v.-irradiated embryos have small organizers whose component cells are of normal potency, and that fragments of marginal zone with reduced dorsalizing activity relative to the DMZ have included a smaller number of organizer cells.

When we consider mesoderm induction we find that inducers of small volume produce smaller inductions than larger pieces, but retain the same dorsoventral character. For example, a single D1 blastomere will induce small amounts of notochord rather than normal amounts of intermediate tissue types. So, in this case, a weakening of the signal is associated only with a reduction of range. This provides further evidence for a qualitative difference between dorsal- and ventral-type mesoderm induction and for the difference between mesoderm induction and dorsalization by the organizer.

References


(Accepted 17 February 1987)