

# Activation of dorsal development by contact between the cortical dorsal determinant and the equatorial core cytoplasm in eggs of *Xenopus laevis*

H. Kageura

Department of Biology, Faculty of Science, Fukuoka University, Fukuoka 814-01, Japan

## SUMMARY

In eggs of *Xenopus laevis*, dorsal development is activated on the future dorsal side by cortical rotation, after fertilization. The immediate effect of cortical rotation is probably the transport of a dorsal determinant from the vegetal pole to the equatorial region on the future dorsal side. However, the identity and action of the dorsal determinant remain problematic. In the present experiments, individual isolated cortices from various regions of the unfertilized eggs and embryos were implanted into one of several positions of a recipient 8-cell embryo. The incidence of secondary axes was used not only to locate the cortical dorsal determinant at different times but also to locate the region of the core competent to respond to the dorsal determinant. The dorsal axis-inducing activity of the cortex

occurred around the vegetal pole of the unfertilized egg. During cortical rotation, it shifted from there to a wide dorsal region. This is apparently the first evidence for the presence of a dorsal determinant in the egg cortex. The competence of the core of the 8-cell embryo was distributed in the form of gradient with the highest responsiveness at the equator. These results suggest that, in the normal embryo, dorsal development is activated by contact between the cortical dorsal determinant and the equatorial core cytoplasm, brought together through cortical rotation.

Key words: *Xenopus*, dorsal determinant, cytoplasm, rotation, axis determination, cortex

## INTRODUCTION

Specification of the dorsoventral axis is an early and important step in amphibian embryogenesis. The unfertilized *Xenopus* egg is radially symmetric and becomes bilaterally symmetric through cytoplasmic rearrangement within the first cell cycle after fertilization (cortical rotation) (Gerhart et al., 1989). The entire egg cortex rotates by an arc of 30° relative to the stationary subcortical cytoplasm (the core) in an animal-vegetal direction. Cortical rotation is normally oriented toward the sperm entry point and the side opposite that of sperm entry becomes the dorsal side of the embryo. However, it is not known what cytoplasmic modifications on the future dorsal side activate dorsal development. Ventral development does not require cortical rotation and occurs where dorsal development is not activated.

Dalcq and Pasteels (1937) pointed out that interaction between the core and cortex of the egg was important for dorsal development. They hypothesized a core factor ( $\beta$ ) in vegetal yolk and a cortical factor ( $\alpha$ ) formed by cortical rotation. They thought that both factors would be present in gradients, and that the concentration of  $\alpha$  would be maximal in the center of the grey crescent and that of  $\beta$  at the vegetal pole (see Pasteels, 1964 and Brachet, 1977). According to their theory, the whole fate map of an embryo is determined by local values of  $\beta$ . The dorsal development is activated at the side where the value of  $\beta$  is greatest, namely, at the equatorial surface position where vegetal yolk and the grey crescent meet. However, it is generally accepted that cortical rotation determines only the 'Nieuwkoop center' that leads to the organizer, and that the

developmental fate of each egg region is determined by the subsequent mesoderm induction (Nieuwkoop, 1973; Smith, 1989; Kimelman et al., 1992; Cornell et al., 1995) and neural induction (Spemann, 1938).

Direct support for the presence of the cortical factor hypothesized by Dalcq and Pasteels was obtained by Curtis (1960, 1962), who reported that a secondary axis was induced when a piece of grey crescent cortex of an early embryo was transplanted in the ventroequatorial region of another early embryo. However, Gerhart et al. (1981) pointed out that, in Curtis' experiments, a control was not done to show that non-grey crescent cortex would not have this effect. Furthermore, they reported that twinning could be obtained simply by exposing eggs to some of the experimental conditions used by Curtis, without transplantation of the grey crescent cortex; i.e., when the egg was removed from the vitelline membrane and allowed to settle onto an agar surface with its ventral side uppermost, twins were produced. In Curtis' experiments, eggs receiving grafts in the ventroequatorial region were turned slightly with this side uppermost to receive the graft. This was the condition for twinning by gravity. Gerhart et al. (1981) suggested that twinning in Curtis' experiments took place by inadvertent rotation of the cytoplasmic contents and extreme flattening of the egg, rather than by action of the transplanted grey crescent cortex.

Black and Gerhart (1986) centrifuged fertilized uncleaved eggs twice in opposite directions and showed that the grey crescent was not an exclusive axis determinant because secondary axis formation could be elicited well after the grey crescent had formed. They concluded that the grey crescent was only an

epiphenomenon produced by cortical rotation. The cortical dorsal determinant was sought somewhere other than the grey crescent.

In blastomere transplantation and recombination experiments, it was suggested that a dorsal determinant occurred in the dorsovegetal region of the normal embryo, but not the dorsoanimal region (Nieuwkoop, 1969a; Gimlich and Gerhart, 1984; Gimlich, 1986; Kageura and Yamana, 1986; Kageura, 1990). The region with the dorsal axis-inducing activity (dorsal activity) differed from the grey crescent region, but the former overlapped with the latter at the dorsoequatorial region. The presence of the dorsal determinant in the dorsovegetal region was confirmed by experiments of cytoplasmic transplantation at the 16-cell stage (Yuge et al., 1990; Fujisue et al., 1993; Holowacz and Elinson, 1993). The dorsal determinant was transplantable from one embryo to another by cytoplasmic injection, to give secondary axes.

Another line of support for the presence of a dorsal determinant was obtained from UV-irradiation experiments. A low dose of UV irradiation to an oocyte does not block the cortical rotation but can effectively abolish axis specification (Holwill et al., 1987; Elinson and Pasceri, 1989; Gerhart et al., 1989). The dorsal development of these UV-irradiated eggs can be restored by injection of cortical cytoplasm taken from the vegetal pole of a donor egg (Holowacz and Elinson, 1993). Since UV irradiation penetrates only 5-10  $\mu\text{m}$  or less into the egg (Grant and Wacaster, 1972), the determinant seemed to be located very close to the surface. Furthermore, the dorsal activity, as assayed by cytoplasmic transfer, shifts from the vegetal pole to the dorsal region in the course of cortical rotation (Fujisue et al., 1993). These findings suggest that the dorsal determinant originating from the vegetal cortical cytoplasm of fully grown oocytes is translocated from the vegetal region to the dorsal equatorial region by cortical rotation. How the dorsal determinant leads to dorsal development at one position of the equator is unknown because the factor of the cytoplasmic core that interacts with the dorsal determinant is unknown.

The present experiments were done to examine the mechanisms of activation of dorsal development. The combination of the core and cortex that activates dorsal development was examined by cortical transplantation (artificial rearrangement of the core and cortex). The incidence of secondary axes was used to locate not only the dorsal determinant but also the region of the core with the greatest competence to respond to the dorsal determinant. The cortical dorsal activity was displaced by cortical rotation from the vegetal pole to a wide dorsal region extending far into the animal hemisphere, and the competence of the stationary core was localized within the equatorial region of the egg. Thus, in the normal embryo, dorsal development is probably activated by contact between the cortical dorsal determinant and the equatorial core cytoplasm, brought together through cortical rotation.

## MATERIALS AND METHODS

### Collection of eggs

To obtain cortices from well-defined regions of eggs and to implant them into well-defined regions, it was essential to have eggs with a symmetrical pigmentation pattern and a regular cleavage pattern. Females that had ovulated a high proportion of such cleavage eggs were selected; males were chosen at random.

Fertilized eggs of *Xenopus laevis* were obtained by natural

amplexus of the males and females after injection of human chorionic gonadotropin (200 IU). The eggs with a symmetrical pigmentation pattern and cleavage pattern were selected. In such eggs, the first cleavage plane coincides with the bilateral plane of the embryo. Unfertilized eggs were obtained by squeezing the females that had laid the fertilized eggs. The fertilized and unfertilized eggs were dejellied in 2.5% cysteine (adjusted to pH 9.0 with 10 N NaOH), washed with 100% Steinberg solution and sterilized with 0.1% sodium p-toluenesulfone chloramide (chloramine T) for 1 minute. They were then transferred to 100% Steinberg solution in a Petri dish coated with 2% agarose and their vitelline membranes were removed manually with forceps.

### Cortical isolation and implantation

Curtis (1960, 1962) removed a small piece of grey crescent cortex from a donor egg and grafted it into a cut, which was made with a tungsten needle, on the surface of a recipient 1- to 2-cell egg. However, opening out a cut on the surface of a recipient egg causes such extensive cytoplasmic leakage that it proved impossible to graft a piece of cortex isolated from a donor egg into a cut as Curtis had done. Therefore, an isolated piece of cortex from a donor was inserted into the peripheral core of a recipient embryo with a glass needle.

Pieces of cortices of various shapes and sizes (0.4 $\times$ 0.4 mm-0.4 $\times$ 9 mm) were cut and stripped from donor eggs and embryos with a glass needle. All operations were done in 100% Steinberg solution. As the isolated cortices were strongly adhesive, the glass needle was coated with 2% agarose before use. The isolated cortices quickly shrank and then relaxed. An isolated cortex was caught on the tip (1-5  $\mu\text{m}$  in diameter) of a glass needle, which was inserted into the fixed position of a recipient 8-cell embryo and then quickly withdrawn. Part of the cortex was thus inserted into the recipient. The part of the cortex left outside was squeezed into the recipient, using the same needle. The small wound quickly healed and there was little leakage of cytoplasm. The cell operated thus cleaved normally.

The cortex of unfertilized eggs was too soft to remove. In the present experiments, pricked unfertilized eggs were used as source of the cortex, prior to cortical rotation. After being pricked, unfertilized eggs underwent activation contraction as do fertilized eggs (Kirschner et al., 1980), and their cortices hardened. Pieces of cortex could be removed from the eggs 10 to 20 minutes after pricking and used for implantation experiments. In the normal embryo, cortical rotation begins 45 minutes after fertilization (Gerhart, 1989).

To prevent eggs from twinning by gravity, 8-cell embryos were used as recipients, in all experiments, because the third cleavage planes prevent rotation of cytoplasmic contents. The recipient embryos were slightly inclined to cortical implantation but only for a few seconds. After cortical implantation, they were quickly transferred to a Petri dish filled with 100% Steinberg solution and dropped into round wells with 2% agarose, using a glass template. They were then returned to their natural orientation, vegetal pole down, in the 1.3 mm diameter wells. As the wells were a good fit for the recipients, flattening of the recipients did not occur. The medium was gradually changed to 10% Steinberg solution (with 50 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin) by midblastula stage, to prevent exogastrulation. All operations and culturing of embryos were performed on a clean bench and under aseptic conditions. When the ventroanimal and ventroequatorial cortices were implanted into the ventroequatorial core of the recipients with competence to the dorsal determinant, secondary axes were not induced. Thus, it was clear that twinning in the present experiments was not caused by the procedures used or by the conditions used for cortical implantation.

When the embryos reached stage 26 (early tailbud stage, about 30 hours after fertilization; Nieuwkoop and Faber, 1967), they were examined macroscopically. The incidence of secondary axes was calculated. In an individual double embryo, the ratio of the length of the secondary axis relative to the primary axis was determined, using an ocular micrometer.

### Histological examination

The major purpose of this histological examination was to measure thickness of the isolated cortices. For light microscopy, cortices were removed from dorsovegetal and animal regions of 2-cell embryos with a glass needle in 100% Steinberg solution and immediately fixed with Bouin's solution for 1 hour. They were stained with carmine, dehydrated through a graded series of ethanol, embedded in paraffin and sectioned perpendicularly at 6  $\mu$ m. The specimens were dewaxed and stained with haematoxylin-eosin.

## RESULTS

### Development of recipient embryos

For each experiment, 50 recipients were prepared (Tables 1-4). Some recipients died during cleavage and blastulation, mainly because of abnormal divisions ('early deaths' in the tables). The remainder developed into 'normal embryos' or 'abnormal ones'. The abnormal embryos were divided into two groups, 'embryos with a secondary axis' and 'other abnormal embryos.' Other abnormal embryos contained three kinds of abnormal embryos such as 'head deformity', 'small head' and 'incomplete invaginated embryo'.

For embryos with a secondary axis, the relative length of the secondary axis varied from 0.40 to 0.95 and there were none under 0.40 and over 0.95. As length of the secondary axis was progressively reduced, dorsal axial structures were sequentially reduced and lost in an anterior-posterior progression. Embryos with a secondary axis were classified into three groups based on the relative length of the secondary axis.

Secondary axes of the largest class (0.80-0.95) had an almost complete set of axial structures. In some cases, eyes and a cement gland were smaller than those of a normal embryo and were absent in some other cases. Secondary axes of the middle class (0.60-0.75) had an incomplete head, though their axial structures were almost complete in the trunk and tail regions. Their eyes were often smaller than those of a normal embryos. Their eyes sometimes fused at the front of the head and sometimes were absent. In most cases, their cement glands were absent. In the small class (0.40-0.55), secondary axes had a small and vacuolated head in some cases, but the head was absent in other cases. Their dorsal axial structures were slender and the protrusion from the host was weak. The segmentation of somites was not clear, but many melanophores developed along the axial structures. In most cases, the axial structures ended at the anus of the host. The tail fin rarely protruded from the belly of the host.

A piece of vegetal cortex peeled from a donor 2-cell embryo

was stained with Nile blue sulfate and implanted into the ventroequatorial region of a ventrovegetal cell of the recipient 8-cell embryo to trace the fate of the transplanted cortices. The implanted cortex did not immediately dissociate and mix with core cytoplasm of the recipient. One or two cells of the 32-cell embryo were labelled with Nile blue sulfate. At the tailbud stage, some labelled cells were found in anterior axial mesoderm of the secondary embryo, but most of labelled cells were found in dorsoanterior and dorsomiddle endoderm of the secondary embryo. This result supported the notion that axial mesoderm was 'induced' by cells that contained the dorsal determinant.

### Localization and displacement of dorsal activity in the egg cortex

Each piece of cortex isolated from various regions of the eggs before and after cortical rotation was implanted into the ventroequatorial core of a recipient 8-cell embryo (Tables 1-3).

A pricked unfertilized egg was subdivided into five animal-vegetal zones (Fig. 1A). The pigmented animal half comprised a circular region around the animal pole (AnI) and a ring-shaped subequatorial zone (AnII). The vegetal half consisted of three zones comprising a ring-shaped equatorial zone (VgI), a ring-shaped subequatorial zone (VgII) and a circular region around the vegetal pole (VgIII). Pieces of cortices were removed from half of each AnI or VgIII region and from a quarter of each AnII, VgI or VgII region. The area of a transplanted cortex was approximately 0.20 mm<sup>2</sup> in AnI and VgIII cortices, 0.29 mm<sup>2</sup> in AnII and VgII cortices, and 0.35 mm<sup>2</sup> in VgI cortex.

When animal cortices were implanted from unfertilized donors into 8-cell recipients, secondary axes were not formed, except in 1 case of AnII implantation (Table 1). In implantations of vegetal cortices, a high incidence of secondary axes (96%) was observed in the VgIII cortex. The VgI and VgII cortices induced secondary axes only in one and two cases out of 50, respectively. The AnII, VgI and VgII cortices induced secondary axes of the small and middle classes. Secondary axes of the largest class were induced only by VgIII implantation, in 8 of 50 cases (Fig. 2A,B). The results are summarized in Fig. 1A. The dorsal activity was strongly localized around the vegetal pole of the unfertilized egg. The average relative length of the secondary axes was 0.69 in VgIII implantation.

With the 2-cell donor embryos, cortices from eight different regions were examined. Each region was named, as shown in Fig. 1B. Isolated cortices were approximately 0.4×0.4 mm in size. For implantations of all kinds of dorsal cortices, the incidence of secondary axes was high, including regions that

**Table 1. Localization of dorsal activity in cortex of the unfertilized egg**

Implanted cortices*	Embryos with a secondary axis				Other abnormal embryos	Normal embryos	Early deaths
	Total	0.40-0.55	0.60-0.75	0.80-0.95			
AnI	0	0	0	0	Head deformity (1)	46	3
AnII	1	1	0	0	0	47	2
VgI	1	0	1	0	Head deformity (2)	44	3
VgII	2	0	2	0	Head deformity (2)	45	0
VgIII	48	2	38	8	Incomplete invaginated embryo (1)	1	1
					0		

\*Cortices were implanted into the VV position of the 8-cell embryo.

**Table 2. Localization of dorsal activity in cortex of the 2-cell embryo**

Implanted cortices*	Embryos with a secondary axis				Other abnormal embryos	Normal embryos	Early deaths
	Total	0.40-0.55	0.60-0.75	0.80-0.95			
DA	27	4	22	1	0	22	1
DE	35	4	26	5	Incomplete invaginated embryo (1)	13	1
DV	45	10	28	7	0	3	2
DLE	1	0	1	0	Small head (1)	48	0
VLE	0	0	0	0	0	50	0
VA	1	1	0	0	Incomplete invaginated embryo (1)	47	0
VE	1	0	1	0	Head deformity(1)	47	1
VV	4	2	2	0	Incomplete invaginated embryo (1)	46	0
					0		

\*Cortices were implanted into the VV position of the 8-cell embryo.

had been inactive in the unfertilized egg (Table 2). However, it was highest for the DV cortex and it decreased in the direction of the animal pole (DV, 90%; DE, 70%; DA, 54%). In implantations of lateral and ventral cortices, only in 7 clear cases were secondary axes of the small and middle classes induced. The results in the 2-cell donor embryos are summarized in Fig. 1B. As the implanted ventral cortices did not induce secondary axes, it is clear that twinning in the present experiments was not caused by gravity or by the operation. Although the dorsal activity was mainly contained in the DV cortex, it also extended into the DE and DA cortices, i.e., well into the animal hemisphere. For these cortices, the average relative lengths of the secondary axes were similar (0.64-0.68). It is noteworthy that the dorsal activity was almost negligible in the VV cortex.

At the 32-cell stage, the donor embryos are bilaterally symmetric and could be subdivided into 32 regions, according to cleavage planes (Fig. 1C). The nomenclature used for each cell was that of Nakamura and Kishiyama (1971). As a cortex taken from one donor cell at the 32-cell stage was insufficient to induce a secondary axis, two cortices were taken from two cells from bilaterally symmetrical positions of a donor embryo and implanted into the ventroequatorial core of one cell of a recipient 8-cell embryo. The area of a pair of transplanted

cortices was approximately 0.21 mm<sup>2</sup> in A1-4 cells, 0.27 mm<sup>2</sup> in B1-4 cells, 0.25 mm<sup>2</sup> in C1-4 cells, and 0.40 mm<sup>2</sup> in D1-4 cells. All kinds of dorsal cortices (column 1) initiated secondary axes (Table 3); however, the incidence varied from one cortical region to the next. The cortex inducing the highest incidence of secondary axes was D1. The incidence of secondary axes became lower in the direction of the animal pole (D1, 82%; C1, 50%; B1, 42%; A1, 14%). In these implantations, most of the induced secondary axes were of small and middle class (Fig. 2C). Secondary axes of the largest class developed only in two cases of D1 implantation. In implantations of dorsolateral, ventrolateral and ventral cortices (column 2-4), secondary axes of the small and middle classes rarely developed (Fig. 2D) except in the A2 cortex implantation (10%). The average relative length of the secondary axes for the A2 cortex (0.56) was small and the dorsal activity was almost negligible in this cortex.

The results in the 32-cell donor embryos are summarized in Fig. 1C. The dorsal activity was widely evident in dorsal cortices (A1, B1, C1 and D1) and it was highest in the D1 cortex. The dorsal activity was reduced in the direction of the animal pole: D1, C1, B1 and A1. The average relative lengths of the secondary axes for these cortices (0.59-0.61) were not so large as those for DV, DE and DA cortices of the 2-cell

**Table 3. Localization of dorsal activity in cortex of the 32-cell embryo**

Implanted cortices*	Embryos with a secondary axis				Other abnormal embryos	Normal embryos	Early deaths
	Total	0.40-0.55	0.60-0.75	0.80-0.95			
A1	7	2	5	0	0	42	1
B1	21	8	13	0	0	28	1
C1	25	8	17	0	0	24	1
D1	41	12	27	2	0	6	3
A2	5	3	2	0	0	44	1
B2	2	2	0	0	0	48	0
C2	0	0	0	0	0	50	0
D2	1	0	1	0	0	48	1
A3	1	1	0	0	0	48	1
B3	0	0	0	0	0	49	1
C3	0	0	0	0	0	48	2
D3	2	2	0	0	0	47	1
A4	0	0	0	0	0	50	0
B4	1	0	1	0	Head deformity (1)	46	2
C4	0	0	0	0	Small head (1)	49	0
D4	0	0	0	0	0	48	2

\*Cortices were implanted into the VV position of the 8-cell embryo.

**Table 4. Localization in the 8-cell embryo of core competence to respond to dorsal determinant**

Implanted positions*	Embryos with a secondary axis				Other abnormal embryos	Normal embryos	Early deaths
	Total	0.40-0.55	0.60-0.75	0.80-0.95			
VP	0	0	0	0	Small head (1)	45	4
V30°	1	0	1	0	Incomplete invaginated embryo (2)	47	0
V45°	11	1	10	0	0	37	2
V60°	35	4	29	2	0	15	0
VV	46	11	33	2	0	1	3
AV	6	3	3	0	Small head (1)	42	0
AP	0	0	0	0	Head deformity (1)	48	0
VD	0	0	0	0	Incomplete invaginated embryo (2)	44	5
					Head deformity (1)		

\*The vegetal cortices of the 2-cell embryos were implanted.

donor embryo (0.64-0.68), but distribution of the dorsal activity in the 32-cell donor embryo (Fig. 1C) was similar to that in the 2-cell embryo (Fig. 1B).

These results suggested that the dorsal activity was displaced by cortical rotation from the vegetal cortex to a wide region of dorsal cortex extending into the animal hemisphere, and was retained in the dorsal cortex at least until the 32-cell stage.

#### Localization of competence to respond to the cortical dorsal determinant in the cytoplasmic core of an 8-cell embryo

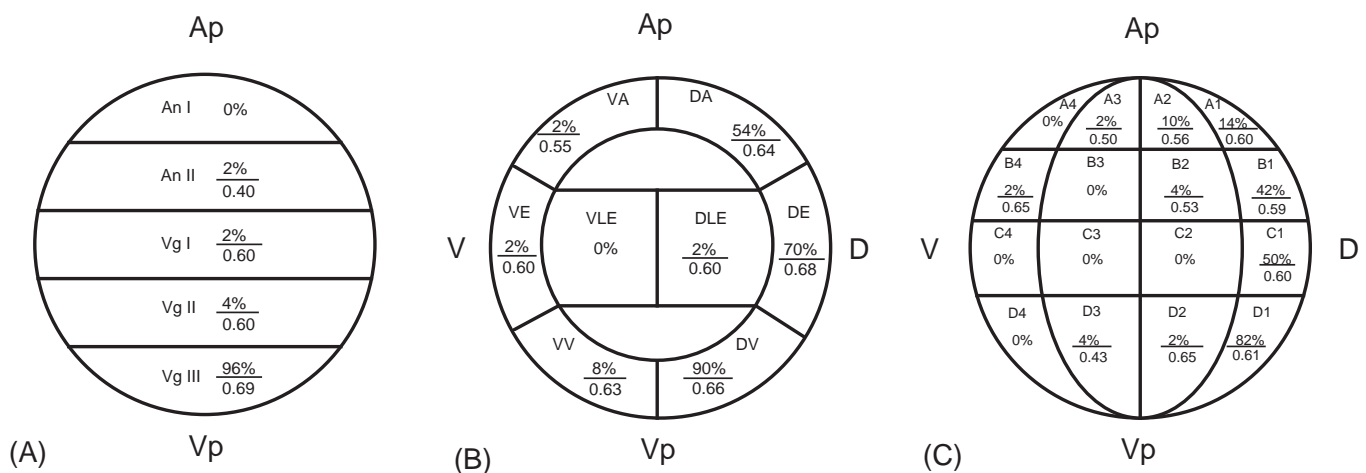
To examine distribution of the competence of the core cytoplasm to respond to the cortical dorsal determinant in the 8-cell recipient embryo, cortices from the vegetal pole of 2-cell donor embryos were implanted into the core at various positions close to the first cleavage plane of 8-cell embryos (Table 4). The implanted cortex had an area of about 0.4 mm $\times$ 0.4 mm. Each implanted position was named as shown in Fig. 3.

When vegetal cortices were implanted into the VP position of the recipients, no secondary axis was induced. In implanta-

tion into the V30° position, secondary axes developed in only one case. In implantations into all equatorial positions, secondary axes were induced (Fig. 4A). The incidence of secondary axes was highest for the VV position and it decreased in the direction of the vegetal pole (VV, 92%; V60°, 70%; V45°, 22%). In these implantations, most of the induced secondary axes were of small and middle class, and secondary axes of the largest class rarely developed.

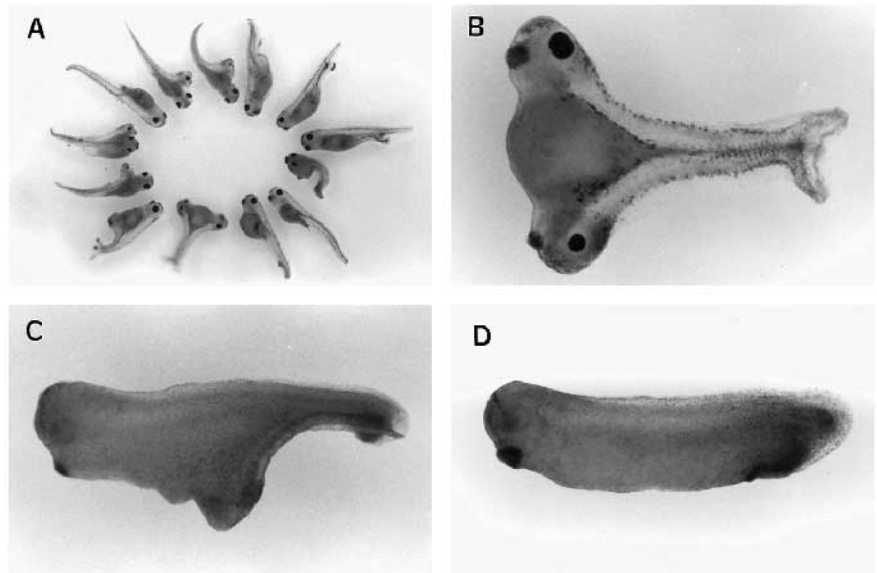
Although some grafts (12%) implanted into the AV position of the recipients induced secondary axes, no graft implanted into the AP position initiated a secondary axis. Implantation into the VD position did not induce dorsalized embryos, and only normal embryos were obtained (Fig. 4B).

The results summarized in Fig. 3 provided information on localization of competence to respond to the dorsal determinant. The competence occurred in the ventroequatorial region of an 8-cell embryo in the form of a gradient and was highest just under the third cleavage plane. Though a weak competence was also found in ventroanimal regions, there was no competence around the animal pole. For implantations into equatorial positions, the average relative lengths of the secondary axes were similar (0.63-0.65).



**Fig. 1.** Localization of dorsal activity in the egg and embryos. Each isolated cortex (0.4 $\times$ 0.4 mm-0.4 $\times$ 0.9 mm) was implanted into the ventroequatorial region of a ventrovegetal cell of an 8-cell embryo. For each region, 50 recipients were prepared by cortical implantation. The incidence of secondary axes is shown by a percentage written above the line, and the average relative length of the secondary axis is indicated by a number written below the line. Ap, animal pole; Vp, vegetal pole; D, dorsal; V, ventral. (A) A pricked unfertilized egg. (B) A 2-cell embryo. (C) A 32-cell embryo.

**Fig. 2.** Embryos derived from the 8-cell embryos into which a piece of cortex of eggs or embryos was implanted. The implantation site was the ventroequatorial region of a ventrovegetal cell. (A) Thirteen pairs of unequal twins derived from the recipients into which a piece of the VgIII cortex of the pricked unfertilized egg was implanted (stage 41; lateral view). (B) Unequal twins derived from the recipient into which a piece of the VgIII cortex of the pricked unfertilized egg was implanted (stage 41; lateral view). This secondary axis is one of the largest induced by cortex implantation. (C) Unequal twins derived from the recipient into which two D1 cortices of the 32-cell embryo was implanted (stage 31; lateral view). (D) A normal embryo derived from the recipient into which two D4 cortices of the 32-cell embryo was implanted (stage 31; lateral view).



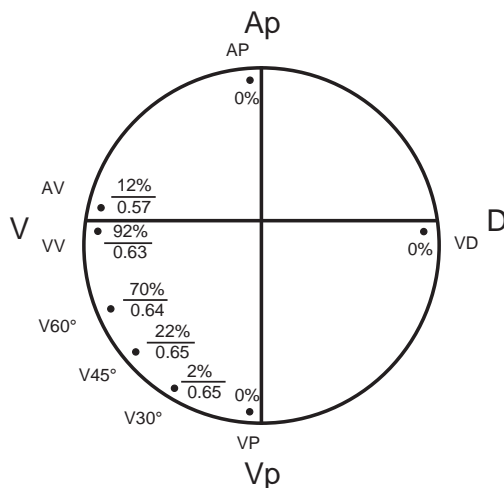
### Structure of isolated cortices

The structure of the isolated animal and dorsovegetal cortices from 2-cell embryos was examined by light microscopy. The structure of both cortices was similar, 4–8  $\mu\text{m}$  thick and consisted of two layers. The outer layer containing many pigment granules was a rigid peripheral unit, about 2  $\mu\text{m}$  thick. The density of pigment granules of this layer was higher in the animal cortex (Fig. 5A) than in the dorsovegetal cortex (Fig. 5B). The 2–6  $\mu\text{m}$  thick inner layer was a yolky layer and

contained few pigment granules. The diameter of the yolk platelets of this layer was 1–3  $\mu\text{m}$  in the case of the animal cortex and 2–6  $\mu\text{m}$  in the case of the dorsovegetal cortex.

The cortex is defined in different ways depending on the method of isolation or observation; an ‘in vivo cortex’ and a ‘peeled cortex’. Cortical rotation was observed in the living egg and it was shown that yolk platelets and microtubules were not associated with the in vivo cortex but rather with the core (Houliston and Elinson, 1991; Larabell et al., 1996). On the contrary, the peeled cortex contained yolk platelets and microtubules (Elinson and Rowning, 1988; Elinson et al., 1993), probably because some yolk platelets and microtubules are pulled off with the in vivo cortex, which is thinner than the peeled cortex.

The present results show that the dorsal determinant is very close to the surface in a thin layer, but there is no evidence that the dorsal determinant actually associates with the in vivo cortex. Observations of Rowning et al. (1997) suggest that the dorsal determinant is in the microtubule layer, at the surface of the core and that during cortical rotation, the determinant is transported from the vegetal pole to the dorsal equator.

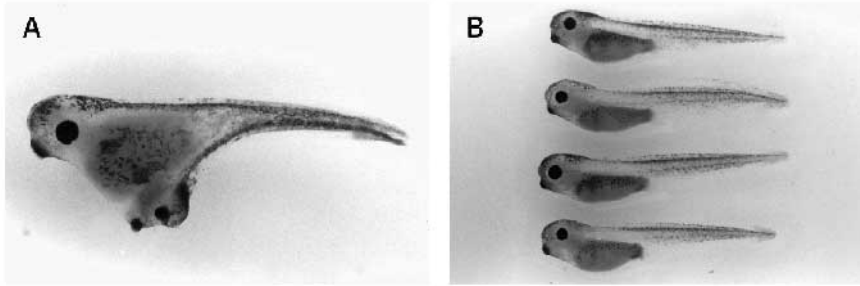


**Fig. 3.** Localization in the 8-cell embryo of the core competence to respond to the dorsal determinant. For each position, 50 recipients were prepared by implantation of the vegetal cortex of the 2-cell embryo. The incidence of secondary axes is shown by a percentage written above the line and the average relative length of the secondary axis is indicated by a number written below the line. Ap, animal pole; Vp, vegetal pole; D, dorsal; V, ventral. VP, at the vegetal pole of the vegetal ventral cell; V30°, at latitude 30° south of the vegetal ventral cell; V45°, at latitude 45° south of the vegetal ventral cell; V60°, at latitude 60° south of the vegetal ventral cell; VV, at the 90° south of the vegetal ventral cell; AV, at latitude 90° north of the animal ventral cell; AP, at the animal pole of the animal ventral cell; VD, at the latitude 90° south of the vegetal dorsal cell.

## DISCUSSION

### Experiments of Curtis

As pointed out by Gerhart et al. (1981), Curtis (1960, 1962) did not perform a control to show that he did not get a secondary axis when a ventral cortex was grafted to the ventral side. In the present experiments, such controls were used with the animal cortex as well as the ventral cortex. Curtis claimed that the 1- and 2-cell embryos were competent to the cortical dorsal determinant, whereas the 8-cell embryo was not. However, it is now known that the 8-cell embryo has competence. The dorsal activity of the grey crescent cortex is weak. Curtis transplanted a small piece of grey crescent cortex (0.15 × 0.15 mm), but he often got a secondary axis as large as the primary one. All these facts support the suggestion of Gerhart et al. (1981), i.e., the twinning in the Curtis experi-



**Fig. 4.** Embryos derived from 8-cell embryos into which the vegetal cortex of the 2-cell embryo was implanted (stage 41; lateral view). (A) Unequal twins. The implanted position is the VV. (B) Normal embryos. The implanted position is the VD.

ments resulted from inadvertent rotation of the cytoplasmic contents and extreme flattening of the egg, rather than the action of the transplanted grey crescent.

### Translocation of the dorsal determinant by cortical rotation

Fujisue et al. (1993) found in experiments of cytoplasmic transplantation that the dorsal activity was displaced from the vegetal pole to the dorsoequatorial region by cortical rotation. However, intracellular localization of the dorsal activity in the donor egg was not clear, because about 30 nl of cytoplasm was withdrawn from each donor and injected into each recipient to detect the dorsal activity. This volume was about 40% of an average vegetal cell volume of the 16-cell embryo.

In the present experiments, peeled cortices were used to locate the dorsal determinant in the egg. The peeled cortex consisted of the *in vivo* cortex, the shear zone and the surface of the core. Hereafter, to distinguish between these two cortices, the peeled cortex is represented as the 'cortical peel'. Though not as thin as the *in vivo* cortex, the implanted cortical peel was remarkably thin (4–8  $\mu\text{m}$  thick) and was 1–4 nl in volume (0.1–0.4% of the whole embryo). Despite the small volume, the dorsal activity of implanted dorsal peel was higher than that of injected dorsal cytoplasm in cytoplasmic transfer experiments (Yuge et al., 1990; Fujisue et al., 1993). The dorsal activity of the cortical peel with a thick yolky layer (20–30  $\mu\text{m}$ ) was identical with that of the cortical peel with a thin yolky layer (2–6  $\mu\text{m}$ ) used in the present experiments (unpublished data). In case of cytoplasmic transfer, only when the surface cytoplasm close to the cortex was withdrawn using a pipette with a flat end at the tip could cytoplasm be obtained with the dorsal activity (Yuge, M., personal communication). Thus, the dorsal determinant is not freely diffusible in the egg core.

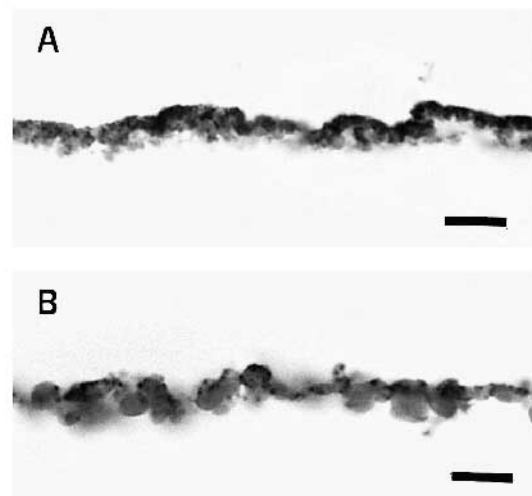
In the unfertilized egg, the dorsal activity of the cortical peel is strongly localized around the vegetal pole. In the 2-cell embryo, the dorsal activity of the cortical peel is mainly localized in dorsovegetal region but also extends into the dorsoequatorial and dorsoanimal regions; it is not present in lateral and ventral regions. The major part of the dorsal activity shifts from the vegetal pole to the dorsovegetal region, but probably not to the dorsoequatorial region by cortical rotation. During the cortical rotation, the entire egg cortex rotates by only 30° relative to the core in an animal-vegetal direction (Gerhart et al., 1989). The dorsal activity in the dorsovegetal region of the 2-cell embryo can be explained by this amount of cortical rotation, but the dorsal activity in the dorsoanimal and dorsoequatorial regions cannot. It is likely that part of the dorsal determinant continues to move for some time after cortical rotation. As distribution of dorsal activity in the 2-cell embryos

is similar to that in the 32-cell embryo, transfer of the dorsal determinant is most likely completed by the end of 2-cell stage.

The distribution of the dorsal determinant in the oocyte and unfertilized egg is similar to those of *Xlirts*, *Xcat2* and *Xwint11* RNAs in stage 4–6 oocytes (Kloc and Etkin, 1995; Forristall et al., 1995), but after cortical rotation the distribution is quite different.

### Activation of dorsal development

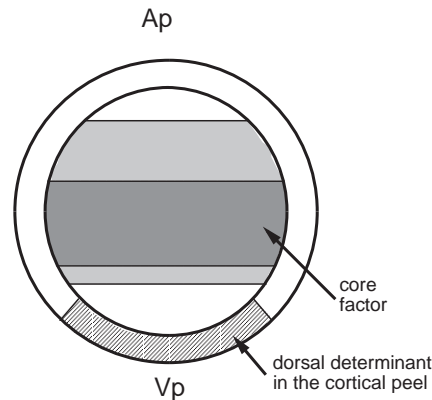
Activation of dorsal development requires a core factor as well as the dorsal determinant. In the present experiments, competence to respond to the dorsal determinant was detected only in the ventroequatorial core of the egg in the form of gradient with the highest responsiveness at the equator. However, the competence also occurs in the dorsoequatorial core, because the primary embryo is formed in this region. It is natural to think that the core competence occurs in a radial-symmetric distribution in the equatorial core, because many experiments indicate that the egg initially has the capacity to develop dorsally at any, several, or even all meridians. Dalcq and Pasteels (1937) thought that a core factor occurred in combination with large yolk platelets of the vegetal region, but the



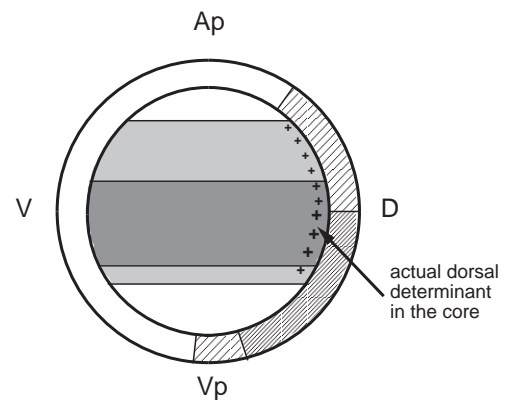
**Fig. 5.** Isolated cortices. The isolated cortices are 4–8  $\mu\text{m}$  thick and consist of two layers. The outer layer is a rigid, parallel peripheral unit, about 2  $\mu\text{m}$  thick. The inner layer is a yolky layer, 2–6  $\mu\text{m}$  in thickness. Bar, 10  $\mu\text{m}$ . (A) The isolated animal cortex. The outer layer contains many pigment granules. The inner layer contains small yolk platelets. (B) The isolated dorsovegetal cortex. In the outer layer, pigment granules are sparse. The inner layer contains large yolk platelets.

**Fig. 6.** A model for activation of dorsal development. Ap, animal pole; Vp, vegetal pole; D, dorsal; V, ventral. (A) An unfertilized egg (before cortical rotation). The dorsal determinant is strongly localized in the cortical peel around the vegetal pole. The cortical peel was remarkably thin and 4–8  $\mu\text{m}$  thick. A ‘core factor’, which interacts with the dorsal determinant for activating dorsal development, occurs within the equatorial zone of the core in the form of a gradient and its maximum is centered on the equator. (B) An early embryo (after cortical rotation). The dorsal determinant has been transported

(A) Before cortical rotation



(B) After cortical rotation



from the vegetal pole to the dorsovegetal region. Although the dorsal determinant mainly occurs in the dorsovegetal region, it also extends into the dorsoequatorial and dorsoanimal regions, in some cases. On the other hand, location of the core factor is not changed by cortical rotation. A hypothetical active factor, that is, an ‘actual dorsal determinant’ is formed in dorsoequatorial region of the core by the interaction between the dorsal determinant and the core factor. Finally dorsal development is activated on the future dorsal side by action of the actual dorsal determinant.

present results suggest that it occurs in combination with medium-sized yolk platelets of the equatorial region.

The spatial distribution of the dorsal activity was also examined in previous blastomere transplantation experiments at the 32-cell stage (Kageura, 1990). The dorsal activity was localized in a comparatively small dorsovegetal region of the 32-cell embryo centered on the C1 pair of cells. Although this distribution of the dorsal activity is similar, in the present experiments, the center of dorsal activity is localized on the D1 pair of cells while, in the blastomere transplantation experiments, it is on the C1 pair of cells).

This discrepancy suggests the existence of a subsequent active determinant, i.e., the actual dorsal determinant, which results from interaction between the cortical dorsal determinant and the equatorial core cytoplasm. This is probably the factor detected in the blastomere transplantation experiments, because the arrangement or combination of the core and cortex could not change in the transplanted blastomeres. In experiments of cytoplasmic transplantation (Fujisue et al., 1993), the dorsal activity suddenly increased in the dorsal cytoplasm at the 4-cell stage. This observation suggests that the actual dorsal determinant appears in the somewhat deep core cytoplasm and that it can be easily withdrawn from the embryo with a pipette.

In the present cortical peel implantation experiments and the previous cytoplasmic transplantation experiments (Yuge et al., 1990; Fujisue et al., 1993; Holowacz and Elinson, 1993, 1995), induced secondary axes were not as large as those in the blastomere transplantation experiments (Kageura, 1990) and no equal twins developed. This means that the actual dorsal determinants produced by cortical peel implantation or cytoplasmic transplantation are fewer than those produced by cortical rotation.

In the blastomere defect experiments (Kageura, 1995), the 32-cell embryos from which all vegetal blastomeres (D1–D4) were removed developed into tadpoles having almost complete axial structures. On the contrary, the 32-cell embryos from which all equatorial blastomeres (C1–C4 and B1–B4) had been

removed, developed into incompletely invaginated embryos. The dorsovegetal information may be located in both prospective mesoderm and prospective endoderm of the equatorial zone, but it may not occur in the prospective endoderm around the vegetal pole (Kageura, 1990). When Nieuwkoop (1969b) combined the animal cap with the whole prospective endoderm in early to late blastulae of *Ambystoma mexicanum*, about 20% of these recombinants developed into embryos with nearly complete axial structures. In his experiments, the whole prospective mesoderm was omitted, but the equatorial endoderm remained.

Based on all these observations, a model for activation of dorsal development can be proposed. The unfertilized egg has a dorsal determinant localized in the ‘cortical peel’ around the vegetal pole (Fig. 6A). It has also another factor in the equatorial core, which interacts with the dorsal determinant to activate dorsal development. This factor is defined as a ‘core factor’. The core factor is distributed in the form of gradient with the highest concentration at the equator and is not distributed around the animal and vegetal poles. As the dorsal determinant is separate from the core factor in the unfertilized egg, dorsal development cannot be initiated.

After fertilization, the cortex rotates in the animal-vegetal direction, upward on the future dorsal side (the side opposite that of sperm entry). The dorsal determinant is transported from the vegetal pole to the future dorsal region by cortical rotation. After cortical rotation, the dorsal determinant mainly exists in the dorsovegetal region, but it also extends into the dorsoequatorial and dorsoanimal regions (Fig. 6B). However, location of the core factor does not change during cortical rotation. A hypothetical active determinant appears in the dorsoequatorial region of the core as a result of interaction between the dorsal determinant and the core factor. By contrast with the dorsal determinant of the cortical peel, this determinant of the core is defined as a ‘actual dorsal determinant’. Dorsal development is activated on the future dorsal side by action of the actual dorsal determinant.



Finally, it is concluded that only the cortical dorsal determinant that comes in contact with the equatorial core cytoplasm is effective in activating dorsal development and that the cortical dorsal determinant transported to the dorsovegetal region remains unused.

In this model, three kinds of molecules may have a role; a dorsal determinant, a core factor and an actual dorsal determinant. The actual dorsal determinant is likely to be a modified dorsal determinant or a modified core factor. The actual dorsal determinant determines 'Nieuwkoop center' leading to the organizer and may be a modifier (such as *wnt* protein, *Noggin* protein and  $\beta$ catenin) of mesoderm inducers (Kimelman et al., 1992; Cornell et al., 1995). Though many molecules active in the ectopic axis formation have been discovered, only  $\beta$ catenin seems to be localized in the subcortical microtubule zone at the dorsal side of the egg at the end of cortical rotation (Rowning, 1997). A molecule localized in the equatorial core has not been described.

I am deeply indebted to Dr K. Yamana for pertinent advice and encouragement and to M. Ohara for helpful comments.

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(Accepted 6 February 1997)