The vegetal determinants required for the Spemann organizer move equatorially during the first cell cycle

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SUMMARY

Embryos with no dorsal axis were obtained when more than 15% of the egg surface was deleted from the vegetal pole of the early 1-cell embryo of *Xenopus laevis*. The timing of the deletion in the first cell cycle was critical: dorsal-deficient embryos were obtained when the deletion began before time 0.5 (50% of the first cell cycle) whereas normal dorsal axis usually formed when the deletion was done later than time 0.8. The axis deficiency could be restored by lithium treatment and the injection of vegetal but not animal cytoplasm. Bisection of the embryo at the 2-cell stage, which is known to restore the dorsal structures in the UV-ventralized embryos, had no effect on the vegetal-deleted embryos. These results show clearly that, in *Xenopus*, (1) the dorsal determinants (DDs) localized in the vegetal pole region at the onset of development are necessary for dorsal axis development and (2) the DDs move from the vegetal pole to a subequatorial region where they are incorporated into gastrulating cells to form the future organizing center. A model for the early axis formation process in *Xenopus* is proposed.

Key words: Xenopus laevis, dorsal determinants, cortical rotation, ligation, cytoplasmic injection

INTRODUCTION

The dorsoventral axis of the *Xenopus* embryo is thought to be specified during the cortical rotation period (Vincent et al., 1986; Vincent and Gerhart, 1987; Gerhart et al., 1989). However, both the location of the determinants and the temporal events that lead to the formation of the dorsal axis remain unclear. It has been suggested that dorsal inducing activity is localized in the vegetal poles at the early 1-cell stage, because the injection of cytoplasm from these vegetal poles into the ventral subequatorial region of recipient embryos results in the formation of a secondary dorsal axis (Fujisue et al., 1993; Holowacz and Elinson, 1993). After the first cleavage, this activity disappears from the vegetal region and reappears in the dorsal region, without any dorsal inducing activity being detected in the ventral subequatorial region at any stage (Yuge et al., 1990; Fujisue et al., 1993). However, it is difficult to judge the relevance of these experiments for dorsal axis formation during normal development.

A more direct approach would be to delete the vegetal region and study the pattern formed in its absence. In this respect, it has been shown that deletions of the vegetal cytoplasm do not interfere with α-actin expression (characteristic of the dorsal mesoderm; Gurdon et al., 1985). This appears to contradict the idea of vegetally localized dorsal determinants. However, their experimental procedure did not allow normal early cleavages, as normal events in development such as gastrulation and dorsal axis formation were not reported.

Thus, we decided to repeat the deletion experiment under milder conditions so as to allow normal early development of the experimental embryos. Surprisingly, the vegetal-deleted embryos lacked the entire dorsal axis, which is contrary to the results of Gurdon et al. (1985). Externally, there were no somites and muscle tissue could not be detected in the histological sections.

MATERIALS AND METHODS

Deletion of cytoplasm

Xenopus eggs were inseminated in 10% modified Steinberg's solution (MS: 100% MS consists of 58.2 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca(NO_3)_2$, 0.83 mM $MgSO_4$ and 3.0 mM Hepes-NaOH, pH 7.4). After 15-20 minutes in diluted sperm suspension, eggs were dejellied using 1% sodium thioglycolate, pH 9-10 and rinsed with 10% MS. The vitelline membrane was removed with fine forceps. All operations were done on an agar-coated plastic dish containing 10% Steinberg's solution (50 µg/ml Gentamycin, supplemented with Hepes to 3.0 mM).

The denuded egg was inclined 90° off its vertical axis. A glass rod (diameter 0.4 mm, length 2 cm) was coated with 0.5% agarose (Type IX Ultra-low-gelling-temperature, Sigma) and then air dried. The glass rod was placed on the egg so as to divide it into animal and vegetal fragments (vegetal deletion) or into a sperm entrance point (SEP) side and an anti-SEP side fragments (vertical deletion). The glass rod pressed the egg down as a result of it's intrinsic weight (Fig. 1A,D). After complete separation, both fragments maintained a spindle shape for a while. The lengths of their long and short axes were measured using a micrometer attached to the eye piece of a microscope. The surface area of the fragments was calculated
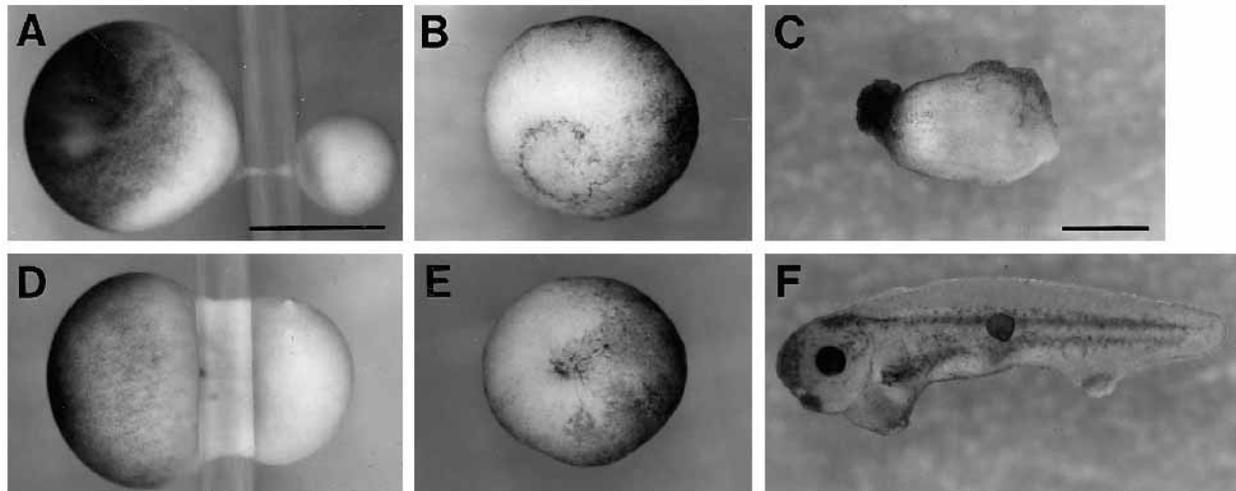
Fig. 1. Development of an early vegetal-deleted fragment (deletion started at time 0.30, A-C) and a late-vegetal-deleted fragment (D-F, time 0.83). (A,D) Just after placing a glass rod. (B,E) Vegetal view at gastrulation. (C,F) At the tadpole stage. Bar in A for A, B, D and E and bar in C for C and F: 1 mm.

assuming that they were spheroids. The degree of deletion was evaluated as being the ratio of the surface area of the deleted fragment relative to the total surface area of the two bisected fragments. After complete separation, nucleus-containing animal or SEP side fragments were placed into round-bottomed wells in 2% agar.

Bisection of the embryo at the 2-cell stage

Demembranated UV-irradiated embryos (Scharf and Gerhart, 1983) and early-vegetal-deleted embryos were placed on a flat layer of agar. When the first cleavage furrow progressed and the embryo took a dumbbell shape (Fig. 2A), each of the blastomeres was gently shifted on the agar into two adjacent wells (Fig. 2B). The two blastomeres separated gradually as gravity pulled them in opposite directions (Fig. 2C).

Lithium treatment

When the early vegetal-deleted embryos reached the 32-cell stage, they were treated with 0.3 M LiCl for 6 minutes. They were then washed thoroughly with 10% MS and were allowed to develop in an agar well until the controls reached stage 35.
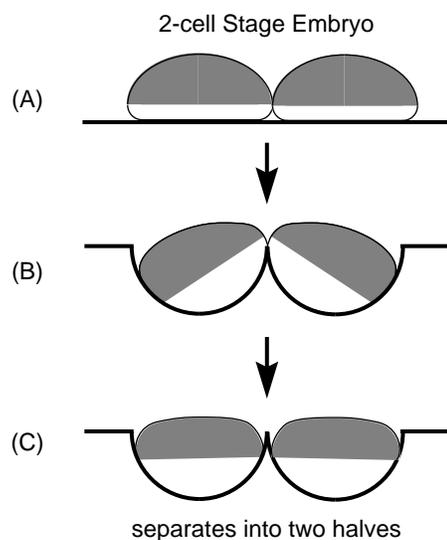

2-cell Stage Embryo

(A)

(B)

(C)

separates into two halves

Fig. 2. Diagrams showing the method of bisection.

Injection of the cytoplasm

The vegetal deletion (20-40% of egg surface) was carried out at a time before 0.5. The deleted egg (only those having completed separation before time 1.0) was injected with cytoplasm (15 to 50 nl) from vegetal or animal fragments following the methods of Fujisue et al. (1993).

RESULTS

Deletion of the vegetal but not the lateral cytoplasm during the early first cell cycle results in the entire loss of dorsal structures

The deletion of the cytoplasm from the vegetal region of the egg before time 0.5 (early-vegetal-deletion) resulted in the loss of dorsal structures (Fig. 1A-C). The early-vegetal-deleted embryos were scored at stage 35 using the Dorsoanterior Index (DAI) (Kao and Elinson, 1988). The loss of dorsal structures was exaggerated in relation to the amount of deleted cytoplasm (Fig. 3). For complete loss (DAI 0), the deletion of 15% of the surface area was sufficient. This surface area corresponds to 6% of the egg volume. It should be noted that the active cytoplasm may be confined to an even smaller part of the embryo since, in one extreme case, the deletion of 7% of the egg surface (2% of the egg volume) yielded entire axis deficiency (Fig. 3). This was specific for vegetal deletions since lateral (vertical) deletions did not cause dorsal deficiency (Fig. 3).

Although the results were mainly derived from examination of the external morphology of the experimental embryos, some were fixed, sectioned and examined histologically (Fig. 4). None of the DAI 0 embryos had any muscle while the DAI 1-5 embryos had striated muscles.

Some of the early-vegetal-deleted embryos were stained in the vegetal pole with a Nile blue spot pattern (Vincent et al., 1986) and the pattern movement was examined. The pattern moved as in the normal embryos (Fig. 5) suggesting that the dorsal defficiency is not due to the absence of the cortical rotation.
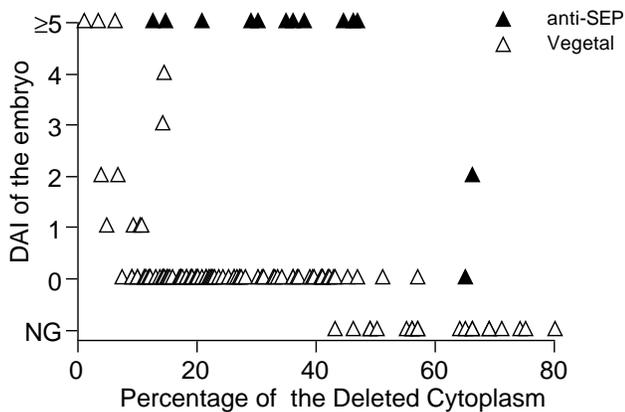
Fig. 3. Effect of early-vegetal and early-vertical (anti-SEP) deletion. Data from individual embryos (DAI of the embryo) are plotted against the deleted area (% of the whole surface). Data from embryos deleted before time 0.5 (start of deletion) are shown. Embryos which completed separation after the first cleavage (time 1.0) were omitted. Open triangles: vegetal deletion. Black triangles: vertical (anti-SEP) deletion. NG denotes the embryos that did not gastrulate, while DAI 0 denotes embryos that did gastrulate.

Deletion of the vegetal cytoplasm does not interfere with gastrulation movement

The vegetal-deleted embryo always formed a blastopore if the deleted part did not exceed 40% of the whole embryo (Figs 1B,E, 3). The size of the blastopore became smaller as more of the embryo was deleted. In extreme cases, invagination occurred from one point and the blastopore looked like a pit (Fig. 1E). In these cases, invaginating cells (bottle cells) appeared to occupy a position close to the vegetal pole, suggesting that, for the formation of the blastopore or bottle cells, most of the vegetal region is unnecessary. The pit-like invagination was observed in both early- and late-vegetal-deletions. This suggests that, just after fertilization, some of the factors necessary for gastrulation might be already localized in the subequatorial circular zone.

Normalized time	Grade of the embryo						
	0	1	2	3	4	≥5	Average
0.2-0.3	7	0	0	0	0	0	0.0
0.3-0.4	22	0	0	0	0	1	0.2
0.4-0.5	20	0	0	0	0	0	0.0
0.5-0.6	11	0	0	2	0	0	0.5
0.6-0.7	12	2	0	0	1	0	0.4
0.7-0.8	2	0	2	1	1	2	2.6
0.8-0.9	4	1	5	2	0	19	3.6
0.9-1.0	1	0	2	2	1	22	4.4

The timing of the deletion in the first cell cycle is critical

We examined the stage dependence of the vegetal-deletion (Table 1). Embryos were staged on a normalized time scale in which fertilization is taken to be time 0.0 and the appearance of the first cleavage furrow in control eggs is taken to be time 1.0. Embryos were classified as to the time of the start of the deletion (time of placing the glass rod), since the cytoplasmic bridge connecting the two fragments becomes very thin soon after placing the rod. Complete separation took 15-40 minutes. Only those embryos that were deleted for 20 to 40% of the vegetal cytoplasm are indicated. In Table 1, the embryos that completed separations after the first cleavage were *not* omitted since most of the late-deleted embryos completed separation after this stage.

As shown in Table 1, the later the onset of the deletion in the cell cycle, the less sensitive the embryos are to loss of the dorsal axis as measured in terms of the DAI. The sensitive period ends at time 0.8. This is similar to the period observed in the data from UV-, cold- and pressure-treatment experiments (Scharf and Gerhart, 1983). The late-deleted embryos, e.g., embryos deleted after time 0.8, developed fairly normally with respect to their dorsal axial structures (an example is shown in Fig. 1F). These results strongly suggest that some factors necessary for dorsal axis formation (dorsal determinants, DDs) must move from the vegetal pole to the subequatorial or blastopore-forming region before the complete sepa-
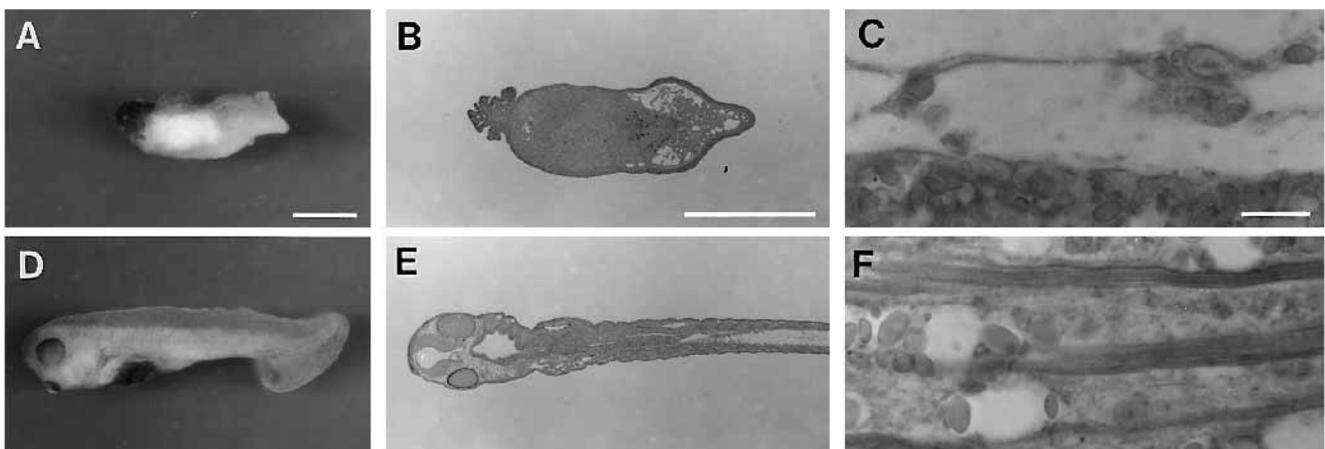

Fig. 4. Histological sections of an early-deleted embryo and a late-deleted embryo. (A-C) An early-deleted embryo deleted 20% of the egg surface at time 0.30. This is the same embryo as shown in Fig. 5. (D-F) A late-deleted embryo deleted 35% of the egg surface at time 0.86. Note striated muscles in F, which was not observed in the early-deleted embryos (see for example, C). Bar in A for A and D: 1 mm. Bar in B for B and E: 1 mm. Bar in C for C and F: 10 μm.
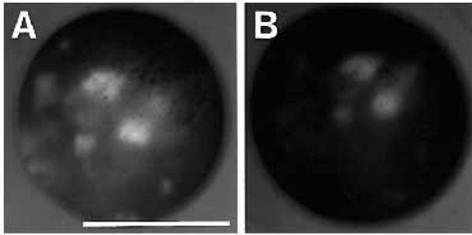
Fig. 5. Cortical rotation in a vegetal-deleted embryo. At time 0.30, the vegetal pole (20% of the egg surface) was deleted. The embryo was then stained with small pieces of Nile red crystal (Kirschner and Hara, 1980) and loaded with minute carbon particles (Norit, 'SX Plus'). The movement of the Nile blue spots were photographed with a Nikon inverted epifluorescence microscope equipped with the filter sets for rhodamine. (A) At time 0.58. (B) At time 1.03. Note the movement of the Nile blue spot relative to the egg surface, which is indicated by the carbon particles (three black dots can be seen in both panels). In this case, the angular distance traveled by the dye spots was 12°. Bar: 1 mm.

ration. The exact timing of the transportation event that leads to the transfer of the necessary amount of DDs is difficult to determine since it took 15 to 40 minutes for complete separation of the embryos. However, separation was almost always complete before the second cleavage even when the deletion started between time 0.9 to 1.0. About 80% of these embryos formed a complete dorsal axis (Table 1). Thus, the DDs sufficient for total axis formation in the vegetal-deleted embryos must be transported before the second cleavage.

Bisection at the 2-cell stage does not restore dorsal structures

We next tested two treatments that are known to restore dorsal structures in UV-ventralized embryos: bisection of the embryo at the 2-cell stage (Fujisue et al., 1993) and lithium-treatment (Kao et al., 1986).

In the UV-irradiated (UV'd) embryo, it has been proposed that the dorsal determinants do not move to the subequatorial region (Fujisue et al., 1993) because cortical rotation is impaired (Scharf and Gerhart, 1983). When the UV'd embryos are bisected before the 8-cell stage, one half of the embryo formed a dorsal axis (Fujisue et al., 1993). This could be caused by mixing of the vegetal and the subequatorial cytoplasm (Fujisue et al., 1993), although wounding as a cause of dorsal development (Gerhart, 1980) could not be excluded.

When the early-vegetal-deleted embryos were bisected along the first cleavage plane, none of the half embryos (*n*=23) formed axial structures (Fig. 6A, Table 2) while the UV'd embryos (Fig. 6B) bisected in our hands (*n*=10) always formed dorsal structures (Fig. 6C, Table 2). These results would appear

Table 2. Effect of bisection on the UV-ventralized and vegetal-deleted embryos

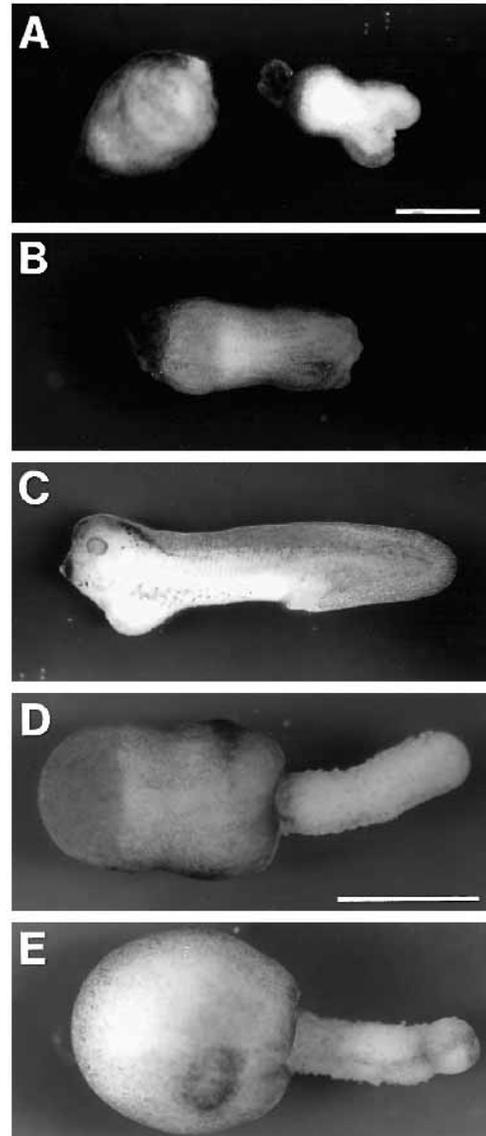| Experiment	Grade of the embryo						
	0	1	2	3	4	≥5	Average
UV	34	1	0	0	0	1	0.2
UV+Bisection	0	0	3	3	4	0	3.1
V-Deletion	34	0	0	0	0	0	0.0
V-Deletion+Bisection	23	0	0	0	0	0	0.0

Fig. 6. Lithium treatment and bisection at the first cleavage. (A) Two examples of bisected early-vegetal-deleted embryos at control stage 35. No axis (DAI 0). (B) An UV-ventralized embryo (DAI 0). (C) An UV-bisected embryo, which has two small eyes and a sucker (DAI 4). (D) An example of a hyperdorzalized early-vegetal-deleted embryo obtained by lithium treatment. (E) A lithium-treated whole embryo. Bar in A for A-C and bar in D for D and E: 1 mm.

to rule out the possibility that wounding by itself causes dorsal axis formation. It seems likely that the UV'd embryos retain DDs in the vegetal pole region whereas our early-vegetal-deleted embryos entirely lacked DDs.

Lithium dorsalizes the early-vegetal-deleted embryos

Lithium is known to respecify the ventral mesoderm so that it differentiates into the Spemann organizer (Kao et al., 1986; Kao and Elinson, 1988, 1989). All of the lithium-treated early-vegetal-deleted embryos (*n*=9) made proboscis (Fig. 6D), as did the whole embryos treated with lithium (Fig. 6E). This
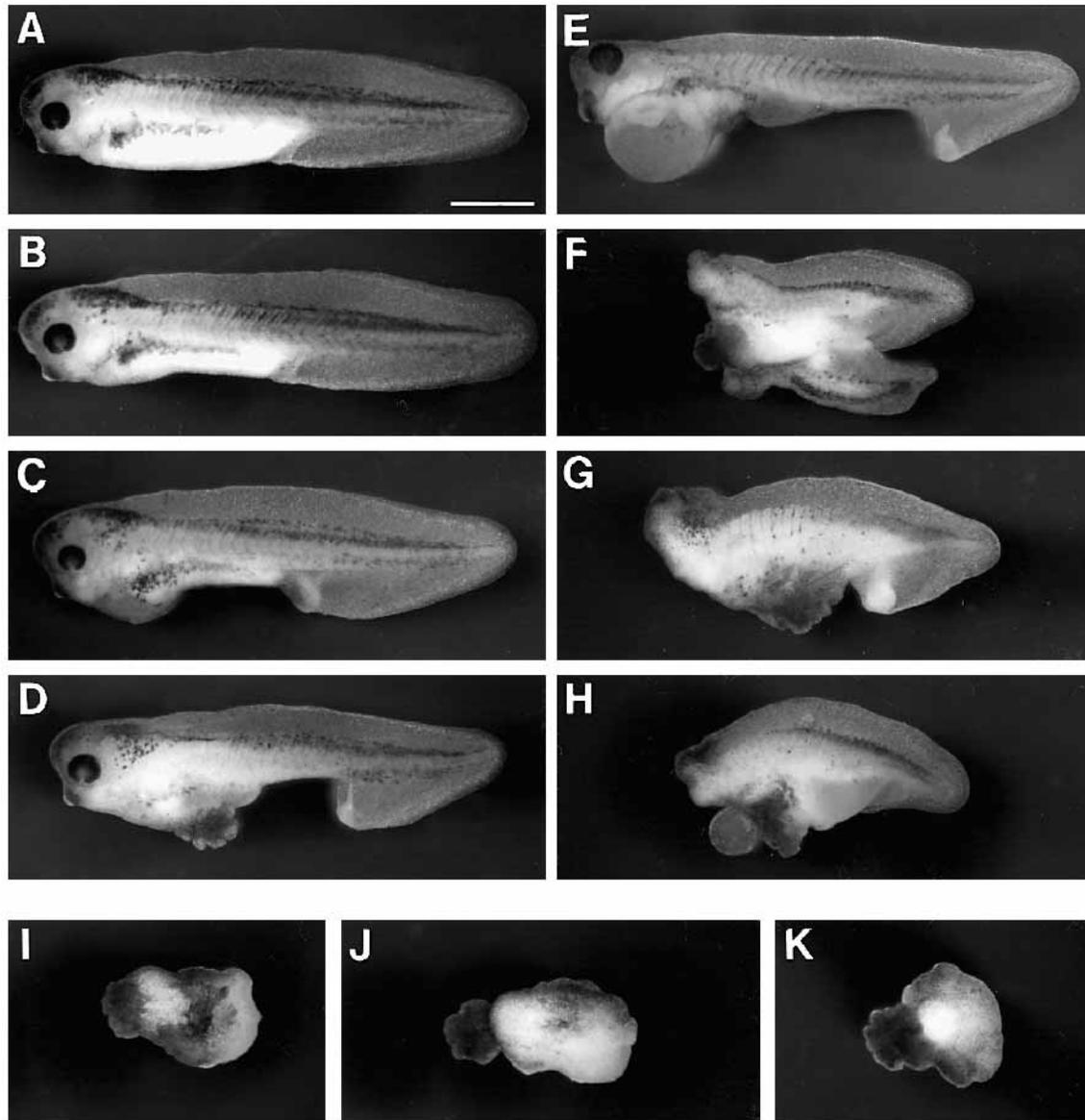
Fig. 7. Injection of vegetal cytoplasm restores dorsal structure. All embryos of one experimental series are shown. (A) A control embryo. (B-H) Vegetal-deleted embryos injected with vegetal pole cytoplasm. The DAI was 5, 5, 5, 5, 2, 2, 2 respectively. (I-K) Vegetal-deleted embryos injected with animal cytoplasm. All have no axis (DAI 0). Bar: 1 mm.

indicates that lithium dorsalizes the vegetal-deleted embryos even though they have no DDs.

Injection of the vegetal but not the animal cytoplasm restores the dorsal structures in the early-vegetal-deleted embryos

The results described above strongly suggest that axis deficiency is due to the lack of the DDs in the early-vegetal-deleted embryos. If this was the case, then injection of the vegetal cytoplasm into the vegetal-deleted egg should restore the dorsal axis.

As shown above, selected early-vegetal-deleted embryos (20-40% of the egg surface deleted by a deletion that started before 0.5 and ended before 1.0) *always* cleaved normally, gastrulated and *never* formed a dorsal axis (see Fig. 3). Injection

of the vegetal cytoplasm restored more or less the dorsal structures (Fig. 7B-H) in 85% (51 out of 60) of the embryos (Table 3). Among these, 18 embryos showed the entire dorsal structures including a sucker and two normal-sized eyes (Fig. 7B-E, Table 3). In the control experiment, the injection of cytoplasm from animal fragments had no effect on axis formation (*n*=18, Fig. 7I-K, Table 3).

Table 3. Injection of vegetal but not animal cytoplasm restores the dorsal axis

Injected cytoplasm	Grade of the embryo						
	0	1	2	3	4	≥5	Average
Animal	18	0	0	0	0	0	0.0
Vegetal	9	4	19	6	4	18	2.8

DISCUSSION

Presence of the dorsal determinants in the vegetal pole

Previously, it has been shown that deletions of the dorsal half (50%) of the early cleavage stage of *Xenopus* embryos do not always result in the loss of dorsal structures (Kageura and Yamana, 1983; Dale and Slack, 1987). In the present study, the deletion of the vegetal cytoplasm completely blocked dorsal axis formation without blocking cortical rotation. This block could be removed by the injection of the small amount of vegetal cytoplasm. Thus, we demonstrate for the first time that the determinants needed for dorsal axis formation in *Xenopus* (and by inference in vertebrates as well) are confined to a very small vegetal region (2% in volume) of the newly fertilized egg.

We show that just after fertilization the dorsal determinants are first of all localized in the *vegetal* region and not in the *future dorsal* region. Afterwards the determinants move to the dorsal subequatorial region. This is the first demonstration that a region of cytoplasm can contain the determinants for certain embryonic structures without actually taking part in or acting as the site for the formation of these structures.

Rather than activating dorsal development, cortical rotation transports the vegetal determinants to the dorsal subequatorial region

At present, there are two opposing ideas concerning the first step of axis formation process in *Xenopus*. (1) The dorsal determinants are localized in the vegetal pole at the onset of development and then move to the future dorsal subequatorial region of the embryo (Fujisue et al., 1993; Holowacz and Elinson, 1993). (2) There are no dorsal determinants. The cortical rotation during the first cell cycle provides a spatial cue that locally activates a region in the egg. This elicits new cues that function in later steps of the axis specification process (Black and Gerhart, 1986).

Black and Gerhart (1986) have argued that the dorsal axis is not specified by the movement of the dorsal determinants during the rotation period but that cytoplasmic displacement acts as a spatial cue that triggers or influences the next step in the axis specification process. According to them, if localization of the axis-specifying molecules was really the cause of dorsal development, then it would be reasonable to speculate that twins form because the first centrifugation forces all the axis-specifying molecules to one side (since a single axis will form at that side if a second displacement is omitted), and then gravity or secondary centrifugation forces one half to the opposite side. They believed this to be unlikely because there is no reason why all the determinants should move during the first displacement, when only half move during the second displacement.

The present deletion-injection experiment showed that only 15 nl of the vegetal cytoplasm (1.5% of the whole egg) is required to restore all the dorsal axis (Fig. 7, Table 3). It seems unlikely that all the dorsal determinants were transplanted when the cytoplasm was sucked out. Rather, the dorsal determinants in the vegetal pole are probably excessive for the formation of one complete axis. The double centrifugation experiment described by Black and Gerhart (1986) may displace the dorsal determinants (or, more likely, displace the

cytoplasm needed for gastrulation) in two different directions. As a result, the dorsal determinants and the gastrulating cytoplasm must meet at the two opposite sides of the embryo where the organizer later forms.

The bisected early-vegetal-deleted embryos did not form any dorsal structures while the bisected UV'd embryos made substantial amounts of dorsal structures. These results exclude the possibility that the dorsal structures made by the bisection of the UV'd embryos are caused by wounding. We conclude that the most vegetal position of the UV'd embryos have DDs while the early-vegetal-deleted embryos lack DDs.

Comparison to a previous deletion study

The cytoplasmic deletion study using precleavage *Xenopus* embryos, carried out by Gurdon's group in 1985 (Gurdon et al., 1985), has lent credence to the notion that dorsal determinants do not exist. Their conclusion was that all the components required for the eventual activation of muscle-specific actin genes (a dorsal marker) are already localized in the subequatorial region of the precleavage embryo. This means that, for dorsal development, the vegetal region of the embryo is unnecessary.

Our results are contradictory to Gurdon et al. (1985). The early-vegetal-deleted embryos made no dorsal structures; all of them resembled the UV-irradiated ventralized embryos. On the contrary, the vertically deleted embryos (bisected in a plane that is parallel to the animal-vegetal axis) developed normally whereas according to Gurdon et al. (1985), this type of deletion resulted in the absence of α-actin expression in half of the operated embryos.

We think that there are two problems in Gurdon et al. (1985) experiments. First, their deletion was carried out between 30 and 60 minutes after fertilization (30%-60% of the length of the first cell cycle) and the results were not presented with regard to the time of deletion. This could have led to an inaccurate interpretation, as the present study shows that the dorsal determinants move from the vegetal to the subequatorial region during this period. Secondly, it seems unlikely that the conditions for rearing embryos were suitable for the study of the early development of *Xenopus* embryos. High salt medium was used and the embryos were reared on a flat agar bed. In our experience, these conditions inhibit the compaction of the early cleavage stage embryos of *Xenopus*. In fact, their embryos seemed to have formed a cell sheet rather than a normal morula; normal gastrulation and morphogenesis were not reported even when the deletion was vertical. In the present experiment, the procedures used were milder since the vertically deleted embryos always cleaved normally and developed into normal tadpoles. Thus, the total lack of dorsal structure in the present early-vegetal-deleted embryos cannot be ascribed to the non-specific injurious effect of the operation.

Gastrulation is independent on the DDs

We also show that, for the formation of the blastopore, the vegetal-localized dorsal determinants are not necessary. The blastopore is able to form autonomously but requires the presence of the dorsal determinants to acquire the dorsal property. On the contrary, the dorsal determinant by itself seems to have no dorsal axis-forming activity, since UV-irradiated embryos make no dorsal structures although they retained the dorsal determinants. In this connection, Holowacz
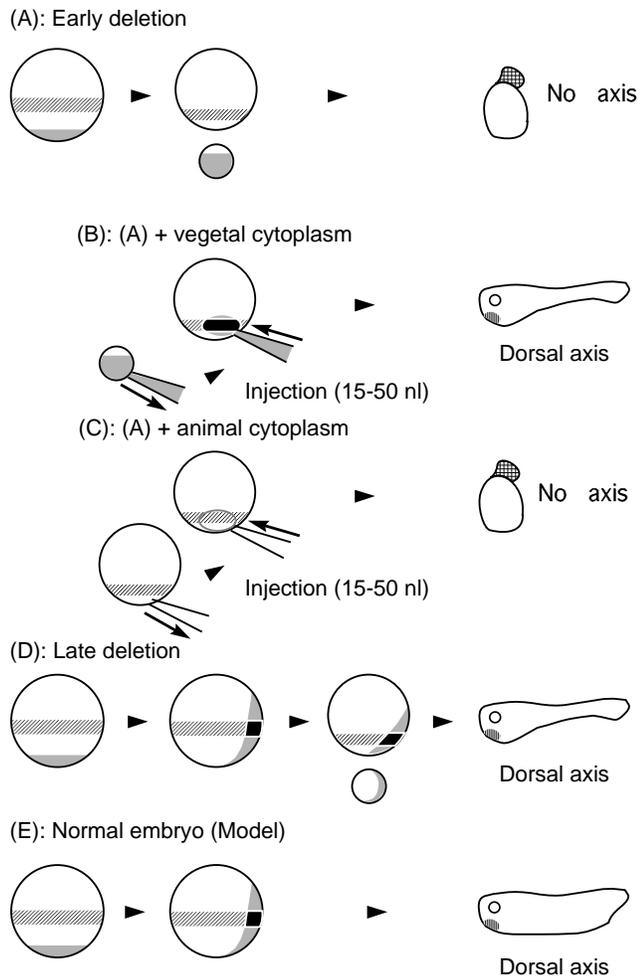
(A): Early deletion

No axis

(B): (A) + vegetal cytoplasm

Dorsal axis

Injection (15-50 nl)

(C): (A) + animal cytoplasm

No axis

Injection (15-50 nl)

(D): Late deletion

Dorsal axis

(E): Normal embryo (Model)

Dorsal axis

Fig. 8. Schematic illustration of the present results and the suggested model for the initial step of dorsal axis formation. (A) Early vegetal deletion. (B) Injection of the cytoplasm of a vegetal fragment after A. (C) Injection of the cytoplasm of animal fragment after A. (D) Late vegetal deletion. (E) Proposed model for normal axis formation. Hatched areas, gastrulating region (blastopore-forming region); stippled areas, dorsal determinants (DDs); black areas, organizer region: this is a part of the gastrulating region that receives the DDs.

and Elinson (1995) have shown that, when the DDs from the vegetal pole is transplanted into the animal blastomeres of the UV-treated embryos, the descendants of the transplanted blastomeres never acquire autonomous dorsal axis-forming activity. It seems likely that the DDs act synergistically with the subequatorial but not with vegetal and animal cytoplasm to form active organizer. Thus, it appears that the cytoplasmic mixing during the first cell cycle in the dorsal subequatorial region is the first and most important step in dorsal axis formation in *Xenopus*.

Proposed model for dorsal axis specification during the first cell cycle

On the basis of the present results, we propose the following mechanism for early axis specification in *Xenopus* (Fig. 8). (1) Just after fertilization, there are two specific regions important for later development; the vegetal pole region, which has DDs

(stippled areas in Fig. 8), and the subequatorial circular region, which has autonomous activity for gastrulation (hatched areas in Fig. 8). (2) During the period of cortical rotation, the DDs move to the subequatorial region along a certain meridian; the part of subequatorial region that receives the DDs will later form a dorsal type blastopore which in turn will induce the dorsal axial structures. (3) The organizer, the core of which is the dorsal blastopore (Shih and Keller, 1992), is formed by the mixing of the DDs and the gastrulation-inducing subequatorial cytoplasm. For the formation of the organizer, 'mesoderm induction' (Nieuwkoop, 1969; Smith, 1993) from the more vegetal region seems to be unnecessary since the dorsal blastopore formed in the absence of the more vegetal region (Fig. 1E,F).

The mechanism by which the DDs interact with the gastrulating cytoplasm is unknown. Thomsen and Melton (1993) speculate a protease activity that cleaves the Vg1 precursor to create the active form, which in turn gives rise to the Nieuwkoop center. Thus, the DDs might be a protease that cleaves the Vg1 precursor to the active form. However, it should be noted that the vegetal pole of the UV'd embryos has no dorsal-axis inducing properties although it possesses both the DDs (Fujisue et al., 1993) and the Vg1 precursor (Thomsen and Melton, 1993). For the formation of the active organizer, some other factors present in the subequatorial region seem to be necessary.

Whatever the nature of the DDs turn out to be, our observation that deletion of the vegetal poles *always* causes an entire dorsal axis deficiency that can be reversed by cytoplasmic injection provides a novel method to clarify the nature of the DDs.

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