Occurrence of dorsal axis-inducing activity around the vegetal pole of an uncleaved *Xenopus* egg and displacement to the equatorial region by cortical rotation

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SUMMARY

Specification of the dorsoventral axis is a subject of great importance in amphibian embryogenesis. We have found that cytoplasm of the vegetal dorsal cells of a 16-cell embryo of *Xenopus laevis*, when injected into the ventral vegetal cells of a recipient at the same stage, can induce formation of a second axis. In the present experiments, using the same assay procedure, we found that the cytoplasm around the vegetal pole of an egg before cortical rotation is also active in inducing a second axis, that the activity decreases throughout the second half of the cell cycle and appears in a presumptive dorsal equatorial region at the 2- to 16-cell stages. This is the first demonstration of the localization of dorsal forming activity in any specific region of an egg. After UV irradiation, a treatment that is known to block cortical rotation and thereby inhibit axis specification, the activity remains near the vegetal pole beyond the first cell cycle and does not appear in an equatorial region, at least at the 16-cell stage. This suggests that cortical rotation or a related force is in some way involved in changes in distribution of the activity.

We also found that UV-irradiated 8-cell embryos can rescue dorsal development when they are cut into halves along the first cleavage plane. Histological examination revealed that the rescued embryos have a neural tube and notochord. In the half embryo, the animal and vegetal regions came into contact during wound healing, an event that enables the activity to localize in the new equator of an embryo. Therefore this rescue suggests that, if the activity is distributed only in the equatorial region, dorsal specification occurs. In fact, the dorsal side of the rescued embryos seems to correspond to the plane through which the embryos have been cut.

Based on our results, we propose (1) that a determinant that carries axis-inducing activity is first present around the vegetal pole, (2) that the determinant shifts from the vegetal pole to an equatorial region by or in close association with cortical rotation and (3) that occurrence of the determinant in the equatorial region is a prerequisite for axis specification.

Key words: *Xenopus laevis*, cortical rotation, dorsal axis determinant

INTRODUCTION

Specification of the dorsoventral axis is the earliest and most important step in amphibian embryogenesis. It is generally accepted that axis specification depends both on the presence of an axis-inducing determinant and on activation by sperm-mediated cortical rotation (Gerhart et al., 1989). Such a view is based mainly on the following observation: when an egg is UV-irradiated, cortical rotation is blocked and axis specification is also prevented. Tilting of such an irradiated egg, however, causes gravity-driven rotation and axis specification is rescued (Scharf and Gerhart, 1980). The gravity-driven rotation is thought to replace cortical rotation. A much lower dose of irradiation to an oocyte does not block the cortical rotation after fertilization but can effectively abolish axis specification. In this case, tilting is not effective in rescuing axis specification. One explanation is that the same determinant that is responsible for axis specification is localized near the surface of the cell at this stage and is destroyed by irradiation (Holwill et al., 1987; Elinson and Pasceri, 1989; Gerhart et al., 1989) However, little is known of the ‘determinant’ or molecule involved in the axis specification and its mode of action.

In previous work, we demonstrated that the cytoplasm of the dorsal, but not ventral, vegetal cells of the *Xenopus laevis* 16-cell embryo caused second axis formation, when injected into vegetal cells of a simultaneous recipient (Yuge et al., 1990). This extends transplantation experiments using a dorsal cell of an early embryo (Gimlich, 1986; Kageura, 1990) and substantiates the idea that cytoplasm of the presumptive dorsal cell of an early embryo contains a determinant that may be responsible for dorsoventral axis spec-
ification. Information concerning the nature and distribution of the determinant in an egg and an embryo is important for the identification of the determinant and for elucidation of mechanisms of axis specification.

The purpose of the present study was to locate the cytoplasmic axis-inducing activity in just fertilized eggs and early cleaving embryos. We found that the activity is present in the vegetal pole of an uncleaved egg and subsequently in a dorsal equatorial region of a 2- to 16-cell embryo, together with a decrease in activity around the vegetal pole. However, in UV-irradiated embryos in which cortical rotation was prevented, the activity remained near the vegetal pole beyond first cell cycle. It is likely that a determinant that is responsible for axis specification shifts from the vegetal pole to an equatorial region in normal eggs during cortical rotation. Furthermore, we obtained results that support the notion that occurrence of determinant in an equatorial region is a prerequisite for axis specification. UV-irradiated embryos can be rescued when cut into halves at the 8-cell stage, along the first cleavage plane. These results provide further insight into the nature and role of the axis-inducing activity in axis specification.

MATERIALS AND METHODS

Collection of eggs and embryos
Embryos of Xenopus laevis were obtained following natural mating. A female and a male were injected the previous evening with 300 IU and 200 IU, respectively, of human chorionic gonadotropin (Gonatropin, Teikoku Zoki Co.). The frogs were kept in a vat filled with well water containing 0.3% NaCl to prevent the jelly of embryos sticking to the wall of the vat. Embryos with jelly were washed thoroughly, the jelly was removed and the embryos were sterilized as described elsewhere (Yuge and Yamana, 1989). Procedures for fertilization were as described by Sakai (1990), and the fertilized eggs were not sterilized further.

We selected embryos whose first cleavage plane bisected the pigmented hemisphere of an egg, irrespective of whether the subsequent cleavage planes were typical or not. Embryos were staged according to Nieuwoop and Faber (1967).

Micropipettes for cytoplasm injection
Micropipettes for cytoplasmic injection were constructed and calibrated according to Gurdon (1974) from capillary tubes (for microhematocrits plain, Drummond), but with some modifications. The tips of micropipettes consist of two sections: a thicker section, about 130 µm in diameter, with calibration marks and a parallel wall for at least 20 mm, and a fine section, 5-6 mm long. The end of tip of 30-50 µm external diameter is cut at the right angles to the pipette for suction of surface cytoplasm. All were siliconized before use (Sigma, Sigma Chemical Co.). A simple manipulator (Narishige Co.) was used.

Cytoplasmic injection and subsequent development of recipients
Throughout the present study, 16-cell embryos were used as recipients for cytoplasmic injection. The ventral vegetal cells of a recipient were injected with 30 nl each per cell of cytoplasm (Yuge et al., 1990). In each series of experiments, donor and recipient embryos were obtained from the same batch of embryos.

Cytoplasm was found to leak from the animal pole of some recipients about one hour after injection of cytoplasm. These embryos were discarded. Most of the injected recipients developed normally until the gastrula stage, and then they became twins, that is, an embryo with a second axis. The twins were scored for second axial structures soon after stage 22. The second axes were assigned according to Cooke’s classification of degrees of completeness in second body plans (Cooke, 1989). Grades 1-5 show second plans of increasing completeness.

The embryos that died before gastrulation were classified as ‘dead’ and included embryos that survived several cleavages, those in which the cytoplasm leaked after gastrulation and those in which invagination was incomplete so that the endoderm remained outside after the neurula stage. These abnormalities seem to be caused by the cell death of the descendants of the injected blastomeres (Kageura, personal communication). Other abnormalities were classified as ‘abnormal’.

Ultraviolet irradiation
The vegetal hemisphere of eggs was irradiated by UV through a quartz slide using a short-wave (253.6 nm) Manasle lamp (Manasle Chem. Co.) within 25 minutes after insemination. Exposure times were determined empirically to give embryos that cleaved normally and gastrulated but which lacked the dorsal axial structure. The dorsal axial reductions were scored using the Dorsalanterior Index or DAI (Kao and Elinson, 1989) between stages 27 and 35. Embryos with no dorsal structure are DAI 0 and the completely normal embryos are DAI 5. When the average DAI value of UV-irradiated control embryos was higher than 0.2, data on experiments with this group of irradiated eggs were discarded.

Bisection of the UV-irradiated embryos
Fifteen minutes after the appearance of the cleavage furrow, the vitelline membrane of embryos of 8- to 256-cell stages was removed with a pair of forceps. An embryo was bisected by repeatedly flipping the first cleavage furrow with a nylon loop or forceps. During the bisecting, care was taken to obtain one of the halves intact. The half embryos were carefully placed in an agar well, and the medium was changed from 100% to 10% modified Steinberg solution (Sakai, 1990) during the next few cleavages. Between stages 27 and 35, the embryos developing from the bisected embryonic halves were scored and assigned a DAI.

We tried to label the side of bisection with ‘crystals’ of Nile Red according to Kirschner and Hara (1980) to examine the relationship between the bisection plane and the dorsal side of rescued embryos. If we applied the dye to the vitelline membrane and then removed the membrane before bisecting the embryo, the spot was too widespread. If we injected the dye solution into an isolated demembranated blastomere, the dye congealed the cytoplasm and then leaked out because the egg did not have a membrane.

For the above reasons, we used a video recorder. Some of the half embryos obtained by cutting irradiated embryos were placed in wells, with the wound in a recognized position, and recording was done with an inverted microscope and a time-lapse VTR recorder at a ratio of 20 seconds to 120 minutes real time. Some rescued embryos were fixed and processed for histological examination as described (Yuge et al., 1990).
RESULTS

The presence of axis-inducing activity around the vegetal pole in an uncleaved egg

The cytoplasm from the vegetal pole region of uncleaved eggs was injected into the ventral vegetal cells of a 16-cell recipient embryo (Fig. 1D), as described (Yuge et al., 1990). The results of seven independent series of experiments, carried out with four batches of eggs, showed that the cytoplasm around the vegetal pole had the same activity as did the cytoplasm of dorsal vegetal cells of a 16-cell embryo (Yuge et al., 1990). Of a total of 58 recipients, 42 (74%) developed into double embryos (Table 1). The developed second axes were scored and assigned according to Cooke’s classification of the degree of completeness in second body plans (Cooke, 1989) (Fig. 1C), the average grade of induced axes being 1.9. This is the first indication that the cytoplasm around the vegetal pole of an uncleaved egg is active in inducing a second axis.

The results obtained are presented in terms of the time of cytoplasmic withdrawal which is normalized to first cell cycle (Table 1). The percentage of second axis formation remains fairly constant throughout the cell cycle, while the average Cooke’s grade decreases in the second half of the cell cycle. At time 0.8 of the cell cycle, the grade is about 0.7 times that at 0.3. Once the cleavage begins (time 1.1), the percentage of second axis formation and the average grade of second axes decrease greatly.

The reverse is seen with the cytoplasm of a region opposite the SEP, i.e., dorsal subequatorial region (Table 1). In the early half of the first cell cycle, the cytoplasm is inactive in inducing a second axis; none of the recipients formed second axes. However, at 0.7, the dorsal subequatorial cytoplasm showed slight activity for the first time; one of 14 recipients becomes a double embryo with a small second axis of grade 2. At 0.9, 20% of the recipients developed into embryos with a second axis, though the second axes were not well-developed. In cleaving eggs at 1.1, the cytoplasm of the presumptive dorsal region was now distinctly active, with respect not only to percentage of the second axes but also to the Cooke’s grade of the second axes.

Increase in axis-forming activity in the future dorsal region during cleavage

The cytoplasm from a dorsal subequatorial region of 2- to 16-cell embryos was assayed as described above. The results of four series of independent experiments are compiled in Table 2. Compared with that of uncleaved and cleaving eggs, the dorsal cytoplasm of the 2-cell embryos became greatly active in inducing a second axis. Some cytoplasmic rearrangement probably occurs in the dorsal equatorial region during the first cleavage and may result in an increase in activity. After the 2-cell stage, not only the frequency of the second axis formation but also the average Cooke’s grades of second axes increased and the two reached maximum level around the 8-cell stage (Table 2). At the 8-cell stage, about 80% of the recipients developed into double embryos. Half were assigned Cooke’s grade 5 or 4 and the second body plans had a head with eye and a cement gland. The activity remained high at the 16-cell stage though the average grade is lower.

Distribution of axis-inducing activity in the 16-cell embryo

We assayed the cytoplasm from four different regions of the 16-cell embryo, and the results are shown in Table 3. The cytoplasm around the vegetal pole is no longer as active as in an uncleaved egg. The average Cooke’s grade is as low as 0.4. Neither the cytoplasm from the ventral region nor that from the dorsolateral region of a 16-cell embryo

![Fig. 1.](image)
is active; in none of the recipients was a second axis formed. The cytoplasm obtained from the supraequatorial region of the dorsalmost animal cells was also active, although the level of activity was lower as compared with the cytoplasm derived from vegetal counterparts. 75% of the recipients developed into double embryos of Cooke’s grade 1.8, a finding consistent with that of Hainski and Moody (1992). Although they detected no activity in the dorsalmost vegetal cell, cytoplasm obtained from the subequatorial region of the dorsalmost vegetal cells is very active and more than 80% of the recipients become double embryos of Cooke’s grade 2.4. This is consistent with the results of the blastomere transplantation experiment by Kageura (1990). Therefore, it seems that a great part of the activity is restricted to a broad equatorial region in the future dorsal side of the embryo, by the 16-cell stage, although some activity remained around the vegetal pole.

**UV-irradiation prevents changes in distribution of axis-inducing activity**

Eggs were UV-irradiated at the vegetal pole as described above and the cytoplasm was withdrawn from the vegetal pole.

### Table 1. Axis-inducing activity of the cytoplasm from vegetal pole and presumptive dorsal regions of uncleaved eggs

<table>
<thead>
<tr>
<th>Time</th>
<th>Total</th>
<th>Total (%)</th>
<th>Average</th>
<th>Cooke’s grades</th>
<th>Normal</th>
<th>Abnormal</th>
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<td></td>
<td></td>
<td></td>
<td>0</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

*Calculated by summing individual grades and dividing by the total number of individuals excluding abnormal and ‘dead’.
†Number of embryos with a second axis.
‡The first cleavage period is normalized to 1.0.
§Tail duplication.

### Table 2. Axis-inducing activity of the cytoplasm from the presumptive dorsal regions of 2- to 16-cell embryos

<table>
<thead>
<tr>
<th>2-cell</th>
<th>32</th>
<th>20(63)</th>
<th>1.8</th>
<th>0</th>
<th>6</th>
<th>6</th>
<th>4</th>
<th>3</th>
<th>0</th>
<th>4</th>
<th>10</th>
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<tbody>
<tr>
<td>4-cell</td>
<td>32</td>
<td>25(78)</td>
<td>2.8</td>
<td>9</td>
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<td>8</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8-cell</td>
<td>31</td>
<td>25(81)</td>
<td>3.1</td>
<td>6</td>
<td>7</td>
<td>7</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>16-cell</td>
<td>32</td>
<td>25(78)</td>
<td>2.2</td>
<td>4</td>
<td>5</td>
<td>7</td>
<td>1</td>
<td>8</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Calculated by summing individual grades and dividing by the total number of individuals excluding abnormal and ‘dead’.
†An embryo with two extra axes is included.
‡Tail duplication in embryos derived from a single batch.

### Table 3. Distribution of axis-inducing activity in a 16-cell embryo

<table>
<thead>
<tr>
<th>Cooke’s grades</th>
<th>Embryos with a second axis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Calculated by summing individual grades and dividing by the total number of individuals excluding abnormal and ‘dead’.
†An embryo with two extra axes is included.
‡Macrocephaly.
pole region at different times before and shortly after the first cleavage. Table 4 shows results obtained in five independent series of experiments with three different batches of eggs. In irradiated eggs, the activity persisted around the vegetal pole and was higher than in unirradiated eggs (see also Table 1). Indeed, 51 (91%) of 56 recipients developed into double embryos most of which were assigned Cooke’s grade 4 and 3, and the average grade was as high as 2.6. This high level of the activity was maintained even into the first cleavage (at 1.1); 80% of the recipients formed a second axes of grade 2.5, during which a marked decrease in activity occurred in unirradiated eggs.

At the 16-cell stage in irradiated embryos, the high level of activity remained in the cytoplasm around the vegetal pole (Table 5). Double embryos arose from more than 60% of the recipients and included second axes Cooke’s grades 5 and 4. This is in contrast to the previous observation that the activity of the cytoplasm around the vegetal pole had already become very low by this stage in unirradiated embryos (Table 3). In contrast, in irradiated embryos, the activity failed to appear and increase in the anti-SEP side equatorial region of embryo and none of the 75 recipients formed a second axes of grade 2.5, during which a marked decrease in activity occurred in unirradiated eggs.

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Thus, it has been shown that UV-irradiation prevents not only the decrease in activity during the first cleavage, but also the increase in activity in an equatorial region after the first cleavage. Figure 2 shows the changes in distribution of activity between the egg and the 16-cell embryo. It is likely that the decrease and increase in activity reflects some shift of activity and that cortical rotation is involved in this shift of activity.
Rescue of irradiated embryos by bisection

We studied the development of embryonic halves obtained by cutting irradiated 8- to 256-cell-stage embryos along the first cleavage plane. The embryos that developed were assigned DAI grades (Kao and Elinson, 1989). The results of two to three independent experiments are shown in Table 6. A total of 46 embryos cut at the 8-cell stage were heterogeneous in response to bisection so that they varied from DAI grades 1 to 5 (Fig. 3D, E), the average being 3.3. This value is high when compared with the grade of 0.2 of the control, and indicates that a substantial rescue had occurred. The rescue is more impressive in Fig. 3E, where 10 embryos developed from the bisected embryonic halves, all became elongated and six have one or two eyes (unirradiated, nonbisected control at the left top, and irradiated, non-bisected control at the right top). Histological examination revealed that most embryos contain a neural tube, notochord and somitic tissue (Fig. 3F), although arrangements of these tissues are often not normal.

After bisection, we visualized the whole process of subsequent development of some half embryos, using a time-lapse video equipped with an inverted microscope. The purpose of this was to examine the geographical relationship between the dorsal side of an developing embryo and the plane through which the embryo had been cut. In all eight cases examined, the developmental processes were similar with no rotation of the half embryos, at least before and during gastrulation. The half embryos were hemispherical immediately after being bisected. When embryos were cut at the 8-cell stage, the wound completely closed within two or three cleavages after bisection, then the embryos became spherical. Cells around the wound converged toward the center of the wound as it closed. Most of these cells were pigmented and therefore had derived from the animal hemisphere. As in irradiated embryos (Scharf and Gerhart, 1980), in irradiated embryonic halves the appearance of the dorsal lip of the blastopore is delayed and invagination occurs abruptly at many points of a circle at roughly the time that formation of a circular blastopore in the gastrulae is complete. The position of the midline of the neural folds was later found to coincide roughly with the cut side. This process was also photographed using an inverted microscope and the same was observed (Fig. 3A-C).

Bisection was also carried out with further developed embryos derived from irradiated eggs. As shown in Table 6, the degree of rescue was maximum when bisection was done at the 8-cell stage, and the later that the embryos were bisected, the less was the degree of rescue. At 128- and 256-cell stages, bisection had little rescuing effect.

DISCUSSION

Axis-inducing activity is located around the vegetal pole in an egg before cortical rotation

We found that the cytoplasm around the vegetal pole of the uncleaved egg is highly active in inducing the second axis, when injected into ventral vegetal cells of a 16-cell embryo. Takasaki and co-workers have recently confirmed our results (the 25th Annual Meeting of the Japanese Society of Developmental Biologists, 1992).

Although it is presumed that the UV-sensitive determinant is responsible for dorsal axis specification and is localized on the vegetal surface in the oocyte (Holwill et al., 1987; Elinson and Pasceri, 1989; Gerhart et al., 1989), there is no direct evidence for existence of activity that induces a second axis in an egg before cortical rotation. This is the first demonstration of the localization of dorsal forming activity in any specific region of an egg.

Cortical rotation shifts a determinant from the vegetal pole to the dorsal equatorial region

It has generally been assumed that the activity appears in a presumptive dorsal region of an embryo as a consequence of some activation by cortical rotation (Gerhart et al., 1989). We have shown that axis-inducing activity is detected around the vegetal pole of the egg before the egg undergoes cortical rotation, the activity decreases during the rotation and appears in the dorsal equatorial region of an embryo (Table 1); that is, the total activity was almost constant and only the distribution changed. Such change of activity was prevented in UV-irradiated embryos; the activity remained around the vegetal pole beyond the first cell cycle (Table 4) and at least to the 16-cell stage (Table 5). This strongly suggests that changes in distribution of the activity are caused by a shift of the determinant from the vegetal pole to the equatorial region. In other words, ‘activation by cortical rotation’ is accomplished by transportation of the determinant. The simultaneous occurrence of cortical rotation and regional changes of the activity sup-

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Table 6. Rescue of UV-irradiated embryos by bisection between 8- and 256-cell stages

<table>
<thead>
<tr>
<th>Total</th>
<th>Average</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tr>
<td>8-cell</td>
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<td>3.3</td>
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<td>2</td>
<td>12</td>
<td>7</td>
<td>9</td>
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<td>2*</td>
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<td>16-cell</td>
<td>29</td>
<td>2.4</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td>11</td>
<td>2</td>
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<td>0</td>
</tr>
<tr>
<td>32-cell</td>
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<td>0</td>
<td>7*</td>
</tr>
<tr>
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<td>1.6</td>
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<td>7</td>
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<td>3</td>
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<tr>
<td>256-cell</td>
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<td>21</td>
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<td>Control†</td>
<td>201</td>
<td>0.2</td>
<td>167</td>
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<td>5</td>
<td>0</td>
<td>0</td>
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</table>

†UV-irradiated, but not bisected.

*Incomplete invagination.
ports the above suggestion. Although extensive studies have been carried out on the movement of cellular organelles and motor proteins as well as cytoskeleton during cortical rotation (Houliston and Elinson, 1991), further information is necessary to elucidate mechanisms of transport, because a 30° displacement of the subcortical cytoplasm relative to the egg cortex during cortical rotation seems insufficient for localization of the determinant in an equatorial region.

Occurrence of the activity in the equatorial region is required for axis specification

The irradiated eggs fail to specify the dorsoventral axis, irrespective of the presence of a high level of activity around the vegetal pole. In unirradiated eggs, the activity disappeared from the vegetal pole of an egg and appears in the equatorial region of an embryo. Thus, it is likely that the determinant in the equatorial region, but not in the vegetal pole, is responsible for axis specification. The importance of determinant in the equatorial region is supported by our bisection experiments with irradiated embryos (Table 6), because the observed rescue can be explained by the localization of determinant from the vegetal pole into the equatorial region during wound healing. From our video recordings, the dorsal side of rescued embryos was found to coincide with the plane through which embryos had been cut. We tentatively conclude that the determinant required for dorsoventral axis specification appears first around the vegetal pole and then shifts to the equatorial region by or at least in connection with cortical rotation and becomes involved in the axis specification.

Identity of the present axis-inducing activity

Recently, many gene products have been identified that may be responsible for axis specification. Among them, a
member of the Wnt family has been hypothesized to be activated first in a dorsal region of the egg by cortical rotation (Kimelman et al., 1992). A member of the family, Xwnt-8, is probably not the endogenous dorsal-inducing molecule in the cleavage-stage embryo, because it is not expressed in the dorsal region of an early embryo (Sokol et al., 1991; Christian et al., 1992). However, it is likely that another member of Wnt family plays an important role in specifying the dorsoventral axis. Therefore, it is possible that the dorsal-inducing activity is mediated by it, although much more work has to be done to address this question.

There is another line of investigation that is worth referring to here. Hainski and Moody (1992) demonstrated that RNA isolated from animal dorsal cells (D1.1) of 16-cell embryos can induce a second axis in normal embryos and rescue the dorsal development in UV-irradiated embryos. We also found that the cytoplasm of animal dorsal cells, as well as vegetal dorsal cells, of the 16-cell embryo causes second axis formation. If activity in the cytoplasm depends on RNAs contained in these dorsal cells, not only RNAs from animal dorsal cells but also those from vegetal dorsal cells should be active in inducing a second axis. However, Hainski and Moody (1992) report that the RNA from vegetal dorsal cells (D2.1) has no activity in eliciting a second axis. Therefore, it seems unlikely that the activity that we describe in the present study is attributable to the RNA contained in dorsal vegetal cells.

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