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The effect of egg rotation on the differentiation of primordial germ cells in *Xenopus laevis*

J. H. CLEINE* AND K. E. DIXON

School of Biological Sciences, The Flinders University of South Australia, Bedford Park, S.A. 5042, Australia

SUMMARY

Eggs of *X. laevis* were rotated (sperm entrance point downwards) either through 90° (1×90 embryos) or 180° in two 90° steps (2×90 embryos) at approximately 25–30 min postfertilization after cooling to 13 °C. The embryos were kept in their off-axis orientation and cooled until the early gastrula stage. Rotation resulted in relocation of egg constituents with slight changes in the distribution of outer cortical and subcortical components and major changes in inner constituents where the heavy yolk and cytoplasm appeared to reorient as a single coherent unit to maintain their relative positions with respect to gravity. Development of rotated embryos was such that regions of the egg which normally give rise to posterior structures instead developed into anterior structures and *vice versa*. Germ plasm was displaced in the vegetal–dorsal–animal direction (the direction of rotation) and was segregated into dorsal micromeres and intermediate zone cells in 2×90 embryos and dorsal macromeres and intermediate zone cells in 1×90 embryos. In consequence, at the gastrula stage, cells containing germ plasm were situated closer to the dorsal lip of the blastopore after rotation – in 2×90 gastrulas around and generally above the dorsal lip. Hence, in rotated embryos, the cells containing germ plasm were invaginated earlier during gastrulation and therefore were carried further anteriorly in the endoderm to a mean position anterior to the midpoint of the endoderm. The number of cells containing germ plasm in rotated embryos was not significantly different from that in controls at all stages up to and including tail bud (stage 25). However at stages 46, 48 and 49 the number of primordial germ cells was reduced in 1×90 embryos in one experiment of three and in 2×90 embryos in all experiments. We tested the hypothesis that the decreased number of primordial germ cells in the genital ridges was due to the inability of cells to migrate to the genital ridges from their ectopic location in the endoderm. When anterior endoderm was grafted into posterior endodermal regions the number of primordial germ cells increased slightly or not at all suggesting that the anterior displacement of the cells containing germ plasm was not the only factor responsible for the decreased number of primordial germ cells in rotated embryos. Other possible explanations are discussed.

INTRODUCTION

The eggs of all anuran amphibians so far studied contain a distinctive region of yolk-free, vegetal cytoplasm which is obvious in histological sections. It is distributed around the vegetal pole of early embryos and during cleavage is segregated in such a way that it is contained within approximately four cells in the

**Author's present address*: Imperial Cancer Research Fund, Mill Hill Laboratories, Burtonhole Lane, London NW7 1AD, U.K.

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vegetal hemisphere of early blastula-stage embryos (reviewed in Dixon, 1981). After gastrulation, these cells lie in the posterior endoderm and shortly before the tadpole hatches they migrate dorsally to enter the developing genital ridges.

We became interested in using the technique of egg rotation to relocate within the embryo the cytoplasmic substance called germ plasm which is segregated to the presumptive primordial germ cells (reviewed by Dixon, 1981). The purpose of these experiments was to see whether relocating the germ plasm would affect the germ cell lineage. If so, the function of germ plasm in the differentiation of primordial germ cells might become clearer. As a preliminary to these experiments, it was necessary to investigate the effects of permanent rotation and inversion on development.

A brief report of these results was presented at the European Developmental Biology Conference, Strasbourg, June 1982 and at the British Society for Developmental Biology Meeting in August 1982.

MATERIALS AND METHODS

Eggs and embryos

Eggs were stripped from *X. laevis* previously injected with pregnant mare serum (Folligon, Intervet) and chorionic gonadotrophin (Chorulon, Intervet). They were fertilized using a macerated testis. After the pigment contracted, normally at 8–12 min after insemination, they were dejellied in 4 % sodium thioglycollate pH 8 in 5 mM-Hepes–NaOH buffer, then rinsed in a number of changes of filtered tap water. All embryos were staged according to the Normal Table of Nieuwkoop & Faber (1967).

Rotation

Eggs were cooled by immersing them in tap water at 13 °C, after the sperm entrance point became visible, normally 15–17 min after insemination. They were transferred to 5–7 % Ficoll in 25 % MMR saline (after Kirschner & Hara (1980) but omitting EDTA) in conical wells of microtiter trays (base of well 1·4 mm) and there rotated with a platinum wire loop, either through 90° or 180°, the latter in two equal steps with 40 min interval, normally with the sperm entrance point facing downwards on the first shift. Rotations were carried out at approximately 25–30 min after insemination and the eggs and trays were held on cooled metal plates during the whole procedure. After rotation, the eggs were kept in Ficoll at 13 °C until about stage 10 when they were returned to 21 °C and transferred to filtered tapwater.

Nile blue staining

Eggs in Ficoll solution were stained at the unpigmented pole with a small crystal of Nile red (Kirschner & Hara, 1980), which was removed after a suitable time. The crystals were applied and removed while the egg was turned briefly with the unpigmented pole upwards (<1 min).

All material for histological examination was fixed in Smith's fixative and embedded in paraffin. Serial sagittal sections of embryos up to stage 25 were stained according to the following procedure: 2 % Aurantia in 70 % ethanol, 10 min; polychrome blue (0·4 % methylene violet, 0·1 % Azur II, 0·1 % potassium carbonate in 50 % (W/V) glycerol, 3–5 min or 0·25 % Azure B in 0·1 M-phosphate buffer pH 8·4, 3–5 min. Germ plasm stained a distinctive deep blue. Serial transverse sections of stage 46–49 tadpoles were stained with haematoxylin and various counter stains for examination of primordial germ cells in the genital ridges.

RESULTS

Embryos which were rotated 90° off-axis will be referred to as 1×90 embryos and those which were inverted will be referred to as 2×90 embryos.

Effect of rotation procedures on survival and development

Preliminary experiments were carried out to test the effect on development of the conditions used to rotate embryos. The results (Table 1) permit the following conclusions: (i) treatment with Ficoll did not have any adverse effects; (ii) cooling without rotation resulted in abnormal development although survival was good; (iii) rotation through 90° abolished the effect of cooling on normal development; (iv) inversion adversely affected survival up to the gastrula stage and development in postgastrula stages was frequently abnormal.

Although these procedures, particularly inversion, had marked effects on normal development, survival was in general better than that reported in other similar studies (Gerhart & Bluemink in Gerhart (1980); Malacinski & Chung, 1981; Chung & Malacinski, 1983) in which embryos arrested at the gastrula stage. Increased survival in our experiments was attributable to cooling during the early cleavage cycles (as noted also by Neff, Wakahara, Jurand & Malacinski, 1984). Cooling extends the early cleavage cycles (first cleavage cycle 180 min at 13 °C) and presumably allows time for the egg constituents to organize into a more or less organized, stable distribution compatible with later processes, particularly gastrulation.

Brief description of the development of rotated embryos

Examination of rotated embryos before first cleavage indicated that the pigment had shifted to a limited extent so that three regions could be distinguished – a relatively light (originally ventral) quadrant, a darker (originally dorsal) quadrant and a lightly pigmented area which was an extension of the pigment cap into the originally unpigmented dorsal region of the egg (Fig. 1A). Examination of sections of rotated embryos fixed during first cleavage indicated that pigment had been displaced internally along the circumference into the unpigmented hemisphere. However, the Nile-blue-stained material did not change its appearance (i.e. become more diffuse) or its location.

Table 1. *Effect of Ficoll, cold and rotation on* X. laevis *development*

Treatment	Temperature (°C)	No. eggs	% gastrulas (st. 11)	% tadpole (st. 42)	% normal (st. 42)
Ficoll	13	72	96	76	11
1×90 Ficoll	13	60	100	100	90
2×90 Ficoll	13	60	73	57	15
Ficoll	21	56	100	100	91
Water	21	56	100	98	91

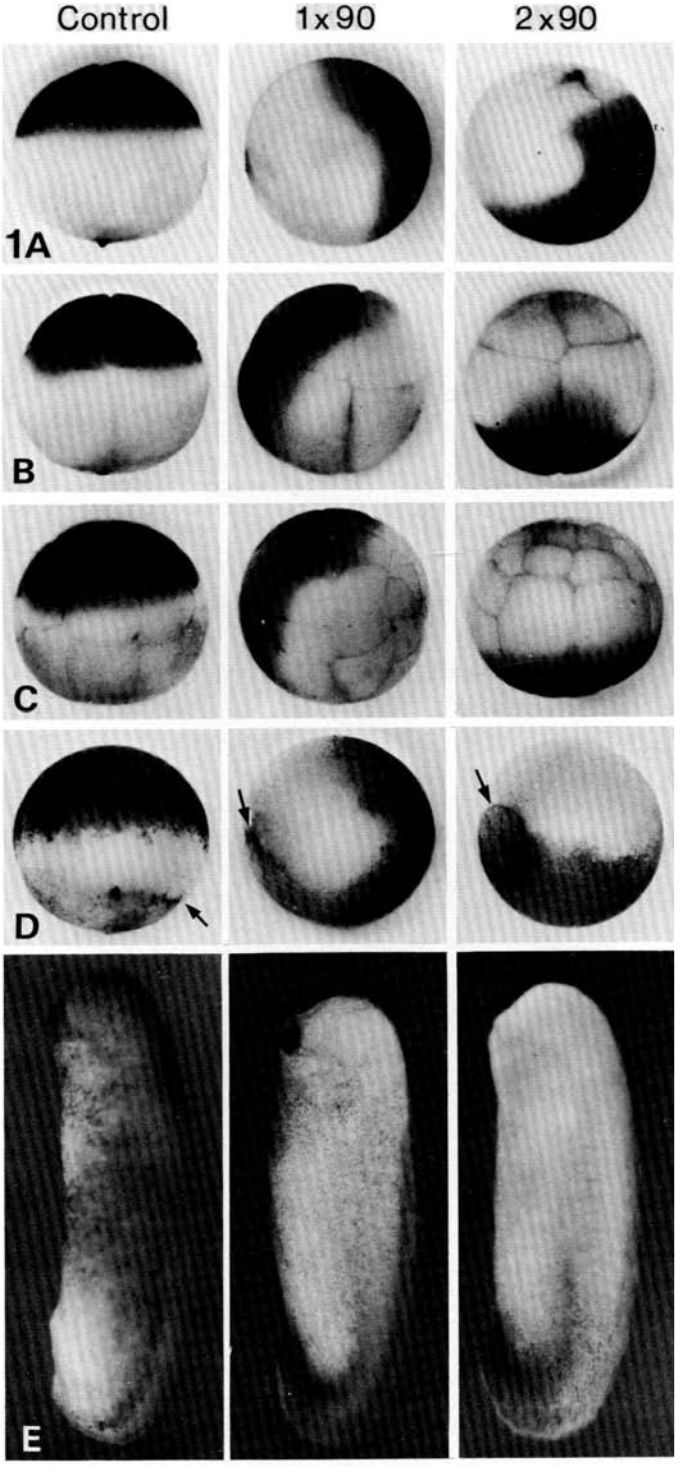

Control
1x90
2x90
1A
B
C
D
E

Table 2. *Displacement of axes and germ plasm in rotated embryos measured as the angular distance (degrees) from the cleavage axis (compare Fig. 2)*

	Displacement from cleavage axis			
	Yolk axis	Pigment axis	Germ plasm	Area of germ plasm
		8-cell stage		
Controls	3·3 ± 2·6	0·0 ± 3·2	3·3 ± 6·8	0·95 ± 0·09
1×90	6·3 ± 13·2	72·3 ± 16·7	70·0 ± 16·4	2·16 ± 0·2
2×90	21·2 ± 11·3	122·4 ± 27·1	131·0 ± 35·6	1·70 ± 0·38
		stage 6½		
Controls	2·0 ± 4·7	3·0 ± 2·3	−2·5 ± 12·9	1·33 ± 0·10
1×90	3·0 ± 7·2	79·2 ± 25·4	48·4 ± 18·9	1·37 ± 0·16
2×90	12·8 ± 7·8	111·7 ± 22·9	111·7 ± 13·7	1·26 ± 0·24

The cleavage axis, specified by the cleavage pattern, was used as the reference axis for measurement of the positions of the other axes. The pigment axis is the line through the centre of the pigment cap and the centre of the egg. The yolk axis is the median through the main body of the heavy yolk and the centre of the egg. The area of germ plasm was measured from its projection onto the surface (see Fig. 3) and expressed as a percentage of the embryo's surface area. Mean ± S.D. of six embryos per group.

The sections also showed that most of the heavy yolk had shifted into the lower hemisphere (see also Table 2) with the exception of a thin, subcortical layer on the dorsal side. The cytoplasm was in consequence located in the upper hemisphere. The sperm trail had a distinct bend close to the cortex but the remainder was straight, suggesting that, after rotation, the heavy yolk mass and the cytoplasm had shifted as a single, coherent unit relative to the outer layer.

We conclude that in rotated embryos, cortical and subcortical elements shifted slightly (the pigment) or not perceptibly (the Nile-blue-stained regions) but the main mass of the yolk and the cytoplasm appeared to have reoriented as one unit to maintain their respective positions relative to gravity.

The first two cleavages were always initiated in the upper hemisphere and micromeres formed in the upper hemisphere and macromeres in the lower

Fig. 1. Development of rotated 1×90 and 2×90 embryos. Embryos were cooled to 13 °C and rotated early in the first cleavage cycle either through 90° or 180°, the latter in two steps each of 90° with 40 min interval. Side view. (A) 1-cell stage. Pigment has shifted into the unpigmented hemisphere in 1×90 and particularly in the 2×90 embryo. The dark spot at the centre of the unpigmented hemisphere is stained with Nile blue. (B) 8-cell stage. Micromeres have formed in the upper hemisphere in all three cases, irrespective of the position of the pigmented cortex. (C) Morula stage (stage 6). Pattern of cleavage conforms to gravity. (D) Onset of gastrulation (stage 10). Dorsal lip of blastopore (arrow) formed at original vegetal pole (1×90) or near marginal zone between pigmented and unpigmented hemispheres (2×90). Lateral lips formed in direction of gravity so that in 1×90 and 2×90 embryos, pigmented surface is invaginated during gastrulation. This does not occur in controls. (E) Tailbud stage (stage 26). Change in pigment distribution in rotated embryos. Control uniformly pigmented, 1×90 with pigment concentrated ventrally, 2×90 with pigment concentrated posteriorly and anterior regions almost devoid of pigment.

hemisphere. The cleavage furrows were often curved or displaced and consequently blastomeres were frequently of irregular shape and size (Fig. 1B,C). There was no apparent correlation between regularity of cleavage pattern and survival and in any case, embryos which had cleaved irregularly during early development appeared normal by late cleavage.

The dorsal lip of the blastopore in 1×90 embryos formed at the unpigmented pole and the lateral lips were directed towards the sperm entrance point (SEP) (Fig. 1D). In 2×90 embryos the dorsal lip formed on the same side as the SEP approximately 100° from its normal position opposite the SEP, measured over the unpigmented pole. The lateral lips extended *away* from the unpigmented pole into the pigmented hemisphere, engulfing the SEP (Fig. 1D), and by stage 12, about one-quarter to one-half of the pigment was invaginated. When gastrulation was completed, the embryos were largely unpigmented except for a small concentration ventrally around the blastopore; 1×90 embryos showed a condition intermediate between this and the uniformly pigmented controls. In early tadpole stages (stage 28 approximately) control larvae remained uniformly pigmented and the Nile blue mark was visible in the posterior endoderm. In 1×90 embryos the anterior regions contained little pigment (Fig. 1E) and the Nile blue mark appeared in the anterior endoderm. In 2×90 embryos, pigment was concentrated towards the anus and the Nile blue mark was located in the anterior ectoderm and along the anterior parts of the neural tube.

In summary, development in rotated embryos proceeded roughly according to the new orientation to gravity but there were some intriguing differences, the principal one being the shift in the position of the blastopore. Overall, however, it can be concluded that regions of the egg which would normally give rise to posterior structures instead develop into anterior structures and *vice versa.* This is particularly obvious for outer (eg. cortical and subcortical) components.

Location of the germ plasm in rotated embryos

The animal–vegetal axis of the embryo is specified by the distribution of yolk and pigment and by the cleavage pattern (Fig. 2). In rotated eggs the 'pigment axis' is uncoupled from the 'yolk axis' and the 'cleavage axis', although all three remained in the plane of bilateral symmetry (Fig. 2; Table 2). We measured the location of the germ plasm in relation to the cleavage axis which was the most definite and hence able to be measured most precisely. In control embryos at the 8-cell stage and at stage 6½, the germ plasm was located in the vegetal subcortical regions about the pigment, yolk and cleavage axes (Table 2; Fig. 2). In broad terms, it was distributed fairly symmetrically about the vegetal pole and therefore was contained solely within the macromeres (Table 3), usually in relatively large, flattened patches (diameter 100–200 μm and 10–50 μm thick) lying at an average distance of approximately 30 μm from the surface.

In rotated embryos, the germ plasm was located about the pigment axis and hence displaced from the coincident yolk and cleavage axes, by approximately 70° in 1×90 embryos and approximately 120° in 2×90 embryos (Table 2) at the 8-cell

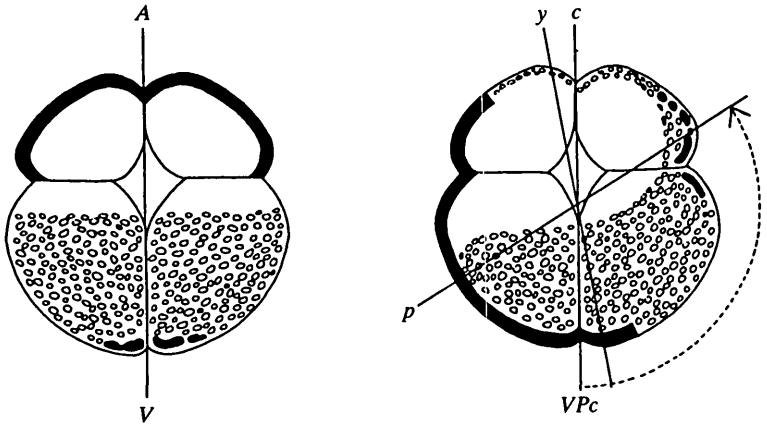


Fig. 2. An egg or early embryo has three visible axes – the pigment axis, the yolk axis and the cleavage axis — all of which are normally coincident with and in part define the animal–vegetal axis (left figure). When early embryos are cooled to 13°C and rotated (right figure; 2×90 embryo) the axes are uncoupled from each other; the cleavage axis (*c*) and yolk axis (*y*) are displaced in the direction of gravity. The pigment axis (*p*) is uncoupled to a much greater extent. Germ plasm (dense, subcortical bodies) remains opposite the pigment cap and becomes localized in the dorsal region. Its displacement is measured as the angle from the vegetal pole as specified by the cleavage pattern (*VPc*).

stage. In Fig. 3, the position of the germ plasm is represented as a projection on to the egg surface. The extent of the displacement is obvious as is the overlap in control and 1×90 embryos on the one hand and 1×90 and 2×90 embryos on the other. The shift in germ plasm was solely in the vegetal–dorsal–animal direction (the direction of rotation) (Table 2) and no lateral displacement was observed

Table 3. *(a) Types of cells containing germ plasm in early cleavage stage embryos. (b) Position of cells containing germ plasm at the onset of gastrulation (stage 10) measured in units of 0·1 radius of the spherical embryo, with coordinates of centre 10, 10, 10 (cf. Fig. 4)*

Stage		Controls	1×90	2×90
		(a) Cleavage stages		
8-cell	No. macromeres	4·0 ± 0·0	2·0 ± 0·0	1·4 ± 0·4
	No. micromeres	0	0·2 ± 0·2	1·6 ± 0·5
6½	No. macromeres	4·2 ± 0·2	2·7 ± 0·3	0·7 ± 0·3
	No. intermediate cells	0	0·3 ± 0·3	1·2 ± 0·3
	No. micromeres	0	0	1·7 ± 0·7
		(b) Gastrula		
10	X(d–v)	9·0 ± 1·6	11·4 ± 1·6*	14·6 ± 2·6*
	Y(a–v)	4·8 ± 2·4	4·3 ± 2·5	5·8 ± 2·4*
	Z(m–l)	10·1 ± 2·0	10·8 ± 6·4	10·6 ± 3·3

X, dorsal–ventral; Y, animal–vegetal; Z, medial–lateral. Mean ± S.D. of pooled values of six embryos per group.

* Significantly different from controls (t-test, $P < 0{\cdot}05$).

(Fig. 3). The area occupied by the germ plasm was similar in rotated and control embryos at stage 6½ (Table 2) but the patches were smaller and more numerous in rotated embryos. Furthermore, the patches in rotated embryos were usually situated closer to the plasma membrane than in control embryos, sometimes just beneath the surface. Hence, although superficially the effect of rotation on the germ plasm seemed to be limited to displacement within the egg, there were some differences which may be significant for the functioning of germ plasm (also see Discussion).

As a consequence of the relocation of the germ plasm away from the vegetal pole of rotated embryos, it was partitioned during cleavage to dorsal micromeres and to intermediate or marginal zone cells whereas in control embryos it was found

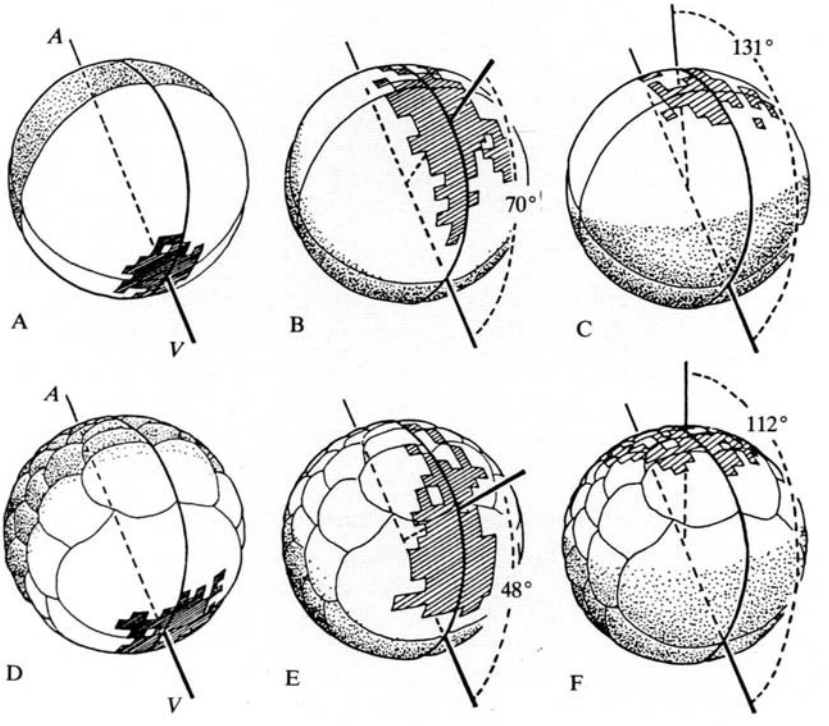


Fig. 3. Germ plasm (cross-hatched) projected on to the surface of the spherical embryo. The animal–vegetal axis (AV) as specified by cleavage, and the dorsal meridian are marked. Upper row: 8-cell embryos; lower: stage-6½ morulas. (A,D) non-rotated controls; germ plasm concentrated around vegetal pole. (B,E) 1×90 embryos; germ plasm distributed more widely and displaced along dorsal meridian 70° and 48° respectively. (C,F) 2×90 embryos; germ plasm scattered over larger area and displaced further along the dorsal meridian 131° and 112° respectively. There is no lateral displacement and the amount of germ plasm is approximately the same in rotated and control embryos (Table 2). Diagrams were constructed from serial sections on which the position of the subcortical germ plasm was measured with an eye-piece protractor and projected on to the surface in a 5°×5° co-ordinate system. Each figure from combined results of six embryos.

only in macromeres (Table 3, Fig. 2). In 1×90 morulas the germ plasm was found in approximately half of the macromeres, those on the dorsal side, and only rarely in intermediate cells. In 2×90 morulas, germ plasm was observed mainly in dorsal micromeres and intermediate-size blastomeres but only rarely in macromeres.

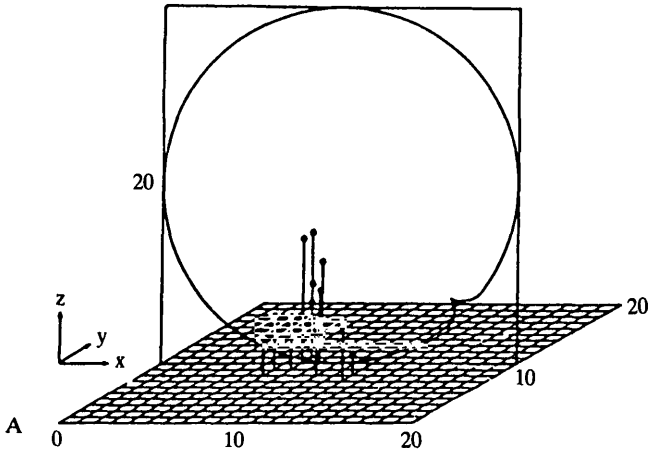
At the beginning of gastrulation, in control embryos the cells containing germ plasm were located around and above the vegetal pole (Fig. 4, Table 3). In 1×90 embryos they were situated closer to the dorsal lip of the blastopore as shown by the higher X-value in Table 3, but in the same position relative to the other axes as in controls. In 2×90 embryos they were situated around and generally above the dorsal lip (Fig. 4) i.e. displaced even further along the X-axis and also along the Y-axis but not in a lateral direction. The position of the cells containing germ plasm at this stage was therefore roughly in agreement with the location of the germ plasm at early cleavage stages although it seems likely that in 2×90 embryos the cells were somewhat closer to the dorsal lip and more internal than might have been predicted.

In stage-25 control embryos the majority of the cells containing germ plasm were located in approximately the middle third of the length of the endoderm (Fig. 5), the mean distance from the anterior end being $0{\cdot}57 \pm 0{\cdot}14$ units (mean, $\bar{M}$, ± S.D.; total endoderm length = 1·0 unit). In 1×90 embryos they were more centrally located, just anterior to the midpoint of the endoderm ($\bar{M} = 0{\cdot}41 \pm 0{\cdot}18$) and in 2×90 embryos still further anteriorly ($\bar{M} = 0{\cdot}38 \pm 0{\cdot}21$). Fig. 5 also shows that in 2×90 embryos the cells containing germ plasm were slightly more dispersed than in controls. There was some overlap in distributions even between controls and 2×90 embryos. The mean values in the two rotated groups were significantly different from that in control embryos (t-test, $P < 0{\cdot}01$).

In summary therefore, rotation of the embryo during the first-cleavage cycle displaced the germ plasm relative to the cleavage axis and as a result, marginal zone cells and micromeres contained germ plasm although normally they would never do so. Consequently, in rotated embryos at the beginning of gastrulation, the cells containing germ plasm were located closer to the blastopore lip and hence were invaginated earlier and thus occupied more anterior regions of the endoderm. Not unexpectedly, the effects were greater in 2×90 embryos than in 1×90 embryos.

Number of cells containing germ plasm

In control cleavage-stage embryos, the number of cells containing germ plasm was approximately four (Table 3). In rotated embryos however the numbers of cells with germ plasm were less because during the early divisions the germ plasm was transected by fewer cleavage planes than in controls. At stage 10, their number had doubled and it increased further at stage 25 (Table 4; Fig. 6), in general agreement with the results of Whitington & Dixon (1975) and Dziadek & Dixon (1978) that the first proliferative division takes place towards the end of cleavage and the second about stage 22–25. At stage 46–48, the number of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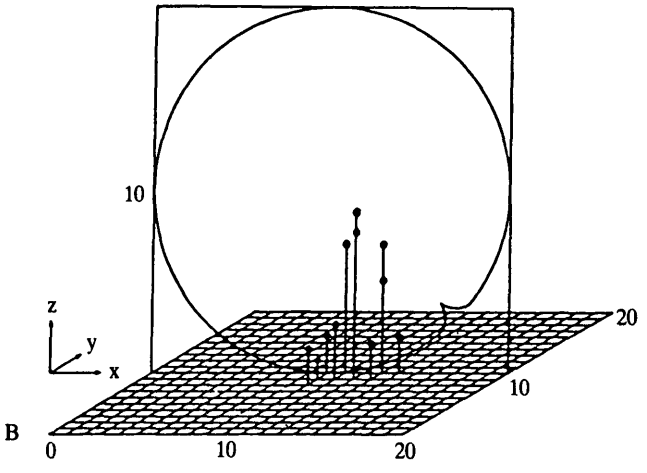
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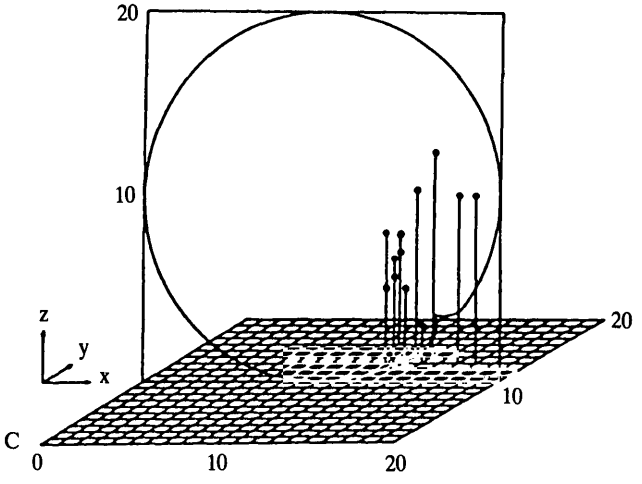
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Table 4. *Number of cells containing germ plasm at different stages of development*

Stage		Control	1×90	2×90	Significance Controls/ 1×90	Significance Controls/ 2×90
8-cell		4·0 ± 0·0	2·2 ± 0·2	3·0 ± 0·5	$P<0.03$	$P<0.03$
6½		4·2 ± 0·2	3·0 ± 0·4	3·7 ± 0·6	$P<0.04$	NS
10		8·0 ± 2·8	6·7 ± 2·3	6·3 ± 3·1	NS	NS
25		10·7 ± 4·7	7·3 ± 2·1(4)	8·2 ± 2·4	NS	NS
46–48	(a)	15·2 ± 5·6	11·2 ± 4·1	6·7 ± 6·0	NS	$P<0.03$
	(b)	12·7 ± 3·7	6·6 ± 6·6	NR*	$P<0.05$	—
	(c)	12·0 ± 2·8	11·7 ± 3·9	5·3 ± 2·9	NS	$P<0.01$

Mean ± S.D. of six embryos per group except for stage-25 1×90 embryos where n = 4. Significance levels from Mann Whitney *U* test.
* NR – no embryos survived.

primordial germ cells in the genital ridges had increased even further (Table 4; Fig. 6).

In rotated embryos, the numbers of cells containing germ plasm were not significantly different from those in controls for all stages from blastula up to and including stage 25 (Table 4). At stage 46–48, the number of primordial germ cells in 1×90 embryos was not different from controls in two of three experiments but significantly reduced in the remaining experiment. In 2×90 embryos the number of primordial germ cells was significantly less than in controls in all experiments. In short, rotation tended to reduce the number of cells containing germ plasm that were able to complete their migration to the genital ridges by stage 46.

We examined a number of rotated tadpoles at stage 49 to ensure that the reduced number of primordial germ cells was not due to a delayed migration from the genital ridges as happens in u.v.-irradiated embryos (Züst & Dixon, 1977; Smith & Williams, 1979; Shirani, 1970, 1972, 1982; Subtelny, 1980). In two experiments (six tadpoles in each group) the numbers of germ cells were not significantly altered (expt 1 – stage 46: 0·3 ± 0·8; stage 49: 0·3 ± 0·8; expt 2 – stage 46: 7·0 ± 5·2; stage 49: 3·5 ± 2·2) (Fig. 6). We conclude there was no late migration of germ cells from the endoderm.

Distribution of primordial germ cells in the genital ridges

The location of the primordial germ cells in the genital ridges of control and rotated tadpoles at stages 46–48 is shown in Table 5 and Fig. 7. In rotated embryos

Fig. 4. Three-dimensional representation of the position of cells containing germ plasm (closed circles) at onset of gastrulation (stage 10). Plane of bilateral symmetry (circle) with dorsal lip of blastopore lower right. Positions were measured in units of 0·1 radius of the spherical embryo on serial sections with a protractor–micrometer mounted in the eye piece. Co-ordinates: X, dorsoventral; Y, animal–vegetal; Z, mediolateral. One example is shown from each experimental group. (A) non-rotated control embryos; cells clustered over vegetal pole. (B) 1×90 embryos; cells displaced towards dorsal side. (C) 2×90 embryos; cells displaced further towards dorsal side and also towards the animal region.

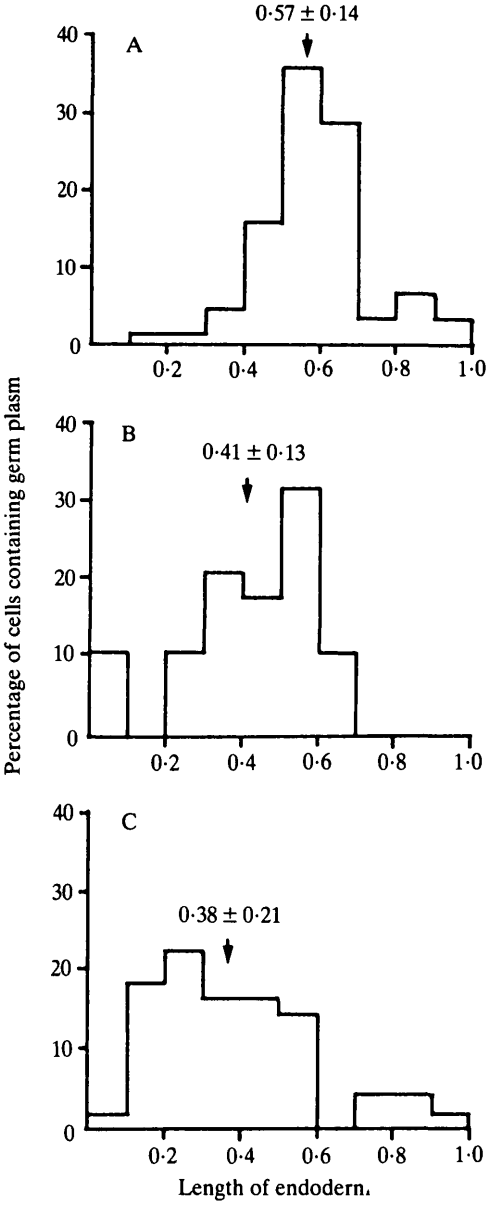

Fig. 5. Anteroposterior distribution of cells containing germ plasm in endoderm of tailbud larvae (stage 25). X-axis represents the length of the endoderm from liver diverticulum to its posterior limit (right). Mean (± S.D.) position of pooled values of cells containing germ plasm in six embryos per group is shown on each histogram. (A) non-rotated control, mean position in posterior half; (B) 1×90 larva, mean position in anterior half; (C) 2×90, mean position further in anterior half. Significance tests (t-test): controls, 1×90–$P \ll 0{\cdot}01$; controls, 2×90–$P \ll 0{\cdot}01$; 1×90–2×90–$P > 0{\cdot}20$ (not significant).

they were on average positioned more anteriorly than in controls and in 2×90 tadpoles further than in 1×90 tadpoles (Table 5). The distribution of germ cells in 2×90 embryos and control embryos, where the differences were greatest, was analysed in more detail. From Fig. 7, the anterior limits of the distributions were very similar in controls and 2×90 embryos. However, the posterior limit of the germ cells in the latter was about 80 μm further anterior than in the former. That is, in 2×90 tadpoles, approximately the posterior 20–25 % of the genital ridges did

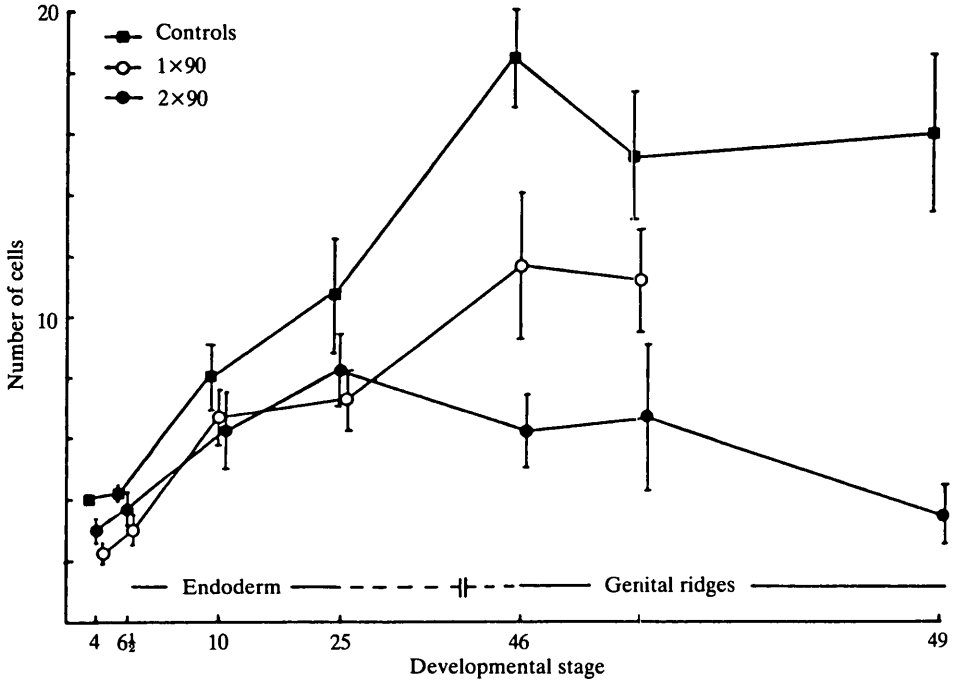

Fig. 6. Number of cells containing germ plasm and number of presumptive primordial germ cells. Mean ± standard error of six embryos in each group except for stage-25 1×90 embryos where n = 4 (see Table 4 for significance values). Number of primordial germ cells in the genital ridges of 2×90 stage-46 and -48 embryos fewer than in controls, although at earlier stages numbers of cells containing germ plasm not significantly different (Mann-Whitney *U* Test).

Table 5. *Distribution of primordial germ cells in the genital ridges at stages 46–48*

Expt No.	Controls	1×90	2×90	Significance Controls/ 1×90	Significance Controls/ 2×90
a	227 ± 100(91)	205 ± 85(69)	291 ± 84(40)	NS	$P \ll 0{\cdot}01$
b	182 ± 76(72)	225 ± 88(70)	241 ± 91(32)	$P < 0{\cdot}01$	$P < 0{\cdot}02$

Results of two experiments. Distance (μm) from the posterior limit of the dorsal mesentery. Mean ± S.D. of pooled values of six tadpoles in each group, number of germ cells in each group in brackets. Significance levels from t-test.

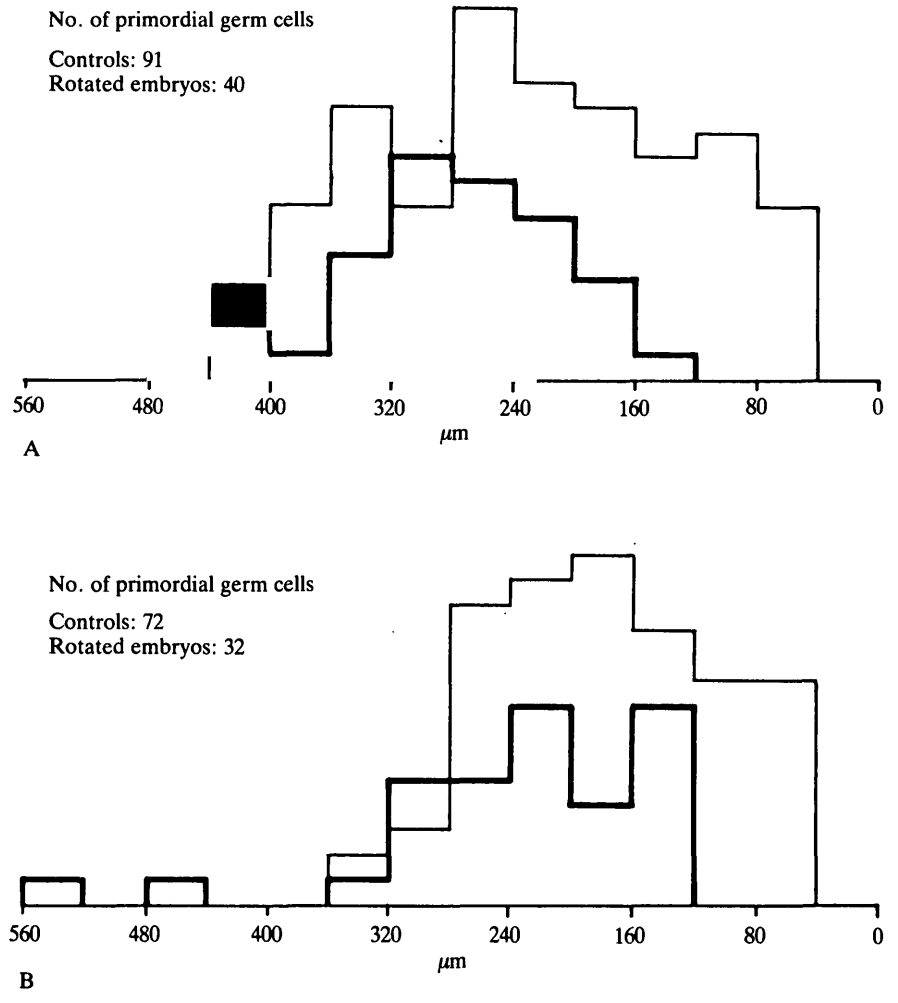

Fig. 7. Anteroposterior distribution of primordial germ cells in the genital ridges of stage-46 to -48 tadpoles. The positions of individual germ cells were measured from the posterior limit of the dorsal mesentery (right). Pooled values of six embryos per group; two experiments are shown (A,B). Fine line: non-rotated controls; thick line: 2×90 tadpoles. The number of primordial germ cells is reduced in 2×90 tadpoles and they are located in the more anterior regions of the genital ridges.

not contain any primordial germ cells, although in controls there were substantial numbers (20–25 %) in this region. No observations were made on 1×90 tadpoles.

These results are consistent with the distribution of cells containing germ plasm in the anterior endoderm of stage-25 embryos. In controls, 75–80 % of the cells containing germ plasm were in the posterior half of the endoderm but in 2×90 embryos only approximately 25 % were in this region. This comparison suggested that the reduction in number of primordial germ cells in the genital ridges of

rotated embryos might be a consequence of their location in the anterior endoderm. This suggestion receives support from the observation that in three 2×90 embryos, cells identified on morphological criteria as (ectopic) primordial germ cells were found on the oesophagus.

Grafting experiments

To test the hypothesis that cells containing germ plasm are unable to migrate from the anterior endoderm to the genital ridges simply because of their position, we grafted anterior endoderm from stage-25 2×90 embryos into the posterior endodermal region of other stage-25 2×90 larvae. The grafting procedure was similar to that described by Blackler & Fischberg (1961). The types of grafts performed and the results obtained are shown in Table 6. The data indicate that grafting increased the number of primordial germ cells slightly (Series 1) or not at all (Series 2). In Series 2, the reduction in number in the 2×90 grafted embryos can be explained as resulting from the grafting procedure. In operated unrotated embryos the number of primordial germ cells was 76% of that in non-operated control embryos, and in 2×90 grafted embryos 76% of that in their respective controls (2×90 non-operated).

Interpretation of these results is not simple. The wide distribution in the endoderm of 2×90 embryos of the cells containing germ plasm means that the grafts do not involve a simple exchange of tissue containing cells with germ plasm for tissue without such cells. The variation between embryos is another complication. Nevertheless, the number of primordial germ cells in the genital ridges of grafted embryos was not sufficient to sustain the hypothesis fully. On the other hand, the increase in Series 1 cannot be ignored. We conclude that the anterior location of the cells containing germ plasm is a factor accounting in part for the reduction in number of primordial germ cells in rotated embryos. This conclusion draws support from comparison between 1×90 and 2×90 embryos which suggests that the more anteriorly the cells containing germ plasm are located in the endoderm the fewer the number of primordial germ cells in the genital ridges.

Table 6. *Number of primordial germ cells in 2×90 embryos after grafting anterior endoderm to the posterior endodermal region*

Experimental series	Controls (non-operated, unrotated)	Controls (operated, unrotated)	2×90 Controls (non-operated)	2×90 Grafted embryos
1	11·6 ± 8·0(20)	10·5 ± 7·9(11)	2·1 ± 3·9(19)	6·4 ± 6·3(14)
2	22·9 ± 6·8(21)	17·4 ± 4·2(13)	9·0 ± 5·1(20)	6·9 ± 3·4(21)

Mean ± S.D.; number of embryos in parentheses. Anterior endoderm from 2×90 embryos was grafted into the posterior endodermal region after the technique of Blackler & Fischberg (1961).

In control grafts posterior endoderm from normal embryos was grafted into the posterior region of another unrotated embryo (operated unrotated column).

DISCUSSION

When embryos are maintained permanently in an off-axis orientation, a proportion are able to develop into swimming tadpoles and a smaller proportion still appear normal. Inversion (2×90) is more detrimental than rotation through 90°, and cooling enhances survival. The results reported here for survival are similar to those of Neff, Malacinski, Wakahara & Jurand (1983) for eggs prevented from undergoing spontaneous rotation after fertilization; they did not report the proportion of surviving embryos which developed normally.

The pattern of cleavage in rotated embryos was frequently irregular in early stages of development. Overall cleavage conformed to gravity in agreement with earlier observations by Gerhart & Bluemink (in Gerhart, 1980), Stanisstreet, Jumah & Kurais (1980), Chung & Malacinski (1982) and Neff *et al.* (1983). We have analysed this result quantitatively however, and show that the cleavage axis at stages 3 and 6½ deviates from the yolk axis to a small extent in 1×90 embryos and to a greater extent in 2×90 embryos (Table 1).

Our observations suggest to us that, with the exception of a thin subcortical layer of yolk on the dorsal side, the internal contents of rotated eggs shifted as a single coherent unit. In contrast, Neff *et al.* (1983) reported that the 'yolk platelets of inverted eggs appear to redistribute as discrete zones' and furthermore their 'density compartment model' is based on changes in relative positions of components of different densities. Neff *et al.* (1983) suggest several experimental tests of their model, the results of which should indicate which interpretation is more tenable.

In rotated embryos, the site of formation of the dorsal lip shifted to new locations within the unpigmented hemisphere (Fig. 7). These observations are similar, in broad principle, to those reported by Gerhart *et al.* (1981) and by Neff *et al.* (1983) and will not be analysed further here. However, a consequence of the relocation of the dorsal lip was that the dorsoventral axis was apparently changed relative to the original animal–vegetal axis as defined by the pigment distribution. Similarly, the observation that the Nile-blue-stained material (from the unpigmented pole) was displaced from the posterior endoderm in controls to the anterior endoderm in 1×90 embryos and to the neural plate and anterior ectoderm in 2×90 embryos indicates that the anteroposterior axes had also been displaced. In order to estimate more closely the extent to which the dorsoventral and anteroposterior axes had changed in rotated embryos, the anterior, posterior, dorsal and ventral limits of a normal stage-18 neurula were mapped back on to the surface of a stage-10 gastrula, using the fate maps of Keller (1975). In this way the future anteroposterior and dorsoventral 'axes', as specified by surface components, can be shown on a normal gastrula (Fig. 8). These axes were then placed on a rotated gastrula at stage-10, keeping their spatial relationship to the dorsal lip. The distribution of pigment and the position of the Nile-blue-stained material in rotated neurula-stage embryos is then similar to that observed by us (Fig. 8). That is, pigment is concentrated ventrally (1×90) or posteriorly (2×90) and the Nile-blue-stained material occupies the anterior endoderm (1×90) or the

anterodorsal regions (2×90). Therefore it seems that in these experiments the anteroposterior and dorsoventral axes maintained their mutual spatial relationship (i.e. remained at the same angle to each other) and together they rotated around the centre of the egg in the plane of symmetry.

This analysis is consistent with the conclusions reached above that the internal components shift as a single unit in response to gravity. However surface-associated and cortical and subcortical components such as pigment granules, dye spots and patches of germ plasm are not so mobile and hence they are relocated within the embryo.

Because of the relocation of the germ plasm dorsal to the vegetal pole of rotated embryos, it was partitioned predominantly between dorsal micromeres and marginal zone cells rather than being confined exclusively to macromeres as in controls. Wakahara, Neff & Malacinski (1984) have observed that in inverted

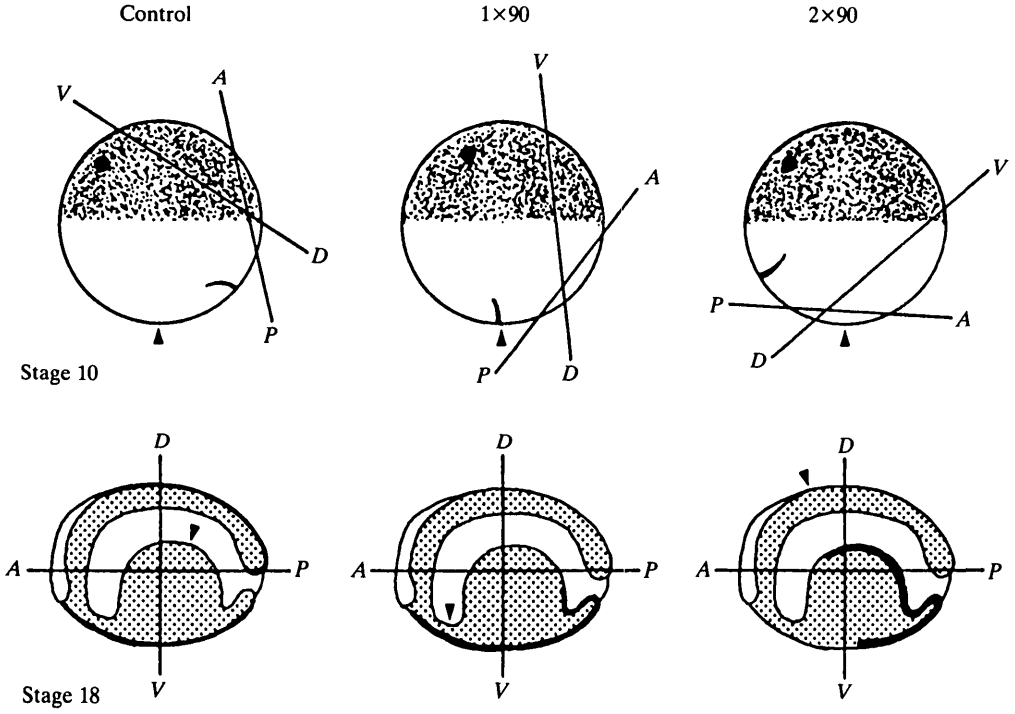

Fig. 8. Diagram representing the shift in anteroposterior and dorsoventral 'axes' due to permanent rotation of embryos during the first cleavage cycle. Control embryos showing the position of the most anterior, most posterior and dorsal and ventral points in a stage-18 embryo mapped back on to the surface of a stage-10 gastrula according to the fate map of Keller (1975). The arrow indicates the position of the Nile-blue-stained material and hence the fate of the unpigmented pole at each stage. SEP at top left hand (spot). Central and right hand panels show same features in rotated embryos. Dorso-ventral and anteroposterior axes defined in control embryos have been superimposed on stage-10 rotated embryos in the same position relative to the dorsal lip. At stage 18 the axes bear the same spatial relationship to the axes of polarity at stage 10 as in control embryos suggesting that rotation displaced the axes as a single unit.

embryos germ plasm is located in micromeres but in their experiments they were unable to distinguish the dorsal side. From their illustrations it appears that marginal zone cells never included germ plasm as in our experiments. In 50 % of their embryos at blastula stage the cells containing germ plasm were located close to the blastocoele (i.e. internally) but in our experiments they were always situated in the outer layer of cells. These differences may be due to differences in procedure: in our experiments embryos were rotated in a standard way in the plane containing the sperm entrance point whereas Wakahara *et al.* (1984) made their observations on embryos held in the orientation in which they were laid. The ability to recognize that the cells containing germ plasm were located on the dorsal side permitted an understanding of their fate after gastrulation. As a consequence of their position closer to the dorsal lip than normal, the cells containing germ plasm were invaginated earlier and further than in controls. Furthermore, there is a correlation between the degree of rotation of the embryo (1×90 or 2×90), the relocation of the germ plasm, and the position at stage 25 of the cells that contain germ plasm. There are no other reports of the number or position of cells containing germ plasm in rotated embryos between blastula and swimming tadpole.

There is an apparent discrepancy between the position of the cells containing germ plasm in 2×90 early gastrulas and their position in cleavage-stage embryos. We expected that since at stage-6½ micromeres and intermediate zone cells contained germ plasm, their daughter cells to which the germ plasm was segregated would have been located above the dorsal lip mainly in the presumptive neural plate, notochord and suprablastoporal endoderm. However, they were located in the endoderm near the dorsal lip. This difference can be explained in two ways. First, the cortical and subcortical components are not fixed but move ('slip') in response to gravity, albeit much less so than the internal components. This tendency can be seen in Table 2 where the difference between yolk and cleavage axes in morulas is less than at the 8-cell stage (1×90 – 3°; 2×90 – 8°). However, this effect is too small to account for the position of the cells containing germ plasm in early rotated gastrulas. Recently it was found that in normal development some of the progeny of the dorsal micromeres (marked with horse radish peroxidase) contribute to cells of the presumptive endoderm located internally to and above the dorsal lip (R. Masho & H. Y. Kubota, personal communication). In an earlier study, Keller (1978) described extensive pre-gastrular movements at the blastula stage which displace cells of the superficial epithelial layer in the animal hemisphere towards the dorsal lip. Early in gastrulation these cells are internalized and subsequently translocated to the anterior end of the embryo (Keller & Schoenwolf (1977) Fig. 1). These findings satisfactorily explain the relocation of the cells containing germ plasm from their initial position above the blastopore to the endoderm and their eventual localization at the anterior end of the embryo.

The numbers of cells containing germ plasm were not significantly different in control embryos and rotated embryos from blastula up to stage 25 (Fig. 6),

suggesting that the normal processes of partitioning and segregation of the germ plasm and proliferation of the cells that contain germ plasm (see Dixon, 1981) had not been affected significantly by rotation of the early embryo. However, the number of primordial germ cells which entered the genital ridges in rotated embryos was significantly lower than in controls and once again the effect was greater in 2×90 tadpoles than in 1×90 tadpoles (Fig. 6). A reduction in number of germ cells in rotated embryos has also been reported by Neff *et al.* (1983), Thomas *et al.* (1983) and Wakahara *et al.* (1984) although their protocols, particularly timing of rotation, temperature for development and duration of rotation were different from ours. At least five possible explanations can be put forward to explain this reduction.

The first, suggested by Wakahara *et al.* (1984), is that germ plasm is lost from the embryo through the external plasma membrane as a result of its close apposition to the surface of the embryo. This loss would presumably mean fewer germ cells were determined. We have not made any observations which support this suggestion although we looked particularly for evidence of it. Our measurements of the amount of germ plasm suggest that at least up to stage 6½ there was no substantial loss of germ plasm, although it would not have been possible to detect small losses. More importantly, the number of cells containing germ plasm was about the same up to stage 25 and although not measured after stage 6½, there did not appear to be any great differences in amount of germ plasm per cell between rotated and control embryos. On these grounds we believe this explanation is not tenable in our case.

The second hypothesis suggested by Thomas *et al.* (1983) is based on the results of temporary 90° rotations only and suggests that the process of partitioning of the germ plasm which normally results in a founder clone of four cells (see Dixon, 1981) is perturbed. They propose that a smaller founder clone (three instead of four cells) is formed which in turn leads to 'a reduced number of germ cells in the embryo at stage 43' (reduction of 25 %). This explanation seems attractive in general terms particularly since in rotated embryos the early cleavage pattern is often irregular. But this hypothesis falls short of explaining why in our study rotated embryos at stage 25 contained a similar number of cells with germ plasm as controls but at stage 46 only 30 % (2×90) or 75 % (1×90) of the number in controls.

The numbers of cells containing germ plasm in our cleavage, gastrula and tail-bud stages also argue against a third hypothesis that in rotated embryos these cells undergo fewer proliferative divisions after cleavage than those in controls.

A fourth possible explanation is that in rotated embryos cells containing germ plasm were displaced too far anteriorly to be able to migrate successfully to the posteriorly located genital ridges. This suggestion draws support from the observation that in rotated embryos the posterior regions of the genital ridges did not contain any primordial germ cells. Furthermore, some ectopic primordial germ cells were identified. We tested this hypothesis and concluded that it could account in part for the reduction in number of primordial germ cells.

The last of the possible explanations suggests that some of the cells containing germ plasm in the endoderm at stage 25 did not migrate because they were not determined (fully) as presumptive primordial germ cells. Jurand & Dixon (1985) have observed that the fine structure of germ plasm in 2×90 embryos differs from that in controls. In inverted embryos mitochondria in the germ plasm are dispersed and their ability to adhere together in branching chains as in controls is much reduced. Structural associations between germinal granules and mitochondria are also less frequent. The aggregates of germinal granules themselves are much less compact and the fine structure of individual components is altered. Jurand & Dixon (1985) suggest that these changes may correlate with the reduction in number of primordial germ cells which enter the genital ridges in inverted embryos. Experiments are planned to test this hypothesis, particularly to see whether in 1×90 embryos the ultrastructure of the germ plasm is less disrupted than in 2×90 embryos, consistent with the differences in numbers of primordial germ cells.

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