Location and behavior of dorsal determinants during first cell cycle in

Xenopus eggs

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SUMMARY

In *Xenopus* eggs, removal of small volumes of cytoplasm along with the surface (2-10% of the entire egg volume) causes very severe dorsal reduction (average DAI=1.4) when made at a site ventrally 30° off the vegetal pole at 20% time of first cell cycle (0.2 NT). The greatest dorsal reduction (average DAI=1.1) occurs when removal is done at the vegetal pole at 0.3 NT, and intermediate reductions (average DAI=2.2-2.6) when done at sites dorsally, dorso-laterally or laterally 30° off the vegetal pole at 0.4 NT. Removal at sites dorsally, dorsolaterally or laterally 60° off the vegetal pole provokes slight dorsal reduction (average DAI=3.5-3.9) when made at 0.4-0.5 NT. Removal at all sites after 0.4 NT causes a steady decrease in the extent of dorsal reduction. By contrast, removal of larger volumes of dorsal

cytoplasm (16-50% of the entire egg volume) causes a steady increase in the extent of dorsal reduction during first cell cycle with its maximum effect at 1.0 NT (average DAI=3.1). The surgery for the cytoplasmic removal does not affect cortical rotation. We conclude from these results that dorsal determinants are concentrated first in a small region ventrally 30° off the vegetal pole by 0.2 NT, then move toward the vegetal pole during the period 0.2-0.3 NT and disperse to a broad region spanning over both the presumptive dorsal and ventral, but mainly the dorsal, hemispheres during the period 0.3-0.8 NT.

Key word: *Xenopus laevis*, dorsal determinants, cortical rotation, localization, axis specification

INTRODUCTION

Establishment of bilateral symmetry that defines the future dorsoventral axis of the embryo is an essential event of early animal development. In *Xenopus,* the egg acquires bilateral symmetry during a rotation of vegetal cortical cytoplasm relative to the egg core, 'cortical rotation' (reviewed by Gerhart et al., 1989). Studies in the past 15 years (Scharf and Gerhart, 1980, 1983; Vincent et al., 1986, 1987; Vincent and Gerhart, 1987) have elucidated an important role of cortical rotation in the egg's acquisition of bilateral symmetry: dorsoventral axis specification. They have shown that not only does the direction of cortical rotation define the future dorsoventral axis but also the extent of the rotation designates the degree of dorsoventral axis formation. Other studies carried out around the same time (Grunz, 1977; Gimlich and Gerhart, 1984; Gimlich, 1986; Render and Elinson, 1986; Kageura, 1990, 1995) have accumulated evidence for possible involvement of cortical rotation in determining the future dorsal side of the embryo and the formation of dorsal axis structures. They have shown that while a cytoplasmic potential to form dorsal axial structures resides in both the future dorsal and ventral sides of the egg before cortical rotation, it is confined to the future dorsal side after cortical rotation.

Recent studies (Fujisue et al., 1993; Holowacz and Elinson, 1993, 1995) have demonstrated more directly, with experi-

ments involving cytoplasmic transfer, that transferable components, capable of producing novel dorsal axial structures, reside in cortical cytoplasm around the vegetal pole before cortical rotation which are displaced to the future dorsal subequatorial region after the rotation. This finding suggests that the cytoplasmic components, or dorsal determinants, are conveyed from the vegetal pole to the dorsal subequatorial region in combination with cortical rotation. Little is known, however, about how cortical rotation is involved in the displacement of the determinants or in what manner the determinants are displaced, i.e. whether they are displaced straightly from the vegetal pole to the dorsal region, keeping themselves as a small aggregate, or whether the two sets of components detected in the two regions before or after cortical rotation are identical or not.

As an approach to answer these questions, we have devised an assay system for following the location and behavior of dorsal determinants during first cell cycle. Various volumes of cytoplasm with or without the surface were removed from the *Xenopus* egg at various sites of the vegetal hemisphere at various points of the first cell cycle by extruding part of the egg out of the fertilization envelope or ligating denuded eggs with a glass rod or a hair loop. If dorsal determinants are localized to a small region around the vegetal pole before cortical rotation and are displaced directly to the dorsal subequatorial region, keeping themselves as a small aggregate,

then removal at sites between the two regions during cortical rotation could cause reduction or deletion of dorsal axial structures. We expect that elucidation of the location and behavior of dorsal determinants during first cell cycle in *Xenopus* eggs would contribute greatly to studies involving the identification of molecules acting as determinants in the egg.

MATERIALS AND METHODS

Preparation of eggs

Adult females of *Xenopus laevis* were injected with 400 IU of human chorionic gonadotropin and kept in a plastic bath at 22-24°C overnight to induce ovulation. About 50 eggs were squeezed into a plastic Petri dish and fertilized with 0.2-0.4 ml of sperm suspension prepared by dispersing finely macerated testis in 2 ml modified De Boer's saline (MDB; see Shinagawa, 1992, for composition). After activation contraction occurred, fertilized eggs were dejellied by gentle agitation in 2.5% L-cysteine dissolved in 50% modified Steinberg's saline (50% MSS; see Shinagawa, 1992, for composition) at pH 8.0. They were washed two times in 50% MSS and prepared for experiments. When necessary, the fertilization envelope was removed with forceps after treatment with 0.2% protease in 50% MSS for 10 seconds. Manipulation of denuded eggs was always done on a 3% agar sheet under 50% MSS.

Removal of small volumes of cytoplasm

Dejellied eggs were transferred to 50% MSS containing 6% Ficoll and kept for 2-3 minutes to remove water from the perivitelline space. Then, the eggs were pricked with a fine glass needle at the vegetal pole (VP), at sites dorsally, ventrally, laterally or dorsolaterally 30° off the vegetal pole (D30°-, V30°-, L30°- and DL30°-sites, respectively), or at sites dorsally, ventrally, laterally or dorsolaterally 60° off the vegetal pole (named D60°-, V60°-, L60°- and DL60°-sites, respectively), as schematically represented in Fig. 1. The dorsoventral axis was designated relative to the site of sperm entry; the side of sperm entry as the ventral side and the opposite side as the dorsal side. Pricking was done at 10-80% of the time duration of the first cell cycle (0.1-0.8 NT). Eggs were left in the Ficoll solution for about 3 minutes to allow the wound to heal. They were transferred to 50% MSS and left for about 5 minutes so that a portion of the vegetal hemisphere was gradually squeezed out of the fertilization membrane by the hydrostatic pressure of the perivitelline space (Figs 2A and 3A). When a desired volume of cytoplasm (usually 2-10% of whole egg volume) was extruded with the surface, eggs were transferred to another 6% Ficoll solution supplemented with antibiotics (71.8 μg/ml streptomycin sulfate and 143 units/ml penicillin G potassium salt). The volume of the extruded egg portion was calculated from its longest and shortest diameters by regarding its shape as spheroidal. For removal of cytoplasm without the surface, dejellied eggs were transferred to a 6% Ficoll solution, pricked at the same sites at the same times as above and slightly squeezed with forceps until a desired volume of cytoplasm, 2-10% of the whole egg volume, was extruded (Figs 2B and 3C). Eggs were then transferred to another 6% Ficoll solution, supplemented with the antibiotics for culturing. The volume of extruded cytoplasm without the surface was calculated from its longest and shortest diameters by regarding its shape as semispheroidal.

Removal of large volumes of cytoplasm

To remove larger volumes of cytoplasm (16-50% of the entire egg volume), eggs were ligated with either a fine glass rod or a loop of new-born human hair (Fig. 2C), following the procedure of Shinagawa (1983). Dejellied eggs were transferred to a plastic Petri dish with a 3% agar sheet and 50% MSS containing protease at 0.2%. After treatment with protease for 10 seconds, eggs were transferred
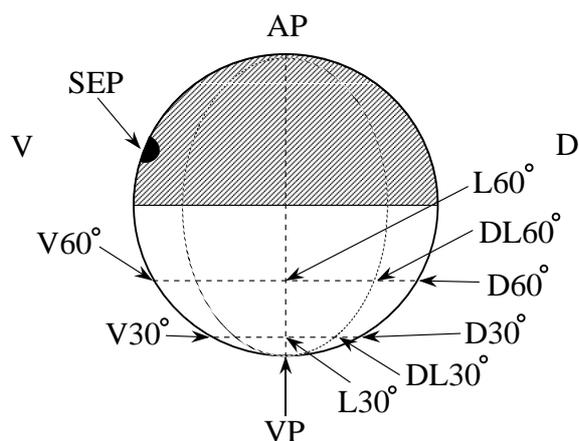
Fig. 1. Schematic representation of the sites of pricking for removal of cytoplasm with or without the surface. Cytoplasm was removed at the vegetal pole (VP) and sites dorsally 30° (D30°), dorsally 60° (D60°), dorsolaterally 30° (DL30°), dorsolaterally 60° (DL60°), laterally 30° (L30°), laterally 60° (L60°), ventrally 30° (V30°) and ventrally 60° (V60°) off the vegetal pole. AP, the animal pole; VP, the vegetal pole; D, the presumptive dorsal side; V, the presumptive ventral side; SEP, sperm entry point.

to another similarly prepared dish, and the fertilization membrane was removed manually with forceps. Denuded eggs at early and late stages of the first cell cycle were ligated with a fine glass rod and a loop of a new-born human hair, respectively. Ligation was made on a plane parallel to the equator, ⅔ of the egg radius away from the vegetal pole (named ⅔ vegetal deletion) or a plane crossing vertically to the future dorsoventral axis full and ½ of the egg radius away from the future dorsal most periphery (named complete and ½ dorsal deletion, respectively). The volumes of the fragments to be removed by ligation on these planes are calculated about 20%, 50% and 16%, respectively, of the entire egg volume.

Irradiation of eggs with ultraviolet light

About 50 dejellied eggs were transferred to a quartz cell (10 mm×10 mm×50 mm) filled with 50% MSS. The open end of the cell was sealed with a piece of parafilm (20 mm×20 mm), and the cell was placed on an ultraviolet light source (UVGL 25, UVP Inc., San Gabriel, CA, USA) with a transparent side of the cell facing the source. Eggs were irradiated with 254 nm light from beneath for 60 seconds at about 0.17 NT. By this irradiation, eggs are expected to receive an energy of 14,000 erg/mm^2, which completely suppresses dorsal development as well as cortical rotation. After irradiation, eggs were transferred to a plastic Petri dish containing 50% MSS and prepared for experiments. When eggs developed to the 8-cell stage, the fertilization membrane was carefully removed with forceps after treatment with 0.2% protease in 50% MSS for 10 seconds.

Transfer of cytoplasm

Transfer of vegetal cortical cytoplasm was carried out following procedures described by Fujisue et al. (1993) and Holowacz and Elinson (1993). When a desired volume of cytoplasm with the surface was extruded after pricking at 30 or 60% time of first cell cycle, about 50 nl of cytoplasm containing pigment granules was withdrawn with a micropipet from the extrusion. About 40 nl of cytoplasm was injected to adjacent two ventral vegetal cells (20 nl per cell) of ultraviolet-irradiated, denuded, 8-cell stage embryos. Injected embryos were placed in depressions on agar sheets under 50% MSS. When wounds due to injection were closed, eggs were transferred to another dish filled with 50% MSS containing antibiotics (71.8 μg/ml streptomycin sulfate and 143 units/ml penicillin G potassium salt) and reared for 2 days.

Culture of eggs and examination of dorsal axis formation

Eggs were cultured first in a 6% Ficoll solution supplemented with the antibiotics (71.8 µg/ml streptomycin sulfate and 143 units/ml penicillin G potassium salt) for 1 day and then transferred to 50% MSS supplemented with the antibiotics and reared for another day at room temperature (21-23°C). At these temperatures, control embryos reached stage 35-37 (Nieuwkoop and Faber, 1967) after 2 days in culture. After a 2-day culture, embryos were fixed by adding 1 ml of 35% formaldehyde to the dish in which the embryos were cultured, and examined for the formation of dorsal axial structures. The degree of dorsal axial development was scored on the dorsoanterior index (DAI) (Kao and Elinson, 1988), and the average DAI for each set of experiment was calculated from scores for usually more than 20 larvae from more than 3 spawnings of eggs.

Examination of cortical rotation

Vegetal cortical cytoplasm was stained using the procedure of Vincent et al. (1986) as follows. Dejellied eggs were placed singly in a depression with a perforation on a thin stainless steel plate. After the excess medium around the eggs was removed with a piece of twisted Kleenex tissue, the plate was floated on a 0.2% Nile blue solution for 3 minutes. Then the plate was removed from the solution, transferred to 50% MSS and quickly submerged in it. Eggs were detached from the plate by gentle streaming, and those with clearly imprinted Nile blue spots were selected for experiments. After the surgery of cytoplasmic removal, eggs were embedded in 7.5% gelatin in a plastic Petri dish and immobilized in it by solidifying the gelatin with iced water. The dish was transferred onto an inverted light microscope and viewed from beneath. Images were photographed at both the start (0.4 NT) and end (0.95 NT) of cortical rotation. To facilitate the examination of the movement of Nile blue spots using the photographs, the images were computer-processed. Pairs of images photographed at the start and end of cortical rotation were scanned (using a GT6500ART2, Epson Co., Suwa, Japan) into a computer (Macintosh Centris 660AV, Apple Computer Inc., Cupertino, CA, USA). One of the pair of images was superimposed onto the other to produce a single image using Adobe Photoshop software (Adobe Systems Inc., San Jose, CA, USA). Each spot was highlighted, and corresponding spots of original images were connected with arrows indicating the direction of their movements.

RESULTS

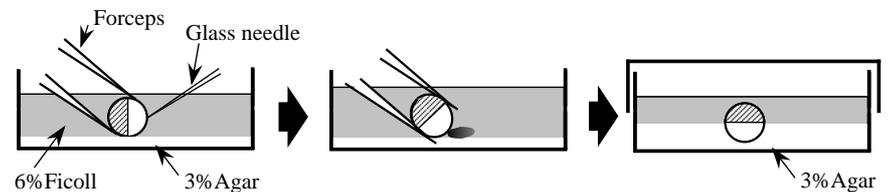### Effect of removal of small volumes of cytoplasm together with the surface at the vegetal pole

As shown in Figs 3B, 4B, 5 and 6, severe reduction of dorsal axial structures (average DAI=1.1) occurred when a small volume of cytoplasm (2-10% of whole egg volume) was removed along with the surface at the vegetal pole (VP) at 0.3 NT. The syndrome of dorsal reduction in these

larvae resemble that in 'ventralized larvae' produced by ultraviolet irradiation (Scharf and Gerhart, 1980, 1983). They suffered from deletion or severe reduction of dorsoanterior structures such as the head, the eyes, the cement gland and the notochord (Fig. 4B,C). Despite such severe reduction of dorsal axial structures, however, the embryos underwent more or less normal gastrulation with a slight delay in the start of gastrulation (data not shown). Surprisingly, removal at the VP at 0.1 NT caused virtually no dorsal reduction at all (average DAI=4.8) (Figs 4A, 5 and 6). The effect of the removal sharply increased during 0.1-0.3 NT and reached its maximum at 0.3 NT (Figs 5 and 6). It then decreased during 0.3-0.5 NT and remained nearly absent during 0.5-0.8 NT (Figs 5 and 6). The effect of removal of cytoplasm together with the surface at the VP thus changed in a V-shape manner during the first cell cycle with its maximum at 0.3 NT (Fig. 6). This result strongly
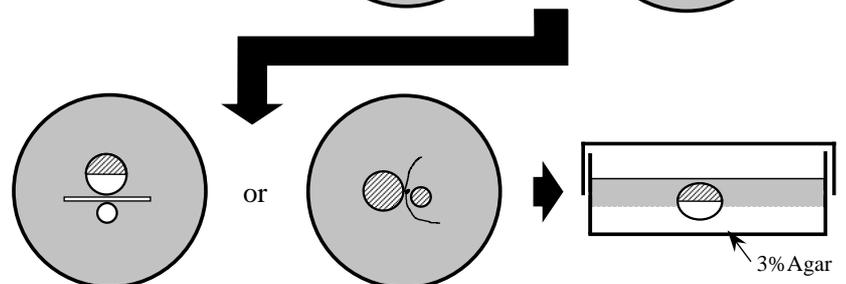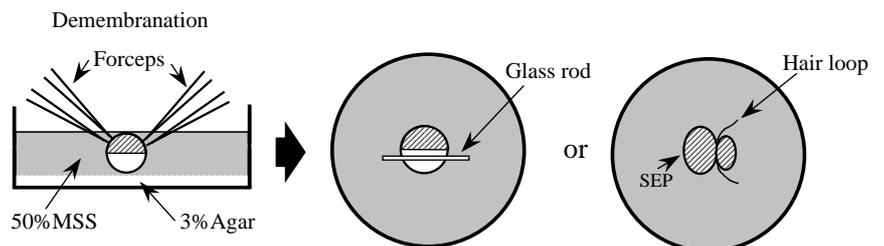
A. Removal of small volumes of cytoplasm with the surface
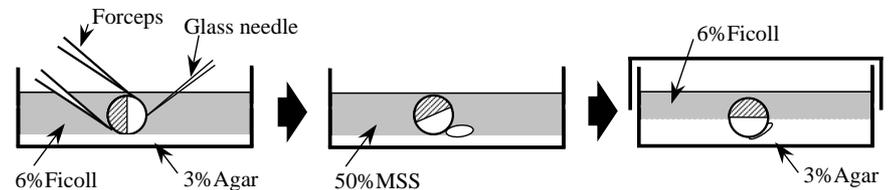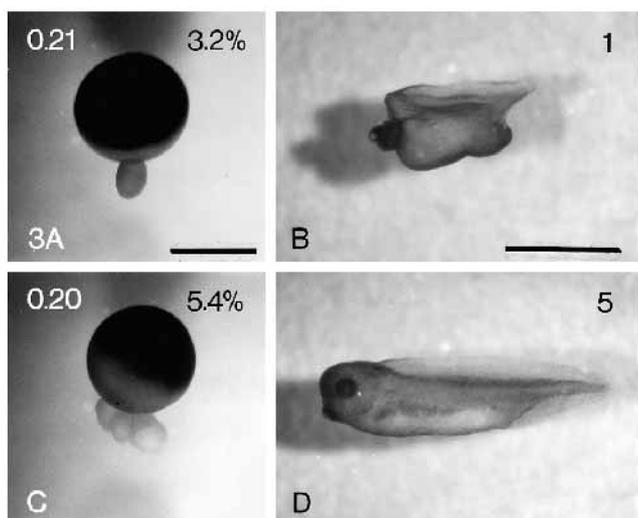

B. Removal of cytoplasm without the surface

C. Bisection of eggs with a glass rod or a hair loop

Fig. 2. Schematic illustration of the procedure for removal of small volumes of cytoplasm together with the surface (A), removal of small volumes of cytoplasm without the surface (B) and removal of large volumes of vegetal or dorsal cytoplasm (C). See text for details.

Fig. 3. Top views of eggs from which cytoplasm with (A) or without (C) the surface are being squeezed out of the fertilization membrane into 6% Ficoll solution. Numbers on the top left of A and C indicate the normalized times of the removal, and those on the top right denote estimated volumes of extruded cytoplasm relative to the entire egg volume. Numbers on the top right of B and D are the DAI for the larvae, which were developed from the eggs shown in A and C, respectively. The scale bar for A and C, 1 mm; B and D, 2 mm.

suggests that dorsal determinants are concentrated around the vegetal pole most extensively at 0.3 NT.

Effect of removal of cytoplasm together with the surface at other sites
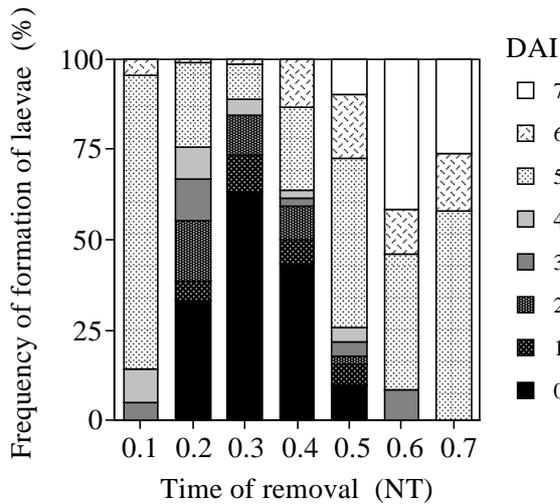
Removal of cytoplasm with the surface at the D30°-site had a similar but overall less significant effect than that of removal at the VP. The effect of removal at the D30°-site, like that of removal at the VP, changed in a V-shape manner during first cell cycle with its maximum at 0.4 NT (average DAI=2.6; Fig. 6). Removal of cytoplasm with the surface at the D60°-site also produced a similar but far less significant effect than that of removal at the VP. The effect of the removal changed likewise in a V-shape manner during first cell cycle with its maximum at 0.4 NT (average DAI=3.9; Figs 4E and 6). Removal of cytoplasm along with the surface at the L30°- and DL30°-sites, like that at D30°-site, had an

Fig. 4. Two-day-old larvae that developed after removal of cytoplasm along with the surface at the vegetal pole at 0.1 (A), 0.3 (B) and 0.7 NT (C), those that developed after removal of cytoplasm with the surface at the site ventrally 30° off the vegetal pole at 0.2 NT (D), or at the site dorsally 60° off the vegetal pole at 0.8 NT (E) and those that developed after removal of cytoplasm without the surface at the vegetal pole at 0.3 NT (F). Numbers close to individual larvae are the DAI scored for the larvae. Numbers on the top right of individual panels indicate the normalized time of cytoplasmic removal. Scale bar, 2 mm.
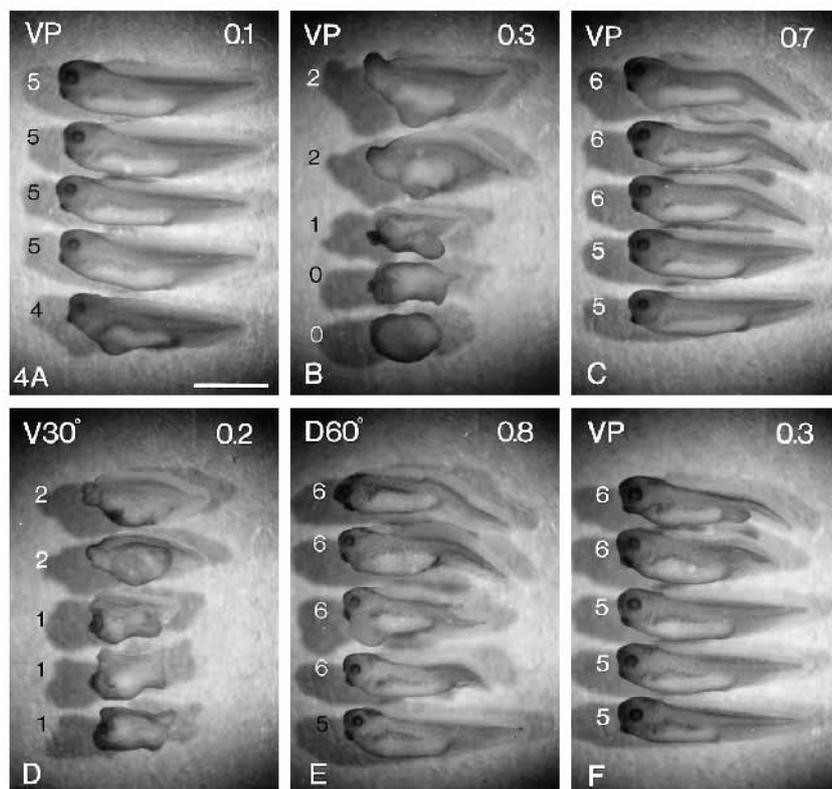
effect of provoking intermediately severe reduction of dorsal axial structures when made at an optimal time. The effects of the removal at the two sites changed in a V-shape manner during first cell cycle with its maximum at 0.4 NT (average DAI=2.6 and 2.2, respectively; Fig. 6). Removal of cytoplasm with the surface at the L60°- and DL 60°-site, like that at the D60°-site, had an effect to induce slight reduction of dorsal axial structures when made at optimal times. The effect of the removal at the two sites changed in a V-shape manner during first cell cycle with its maximum at 0.4 and 0.5 NT, respectively (average DAI=3.5 and 3.6, respectively; Fig. 6). Surprisingly, unlike removal at those sites, removal of cytoplasm with the surface at the V30°-site evoked as heavy reduction of dorsal structures as provoked by removal at the VP at 0.3 NT when made at 0.2 NT (average DAI=1.4), and its effect decreased steadily to its minimum at 0.8 NT (average DAI=6.1) (Figs 4D and 6). Removal of cytoplasm with the surface at the V60°-site had a similar but much less significant effect than that of removal at V30°-site, which decreased steadily from its maximum at 0.2 NT (average DAI=3.4) to its minimum at 0.8 NT (average DAI=5.9; Fig. 6). Gastrulation in these embryos appeared normal with a slight if any retardation of development (data not shown).

It should be noted here that removal at the VP, V30°-, L30°-, DL30°- and D30°-sites at 0.4 NT caused dorsal reduction to nearly the same extent. This result indicates that the concentration of dorsal determinants is nearly the same at these sites at 0.4 NT. It should also be noted here that the effect of the removal at the V30°-site dropped definitely more at 0.5 NT than that of removal at the L30°-, DL30°- and D30°-sites. This result suggests that dorsal determinants are being displaced from the ventral region to the dorsal around 0.5 NT.

Fig. 5. Frequency of production of larvae with differently reduced or enlarged dorsal structures after removal of a small volume of cytoplasm along with the surface at the VP at the indicated times of first cell cycle. Data for the effect of the removal at the indicated times were obtained from 21 (0.1 NT), 78 (0.2 NT), 71 (0.3 NT), 44 (0.4 NT), 51 (0.5 NT), 24 (0.6 NT), 19 (0.7 NT) larvae from 8 females. Average DAI for 45 control larvae from the same females was 5.0. Note that the frequency of production of larvae suffering from severe dorsal reduction (DAI 0-1) changes remarkably: it increased sharply after fertilization, reached its maximum at 0.3 NT and decrease sharply after 0.3 NT. More than 240 larvae from intact eggs from the 8 females were examined as the control for formation of dorsal axial structures (the average DAI= 5.0).

Effect of removal of small volumes of cytoplasm without the surface

Removal of small volumes of cytoplasm without the surface at the VP-, D30°- or D60°-site, as expected from the results of Holowacz and Elinson (1993), had no significant effect on dorsal structures (Figs 3D, 4F and 7). In fact an overwhelming majority of eggs developed to normal (DAI 5) larvae after this removal, showing apparently normal gastrulation, while a minority of eggs developed into larvae suffering from slight reduction of the abdominal region due to reduction of vegetal yolk platelets by the cytoplasmic removal (Fig. 4F). Embryos subjected to the cytoplasmic removal at these sites underwent apparently normal gastrulation with a slight if any delay in development (data not shown). These results, together with above ones, indicate that dorsal determinants are present mostly in the cortical but not deep cytoplasm, as suggested by Holowacz and Elinson (1993).

Cortical rotation in eggs subjected to removal of cytoplasm together with the surface

According to previous studies (Vincent et al., 1987; Elinson and Rowning, 1987; Houliston and Elinson, 1988), irradiation with ultraviolet-light from beneath before cortical rotation prevents *Xenopus* eggs from undergoing cortical rotation by depolymerizing vegetal cortical microtubules serving as the track for the rotation. Deletion and severe reduction of dorsal axial structures in uv-irradiated larvae is thought to be caused by prevention of cortical rotation through depolymerization of microtubules. To test if this is
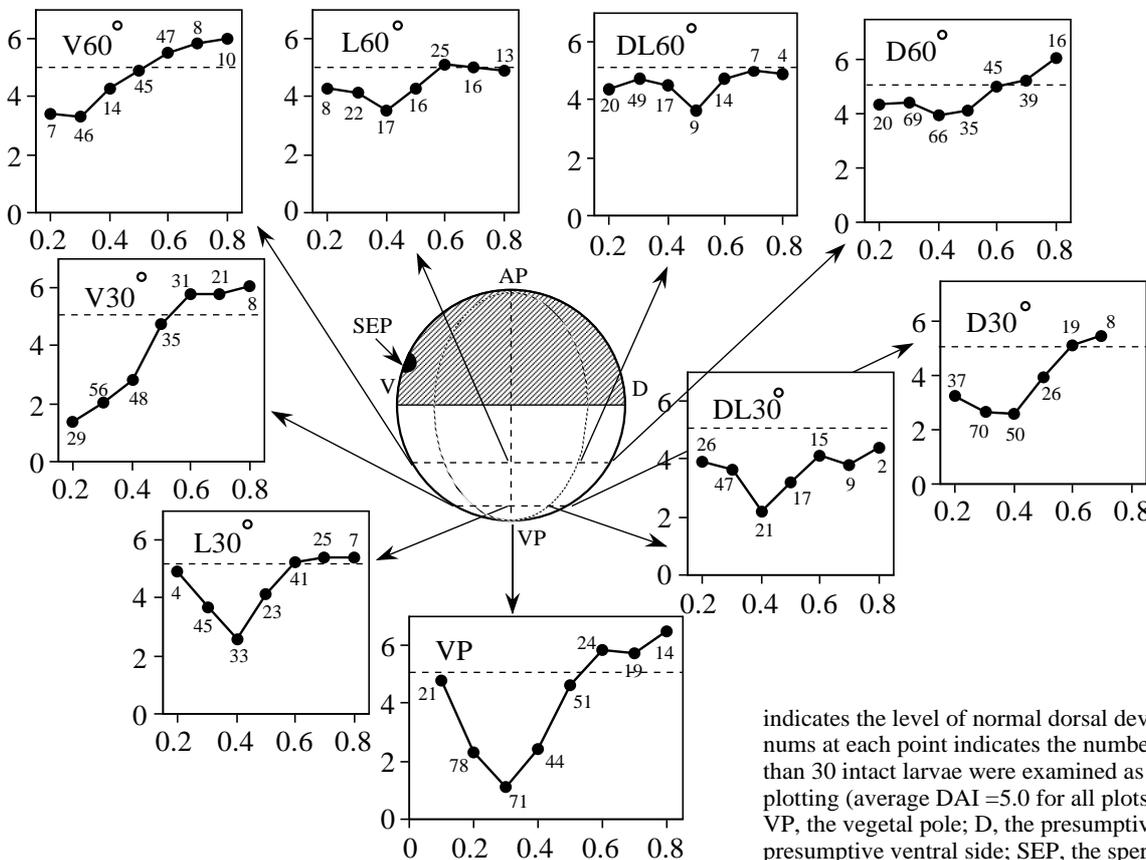
Fig. 6. Summary of temporal changes in the effect on dorsal development of removal of cytoplasm with the surface at different sites. Average DAIs (y axis) for larvae from eggs subjected to removal of cytoplasm together with the surface are plotted against the normalized times (x axis) of the removal. The broken line in each plot indicates the level of normal dorsal development (DAI 5). The nums at each point indicates the number of larvae scored. More than 30 intact larvae were examined as the control for each plotting (average DAI =5.0 for all plots). AP, the animal pole; VP, the vegetal pole; D, the presumptive dorsal side; V, the presumptive ventral side; SEP, the sperm entry point.
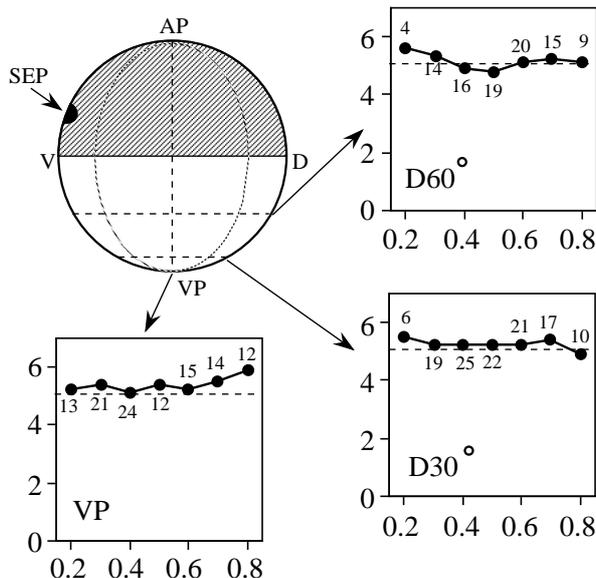
Fig. 7. Summary of the effect on dorsal development of removal of cytoplasm without the surface at different sites and at different times. Average DAIs for larvae from eggs subjected to removal of cytoplasm without the surface are plotted against the normalized times of the removal. The broken line in each plot denotes the level of normal dorsal development (DAI 5). The numbers at each point is the number of larvae scored for obtaining the average DAI. More than 30 intact larvae were examined as the control for each plotting (average DAI =5.0 for all plottings). AP, the animal pole; VP, the vegetal pole; D, the presumptive dorsal side; V, the presumptive ventral side; SEP, the sperm entry point.

the case for dorsal reduction in our study, we examined cortical rotation in eggs subjected to removal of cytoplasm along with the surface at the VP at 0.3 NT or V30°-site at 0.2 NT, which we found causes the heaviest reduction of dorsal axial structures.

Surprisingly, cortical rotation in those eggs was not heavily suppressed or prevented; it occurred through as large degrees as in control eggs (20°-31° and 20°-33° for eggs subjected to the removal at the VP at 0.3 NT and at the V30°-site at 0.2 NT, respectively, and 23°-37° for control eggs; Fig. 8 and Table 1). Although the degree of cortical rotation in those eggs varied slightly and in some eggs it appeared to be suppressed slightly, there was no obvious correlation between the degree

Table 1. Relationship between the degree of cortical rotation and the extent of resulting dorsal axis deficiencies in eggs subjected to removal at the VP- and V30°-sites at 0.25 NT

	Removal at VP		Removal at V30°-site		Control	
	cortical rotation (°)	DAI	cortical rotation (°)	DAI	cortical rotation (°)	DAI
	31	0	33	0	37	5
	29	2	31	1	33	5
	24	3	28	3	30	5
	21	2	25	0	29	5
	21	0	24	4	25	5
	20	2	20	0	23	5
Mean	24.3	1.5	26.8	1.3	29.5	5.0
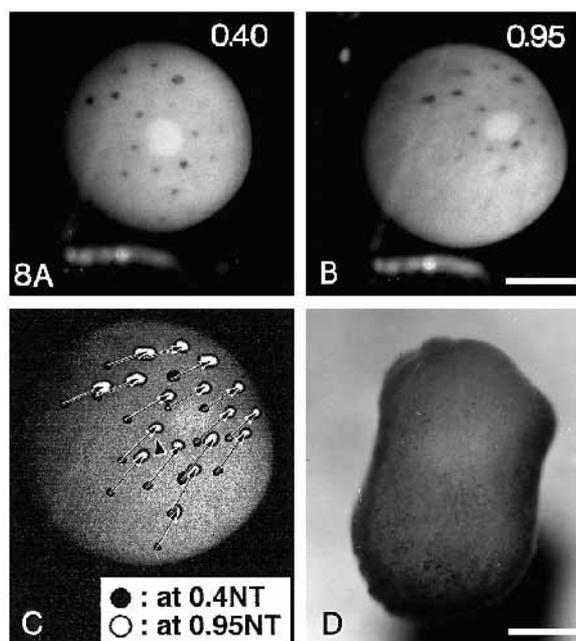
Fig. 8. Cortical rotation in an egg from which cytoplasm was removed along with the surface at the vegetal pole at 0.3 NT (A-C) and the resulting two-day-old larva (D). Vegetal views at 0.4 NT (A) and 0.95 NT (B) of the egg are presented. Arrowheads indicate the point of extrusion for cytoplasmic removal. C is a computer generated composit image fo A and B, showing the movement of Nile blue spots. The pigment-free portion near the site of extrusion was formed after the removal of cytoplasm with the surface, suggesting that a cortical layer containing pigment granules was removed by this surgery. Note that dorsal axial structures are heavily reduced in the larva even though cortical rotation occurs normally. Scale bars in panels A and D equal 0.5 mm.

of cortical rotation and the extent of dorsal reduction (Table 1). Obviously, reduction or deletion of dorsal structures in larvae subjected to cytoplasmic removal is not due to suppression or prevention of cortical rotation by damage caused by cytoplasmic removal.

Transfer of cytoplasm from extrusions at the vegetal pole at 0.3 and 0.6 NT

To confirm that reduction and deletion of dorsal structures after cytoplasmic removal are caused by removal and deletion of dorsal determinants, we examined if cytoplasm removed at a particular site at a particular time really contains dorsal determinants by transferring part of the cytoplasm to adjacent two ventral vegetal cells of UV-irradiated, 8-cell stage embryos. As shown in Fig. 9 and Table 2, larvae that received cytoplasm from extrusions formed at VP at 0.3 NT developed

Table 2. Axis formation in uv-irradiated larvae that received cytoplasm from extrusions formed at the vegetal pole at 0.3 or 0.6 NT

Received cytoplasm from	n	DAI						Mean
		0	1	2	3	4	5	
extrusions at 0.3 NT	16	1	13	2				1.1
extrusions at 0.6 NT	12	11	1					0.1
None	20	20						0.0
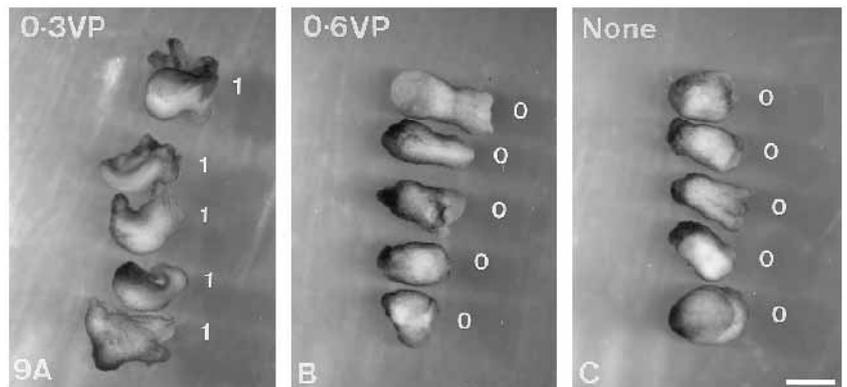
Fig. 9. 2-day-old larvae from a single spawning of eggs that were irradiated with 254 nm light at 14,000 erg/mm^2 and given cytoplasm from extrusions formed at the vegetal pole at 0.3 NT (A) and 0.6 NT (B) or no cytoplasm (C). Note that dorsal axial structures are detectable only in larvae that received cytoplasm from an extrusion formed at 0.3 NT (A). Numbers close to individual larvae are their DAI score. Scale bar equals 1 mm.

detectable, though incomplete, dorsal axial structures (average DAI=1.1). By contrast, those that received cytoplasm from extrusions formed at VP at 0.6 NT did not develop any detectable dorsal structures (average DAI=0.1). These results, in agreement with previous results (Fujisue et al., 1993; Holowacz and Elinson, 1993), indicate that cytoplasm of extrusions formed at the vegetal pole at 0.3 NT but not at 0.6 NT contains dorsal determinants. Thus, reduction and deletion of dorsal structures in larvae subjected to the cytoplasmic removal at the vegetal pole at 0.3 NT are ascribed to reduction and deletion of dorsal determinants, not other inhibitory side effects.

### Effect of removal of large volumes of cytoplasm by ligation

The almost complete absence in the effect of removal of cytoplasm at all sites at 0.6 NT (Fig. 6) suggests possible dispersion of the dorsal determinants from the vegetal pole region to a broader region of the eggs. To examine how broadly the determinants are dispersed in the egg, we tested the effect of removal of larger volumes of egg cytoplasm by ligating eggs on a plane parallel to the equator ⅔ of the egg radius away from the vegetal pole (⅔ vegetal deletion). By this ligation a volume of cytoplasm amounting to 20% of the entire egg volume was expected to be removed. The ⅔ vegetal deletion, as expected from the result of removal at VP, had the effect of severely reducing dorsal structures that occurred in a V-shape manner with its maximum at 0.3 NT (average DAI=1.1) (Fig. 10). It is noticable that the ⅔ vegetal deletion at 0.1 and 0.6 NT, at which time removal of a small volume of cytoplasm with the surface at VP had no significant effect on dorsal reduction, produceed rather severe reduction of dorsal structures (average DAI=2.1 and 2.9, respectively). Dorsal determinants may reside within ⅔ of egg radius of the vegetal pole at 0.1 and 0.6 NT. Unlike UV-irradiated embryos, these embryos usually underwent gastrulation quite normally with a slight delay in the start though some failed it and developed to exogastrulae (data not shown).

To further examine overall displacement of dorsal determinants toward the presumptive dorsal region, we tested the effect of the removal of larger volumes of dorsal cytoplasm,16-50% of the entire egg volume, by ligating eggs on planes that vertically crossed the plane of the future dorsoventral axis at ½, or at the egg radius, away

from the dorsal most periphery (named ½ and complete dorsal deletion, respectively). Both ½ and complete dorsal deletion, unlike removal of small volumes of cytoplasm, resulted in a steady decrease in dorsal structures when done at various stages during the first cell cycle, with its maximum effect at 1.0 NT (average DAI=3.8 for ½ dorsal deletion, 3.1 for complete dorsal deletion; Fig. 10). These results suggest that dorsal determinants are totally shifted to the future dorsal hemisphere during first cell cycle. Gastrulation in ligated embryos was apparently normal, except for a slight delay in the start of gastrulation (data not shown).
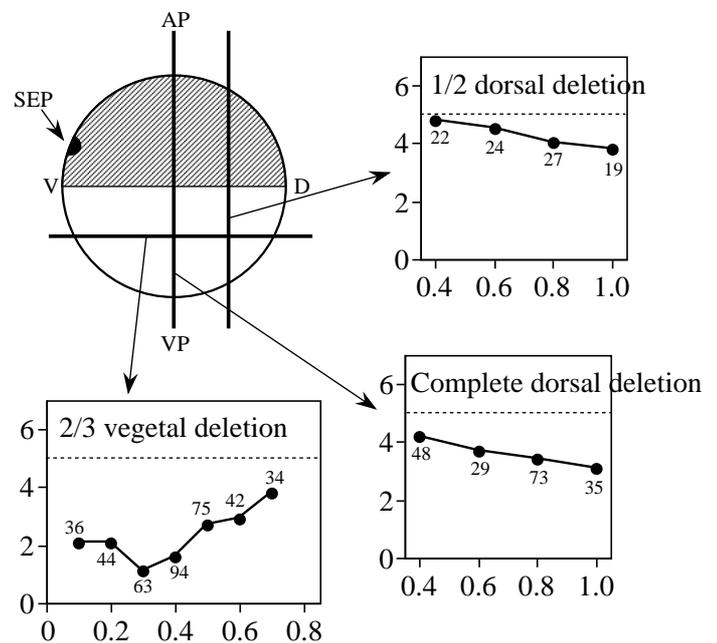
Fig. 10. Summary of the effect on dorsal development of removal of large volumes of dorsal or vegetal egg cytoplasm by egg ligation at varying times. Average DAIs for larvae from eggs subjected to the removal are plotted against the normalized times of the removal. The broken line in each plot depicts the level of normal dorsal development (DAI 5). The number close to each point is the number of larvae scored at that point. More than 30 intact larvae were examined as the control for each plot(average DAI =5.0 for all plots). AP, the animal pole; VP, the vegetal pole; D, the presumptive dorsal side; V, the presumptive ventral side; SEP, the sperm entry point.

Fertilization

First cleavage

0.0   0.1   0.2   0.3   0.4   0.5   0.6   1.0

Time (NT)

SEP   AP

VP
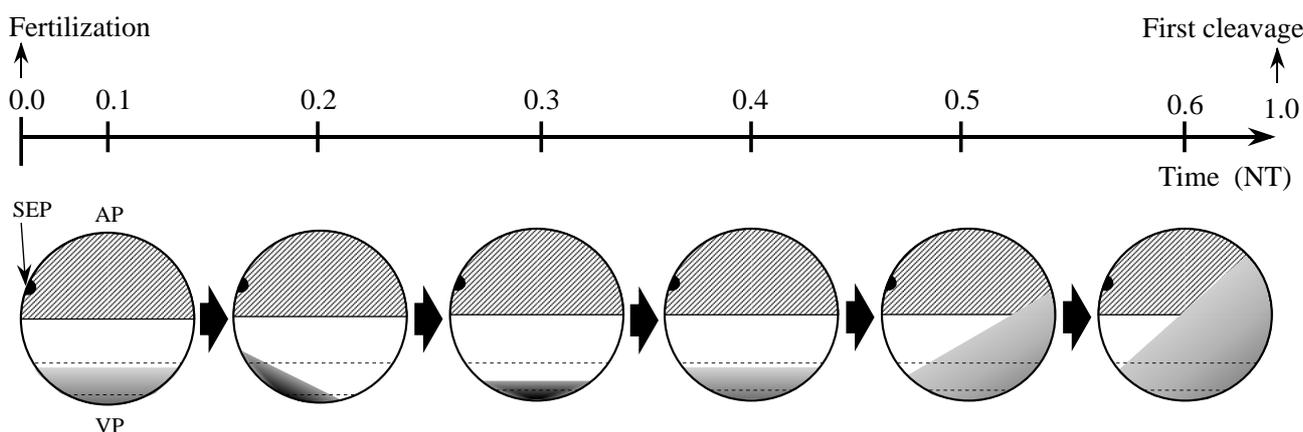
Fig. 11. Illustration of presumed behavior of dorsal determinants during first cell cycle based on the present results. The scale indicates relative times normalized with 0.0 as the time of fertilization and 1.0 as the time of the first cleavage. The darkness of the shaded portion indicate the concentration of localized dorsal determinants. Broken lines indicate latitudes 30° and 60° off the vegetal pole. AP, animal pole; VP, vegetal pole; SEP, sperm entry point.

DISCUSSION

Presence of dorsal determinants in association with the egg surface

Previous studies by Holowacz and Elinson (1993) and Fujisue et al. (1993) have revealed that cytoplasmic components capable of producing novel dorsal axial structures in recipient embryos reside in cortical cytoplasm of the vegetal hemisphere of *Xenopus* eggs. We have shown that removal of cytoplasm along with the surface at a specific site of the vegetal hemisphere at a specific time of the first cell cycle can induce considerable reduction in dorsal axial structures while removal without the surface at any site at any time cannot. We have also shown that cytoplasm from extrusions including the surface, at the vegetal pole at 0.3 NT, has the ability to reactivate dorsal axial development in UV-irradiated, recipient embryos. These results agree with those of Holowacz and Elinson (1993) and support their suggestion that the cortical layer containing pigment granules is most likely to contain dorsal determinants. In fact, what was removed in our study by removal of the surface was the layer containing pigment granules (see Fig. 8). We conclude that at least a major part of components necessary for differentiation of dorsal axial structures must reside close to, and/or in association with, the surface. Recent studies at the molecular level (Smith and Harland, 1991, 1992; Sokol et al., 1991; Thomsen and Melton, 1993; Pierce and Kimelman, 1995) have shown that several factors such as members of Wnt family, noggin, processed Vg1 and unknown inhibitors of the intracellular kinase Xgsk-3 could work as dorsal determinants in *Xenopus* embryos. We presume that among these molecules, those residing mainly in the vegetal cortical layer containing pigment granules must be the most likely candidate for the real dorsal determinants.

Concentration of dorsal determinants to the vegetal pole before cortical rotation

As summarized in Fig. 6, removal of a small volume of cytoplasm with the surface at V30°-site at 0.2 NT elicited severe reduction of dorsal axial structures, whereas removal at other sites at this time had little or no effect. This implies that

dorsal determinants are localized to around the V30°-site at 0.2 NT. It is, however, impossible that dorsal determinants are already localized to the V30°-site before fertilization because the future ventral side, namely the side of sperm entry, has not been designated before fertilization in *Xenopus* eggs. Conceivably, dorsal determinants become concentrated from a rather broad region of the vegetal hemisphere to around the V30°- site during 0.0-2.0 NT, as schematically shown in Fig. 11. Since the ⅔ vegetal deletion at 0.1 NT caused considerable dorsal reduction (Fig. 10) most dorsal determinants must reside within ⅔ of the egg radius of the vegetal pole at 0.1 NT (Fig. 11). Severe dorsal reduction by removal of cytoplasm at the VP at 0.3 NT, together with the result that cytoplasm from extrusions formed at VP at 0.3 NT has an ability to produce novel dorsal structures, suggest that dorsal determinants move from the V30°-site to the vegetal pole during 0.2-0.3 NT (Fig. 11). The actual dorsal determinants might be identified as those molecules first concentrated around the V30°-site at 0.2 NT and then moved to the vegetal pole at 0.3 NT.

Although so far we have no information about the mechanism by which the determinants are concentrated to the V30°-site and moved to the vegetal pole during 0.0-0.3 NT, we could draw an analogy for it from the phenomenon known as 'ooplasmic segregation' in ascidian eggs: it is well known that microfilaments present in the cortex of the egg mediate concentration of cytoplasmic components necessary for gastrulation and muscle differentiation from a broad region of the egg to the vegetal pole region (Sawada and Schatten, 1989; Bates and Jeffery, 1987, 1988). In *Xenopus* eggs also, a similar movement mediated by microfilaments might be involved in localization of the determinants to the V30°- and VP-sites.

Dispersion of dorsal determinants from the vegetal pole during cortical rotation

As shown in Fig. 6, removal of cytoplasm at the VP-, D30°-, DL30°-, L30°- and V30°-sites at 0.4 NT all evoked dorsal reduction to nearly the same extent (average DAI=2.5±0.3) and the effect of the removal at all sites steadily decreased after 0.4 NT. These results suggest that dorsal determinants are distributed fairly uniformly in an area covering these sites at 0.4 NT

and continue to be dispersed to a broader region after 0.4 NT. No rescue of dorsal axial structures in u.v.-irradiated larvae that received cytoplasm taken from the vegetal pole region at 0.6 NT, indicates that a major part of the dorsal determinants have been lost from the vegetal pole cytoplasm at 0.6 NT. Heavy dorsal reduction by the ⅔ vegetal deletion at 0.5-0.6 NT suggests that they, however, continue to reside within ⅔ of the egg radius of the vegetal pole at 0.5-0.6 NT.

Overall displacement of dorsal determinants to the dorsal hemisphere

As shown in Fig. 10, the effect of removal of larger volumes of dorsal cytoplasm, by the ½ and complete dorsal deletion, increased steadily throughout first cell cycle with its maximum at 1.0 NT. In contrast, removal of a small volume of cortical cytoplasm at ventral sites resulted in the effect to reduce dorsal structures earlier than that at dorsal and lateral sites (Fig. 6). These results suggest that overall location of dorsal determinants is gradually shifted to the future dorsal side of the egg during cortical rotation (Fig. 11). It is, however, evident that the dorsal determinants are not localized to a small region within the dorsal hemisphere because removal at D30°-, DL30°-, D60°- or DL60°-site did not cause significant reduction of dorsal structures and because even complete dorsal deletion could not provoke complete dorsal deletion in resulting larvae. We therefore conclude that dorsal determinants become distributed during cortical rotation to a rather broad vegetal cortical region that spans over both the future dorsal and ventral, but mainly the dorsal, hemispheres of the egg (Fig. 11). This conclusion agrees well with results of previous studies (Grunz, 1977; Render and Elinson, 1986; Kageura, 1990, 1995; Fujisue et al., 1993), which demonstrated that a cytoplasmic component capable of producing dorsal axial structures in recipient eggs resides mostly but not exclusively in the future dorsal region after cortical rotation.

Role of cortical rotation in displacement of dorsal determinants

According to studies by Holowacz and Elinson (1993) and Fujisue et al. (1993), the determinants of dorsal axis formation begins to disappear from the vegetal pole and to appear instead in the future dorsal region during cortical rotation in normal *Xenopus* eggs. In contrast, they have shown that when eggs are irradiated with uv from beneath before cortical rotation, the activity continues to stay around the vegetal pole region throughout first cell cycle. These findings apparently suggest that cortical rotation plays an essential role in displacement of dorsal determinants from the vegetal pole to the dorsal region.

We have shown, however, that removal of cytoplasm at any of the sites between the VP and D60° during cortical rotation does not produce as great a reduction of dorsal axial structures as does removal at the VP at 0.3 NT. This result indicates that dorsal determinants are not displaced directly from the vegetal pole to the future dorsal subequatorial region, keeping themselves a small aggregate. This suggests that cortical rotation does not act as a transportation system for dorsal determinants.

Our results do rather appear to agree with those of Vincent et al. (1986)who reported that *Xenopus* eggs can form dorsal axial structures on the normal side predicted by the direction of rotation of subcortical cytoplasm relative to the surface,

even though their surface is immobilized by embedding in gelatin. In those eggs, only a thin layer of subcortical cytoplasm was allowed to move, and its direction was toward the future ventral side, not the dorsal side. How can such a movement of subcortical cytoplasm toward the future vegetal side displace surface-associated dorsal determinants toward the dorsal side? In addition, the average degree of cortical rotation in normal eggs appears smaller than the degree that dorsal determinants travel through. The former is as little as 30° on average, while the latter is as much as 60°. These facts and results suggest that cortical rotation itself is not the event that conveys dorsal determinants to dorsal subequatorial regions.

Since depolymerization of vegetal cortical microtubule by u.v.-irradiation prevents displacement of the determinants to the future dorsal region (Fujisue et al., 1993; Holowacz and Elinson, 1993, 1995), the system for transporting dorsal determinants must be mediated by vegetal cortical microtubules. Although we have no information about the transportation system, a recent report by Rowning et al. (1995) is of interest in this context. They have found a novel rapid transportation of organelles from the vegetal pole region to the future dorsal region along the array of cortical microtubules in uncleaved *Xenopus* eggs. It reportedly occurs during cortical rotation and needs polymerization of cortical microtubules. Such a transportation system might be involved as well in displacement of dorsal determinants from the vegetal pole to the future dorsal subequatorial region during cortical rotation.

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