Evidence for a functional role of the cytoskeleton in determination of the dorsoventral axis in *Xenopus laevis* eggs

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SUMMARY

A normal table of events of the first cleavage period in the fertilized egg (cf. Gerhart, 1980) has been completed (cf. Table I) by studying external and internal features. Through a cytological study of eggs fixed after video time-lapse observation such features can directly be correlated and it has been shown that the first postfertilization wave (PFW) reflects spermaster growth, which causes rearrangements of animal yolk material. This may, in conjunction with the interaction of the spermaster rays with the cortex, define, in time as well as in space, the asymmetric cortical contraction which we suppose to evoke asymmetry in the animal hemisphere by formation of the vitelline wall (Pasteels, 1964) and in the vegetal hemisphere by formation of the Vegetal Dorsalising Centre (Kirschner et al. 1981).

Neither prick-activated eggs nor fertilized eggs incubated in vinblastine develop a spermaster. Under these conditions abnormal cytoplasmic segregation may be directed by gravity alone. For normal development the activated egg must in some way, for instance through the sperm centriole, organize microtubule assembly into a monaster. The centriole acts as a microtubule-organizing centre in structuring the egg's cytoskeleton, and through this directs localization of the various yolk components, in time as well as in space. In egg rotation experiments performed under appropriate conditions, the cytoskeleton is disturbed and yolk rearranges under gravity till a new equilibrium is established which determines a new dorsoventral polarity. Such experiments also show that neither the dorsal cytoplasm nor the grey crescent cortex act as the ultimate dorsal determinants, since their localization is unaltered upon rotation, whereas the overall yolk distribution is significantly changed.

INTRODUCTION

As in most amphibian eggs the unfertilized egg of *Xenopus laevis* is radially symmetrical about its animal–vegetal axis, as expressed by the pigment pattern and the arrangement of the yolk granules. However, fertilization causes cortical and internal cytoplasmic movements which induce bilateral symmetry by the

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time of first cleavage (Ubbels, 1977; Kirschner, Gerhart, Hara & Ubbels, 1980; Kirschner et al. 1981; Gerhart et al. 1981). Upon fertilization cortical granules are extruded, a perivitelline space forms and the egg rotates under the influence of gravity until the unpigmented, dense yolk-rich vegetal half of the egg faces downward and the animal pole turns up. After passage of the Activation Wave (AW) (Hara & Tydeman, 1979) the pigment contracts in the animal pole region. This ‘activation contraction’ is followed by a period of general pigment dispersion, although some of the pigment then concentrates at the Sperm Entrance Point (SEP), which becomes clearly visible as a dark spot on the now lighter animal cap (Paleček, Ubbels & Rzehak, 1978). As in other anuran species, at gastrulation the blastopore forms opposite the original position of the SEP. The place where in normal development cells of the blastopore start to invaginate marks the dorsal side of the future axial mesoderm of the embryo (cf. Spemann, 1936; Nieuwkoop, 1973; Gerhart, 1980). Thus the SEP can be considered as a reasonably good and early recognizable marker of the ventral side of fertilized *Xenopus* eggs (Kirschner et al. 1980) and indicates that the egg is demonstrably bilaterally symmetrical after fertilization. It is our aim to reveal the possible structural causes of bilateral symmetry in normal development.

In fertilized eggs internal cytoplasmic movements occur simultaneously with pigment shifts: a yolk-poor area, the Central Cytoplasm (CC), at first located centrally, is ultimately found opposite the SEP. It is then called the Dorsal Cytoplasm (DC). In addition the characteristic axially symmetrical arrangement of the yolk granules in the unfertilized egg becomes bilaterally symmetrical (Ubbels, 1977). The results presented here strongly suggest that these yolk shifts are directed by the spermaster and that in normal development this bilaterality indeed foreshadows that of the gastrula.

In the period from fertilization until first cleavage two sorts of surface waves, visualized by time-lapse cinematography, show dorsoventral polarity (Hara, Tydeman & Kirschner, 1980). It is assumed that the Activation Wave (AW) spreading from the SEP, reflects the extrusion of the cortical granules (Hara & Tydeman, 1979). In fertilized eggs the AW is followed by the Post Fertilisation Waves (PFWs; Hara, Tydeman & Hengst, 1977), which also spread from the SEP and coincide temporally with the internal cytoplasmic movements (Ubbels, 1977). The two events show an identical polarity. Since the PFWs do not occur in prick-activated eggs or in fertilized eggs incubated in vinblastine, we have suggested that they may have a common basis, in which microtubule action might be involved (Kirschner et al. 1980). The question remains whether these waves are instrumental in, or only secondarily reflect, some primary events concerned with the origin of dorsoventral polarity. We will show that the PFWs reflect the extension of the spermaster, which in its turn is indispensable in orienting the symmetrization of the normally developing egg. However, a grey crescent is found in the absence of both and therefore the aster is not indispensable for formation of the crescent (Manes & Barbieri, 1977).
Determination of dorsoventral axis in *Xenopus* egg

The DC is a characteristic of normal development and together with the Grey Crescent (GC), which develops opposite the SEP, it marks the dorsal side of the fertilized egg. One way to test whether it functions as a dorsal determinant is a cytological analysis of fertilized eggs which had been rotated through 90 degrees with either their SEP up, in order to induce axis reversal, or down, which would have led to normal axis development (Kirschner et al. 1980; Gerhart et al. 1981). The results presented here show that roughly speaking the yolk patterns in these two groups mirror each other (Ubbels, Gerhart, Kirschner & Hara, 1979; Gerhart et al. 1981). The dorsal cytoplasm does not change place upon rotation and we therefore conclude that it does not act as the ultimate dorsal determinant (Ubbels et al. 1979), a conclusion which also holds for the GC (Gerhart et al. 1981), although the localization of the latter is likewise well correlated with that of the SEP.

**MATERIALS AND METHODS**

**Egg material**

Unfertilized eggs were stripped from the female into doubly modified amphibian Ringer solution (MMR): 100 mM NaCl, 2 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, 5 mM HEPES, 0.1 mM EDTA, pH 7.8 (Kirschner et al. 1980). Testes were kept at 4°C in MMR containing 10% foetal calf serum and 50 μg/ml gentamycin. After a short wash in 25% MMR, eggs were artificially fertilized by rubbing a piece of testis over a sample of eggs. After 5 min the eggs were rinsed in 25% MMR and allowed to develop in 25% MMR. They were dejellied at the proper time with a solution of 10 mM-dithiothreitol in 0.05 M-Hepes buffer adjusted to pH 8.3, followed by an extensive wash in 25% MMR (8 mg DTT was dissolved in 5 ml distilled water, containing 0.25 ml 1 M-Hepes buffer, pH 8.8).

**Choice of fixation times**

A relative time scale was used throughout. The first cleavage period is normalized to 1.0 and the time of artificial fertilization represents time 0 (Kirschner et al. 1980) (see Fig. 1) – thus halfway through the time cycle is referred to as to.5. Sixty-five eggs were fixed after video time-lapse observation of the PFWs, to study their relation to spermaster growth; others belonged to batches from which eggs, developing synchronously with those to be fixed, were recorded by time-lapse cinematography; the relative ages of the remaining eggs were calculated from the cleavage time of the controls.

**Histology**

Dejellied eggs were fixed for 2–3 h in Bouin d’Hollande (Romeis, ed 1968, §308) followed by alcoholic dehydration and embedding in Histowax Tissue Embedding medium (HW-0040; Histo-Lab Ltd, Göteborg, Sweden) (m.p.
58–60°C) via amyl acetate; 6 μm serial sections were cut on an AO 820-Spencer Precision Rotary Microtome, arranged on slides, and carefully flattened on a hot plate at 37°C. After deparaffination, sections were stained for 6 min in sol. A, and rinsed several times in distilled water; stained for 3 min in sol. B and rinsed once in distilled water; stained for 5 min in sol. C, and then dehydrated via 96% and absolute ethanol cleared in Xylol and mounted in Depex (sol. A: 1% azofuchsin G (chroma 10135) in 1% acetic acid. Sol B: 2.5 g aniline blue (Merck C.I. 42755, art. 1275), dissolved in 500 ml distilled water and 10-0 g orange G (chroma 1B 221); 40 ml acetic acid was added; this mixture was then boiled, cooled and filtered. Sol. C: 0.5% aqueous solution of aniline blue).

Antimitotic drugs
To test the possible role of microtubules in symmetrization, fertilized *Xenopus* eggs (which are slightly permeable to vinblastine-sulfate (VB)) were dejellied and incubated in VB solutions of 1.0, 0.2 or 0.04 mg/ml MMR (25%) for various time intervals before first cleavage, or microinjected with 20 nl of 500 μM-colchicine.

Artificial activation
To study cytological changes in eggs activated in the absence of the sperm, unfertilized eggs were stripped from the female, dejellied, activated by pricking through the vitelline membrane in the equatorial region with a thin glass needle, and fixed at different time intervals, viz. 0, 20, 40, 60 or 85 min after pricking. During this experiment eggs were kept in 5% Ficoll in 25% MMR (Kirschner et al. 1980) to avoid extrusion of cytoplasm through the pricking hole in the elastic vitelline membrane.

Egg rotation
Fertilized eggs selected for equatorial Sperm Entrance Points (SEP’s) were rotated through 90 degrees so that the animal–vegetal axis was horizontal for various periods of time with their SEP either at the top or at the bottom in 25% MMR containing 5% Ficoll, which dehydrates the perivitelline space and thus prevents the eggs from reorientation under gravity (Kirschner et al. 1980). They were subsequently processed for histology. In some cases the rotation experiment was performed during vinblastine incubation, to find out whether microtubules affect the action of gravity on yolk distribution.

Localization of the centre of the grey crescent
The centre of the grey crescent was determined visually in relation to the SEP by placing a Nile Red pseudocristal (Kirschner & Hara, 1980) slightly under the SEP, or slightly above the equatorial region at the 180° meridian in those cases where the SEP was localized at 0°.
Table 1. Normal table of events from fertilization to first cleavage*

(After Gerhart et al., 1980; modified or extended entries for Xenopus laevis: 5–15.

<table>
<thead>
<tr>
<th>Event</th>
<th>Xenopus laevis</th>
<th>Rana pipiens, R. fusca and R. nigromaculata</th>
<th>Triturus palamatus and T. viridescens</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Egg–sperm contact</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2. Activation wave (cortical granule breakdown)</td>
<td>0.08–0.12</td>
<td>0.10–0.15</td>
<td></td>
</tr>
<tr>
<td>3. Activation contraction</td>
<td>0.12–0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Rotation (perivitelline space fills with liquid)</td>
<td>0.12–0.15</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>5. 2nd polar body extruded</td>
<td>0.16–0.25</td>
<td>0.12</td>
<td>0.16</td>
</tr>
<tr>
<td>6. Cortical rigidity increases</td>
<td>0.18–0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. Spermaster enlarges†</td>
<td>0.16–0.65/0.8</td>
<td>0.18–0.66</td>
<td>0.25–0.65</td>
</tr>
<tr>
<td>8. 1st postfertilisation wave</td>
<td>0.16–0.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. DNA synthesis‡</td>
<td>0.32–0.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. Grey crescent clearly visible</td>
<td>0.50</td>
<td>0.50</td>
<td>Variable?</td>
</tr>
<tr>
<td>11. Pronuclei in contact</td>
<td>0.45</td>
<td>0.58</td>
<td>0.58</td>
</tr>
<tr>
<td>12. Yolk-free cytoplasm to dorsal side</td>
<td>0.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13. Pigment granules leave cortex, then return§</td>
<td>0.17–0.61/0.62–0.71</td>
<td>0.66</td>
<td>0.63</td>
</tr>
<tr>
<td>14. Spermaster disintegrates ventrally</td>
<td>0.53</td>
<td>0.66</td>
<td>0.63</td>
</tr>
<tr>
<td>15. Bipolar spindle appears</td>
<td>0.70–0.80</td>
<td>0.72</td>
<td>0.65</td>
</tr>
<tr>
<td>16. Accessory sperm nuclei degenerate</td>
<td>N/A</td>
<td>N/A</td>
<td>0.68</td>
</tr>
<tr>
<td>17. Mitotic prophase</td>
<td>0.77</td>
<td>0.72</td>
<td>0.68</td>
</tr>
<tr>
<td>18. Pigment granules aggregate</td>
<td>0.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19. 1st surface contraction wave</td>
<td>0.80–0.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20. Mitotic metaphase</td>
<td>0.85</td>
<td>0.83</td>
<td>0.79</td>
</tr>
<tr>
<td>21. 2nd surface contraction wave</td>
<td>0.88–1.0</td>
<td>0.92</td>
<td>0.92</td>
</tr>
<tr>
<td>22. Mitotic telophase, appearance of nuclear vesicles</td>
<td>0.92</td>
<td>0.92</td>
<td>0.92</td>
</tr>
<tr>
<td>23. DNA synthesis starts on 2nd cycle</td>
<td>0.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24. Cleavage furrow appears</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Approximate interval (min) at 20°C: 90, 180, 270

*Entries for each species indicate time on a relative scale from fertilisation (0:0) to first cleavage (1:0). Figures are accurate to one decimal place only. N/A indicates event does not occur in this species. Absence of an entry indicates no datum available. Entries for *Xenopus laevis* were obtained from Ubbels (1977), Hara et al. (1977, 1980), Paleček et al. (1978) and Graham (1966); for *Rana pipiens* from Subtelny & Bradt (1963), Elinson & Manes (1978), Elinson (1975), and Malacinski, Benford & Chung (1975); for *Rana nigromaculata* from Kubota (1966, 1967, 1969); for *Rana fusca* from Ancel & Vintemberger (1948) and Ancel and Calame (1959); for *Triturus palamatus* from Fankhauser (1934, 1937); and for *Triturus viridescens* from Fankhauser & Moore (1941).

† 0.16–0.65 first, 0.65–0.80 second growth (see text).
‡ Modified after Kirschner et al. (1981).
§ 0.70–0.71 aggregation of the returned pigment granules.
Fig. 1. *Xenopus laevis*. Summary of stages and numbers of various groups of cytologically analysed artificially fertilized eggs (* fixed after video time-lapse observation of the first PFW). One dot represents one egg.

Fig. 2. Timing (on relative scale; see Fig. 1 and Materials and Methods) of characteristic features of the first cleavage period in *Xenopus laevis*, AW: activation wave; PFW: postfertilization wave(s); SCW: surface contraction wave; GC: grey crescent, ↓ clearly apparent; M, A, T: meiotic metaphase, anaphase, telophase respectively; n + n: nuclei in conjunction; 2n: nuclei fused. ¹ From Gerhart *et al.* (1980), ² Kirschner *et al.* (1981).
RESULTS

External and internal events of the first cleavage period

Earlier observations (Ubbels, 1977; Paleček et al. 1978; Kirschner et al. 1980; Gerhart et al. 1981) were extended and are summarized in Table 1 (cf. table II in Gerhart, 1980, entries 5–15). The relative time scale (see Materials and Methods) enabled us to summarize data from different egg batches and experiments in a ‘normal table’. Fig. 1 shows the ‘timing’ of nearly 250 eggs cytologically analysed through the first cleavage period. To our knowledge this is the first time that the correlation between internal and external features of the first-cleavage period was studied in the amphibian egg.

a) Asymmetry in yolk distribution forms during the first cleavage period. Formation of the second polar body started around to 16 and was always completed by

![Diagram of PFW and spermaster growth in Xenopus laevis](image)

Fig. 3. Postfertilization wave (PFW) and spermaster growth in artificially fertilized eggs (relative time scale). A: the heavily dotted arc indicates the level of the PFW as observed by video time-lapse observation in top-view, the thinly dotted line delineates the animal pole region. The dotted line in the semischematic drawings of median histological sections (B) delineates the boundary of the aster region. a: small, b: medium, c: coarse yolk, d: central cytoplasm. Further explanation in text.
to-25 (Fig. 2). The most striking features after its extrusion were spermaster growth and accompanying yolk shifts in the animal part of the egg, and pronuclear movements (Figs 2, 3, 4).

The sperm head looked swollen until time t₀.₁₉ (Fig. 8) because it was surrounded by a basophilic cloud, but then the head decreased markedly in size. A tiny granule located centrally to the sperm nucleus was often visible in the centre of the centripetally growing spermaster and is tentatively considered here as the sperm centriole. The aster 'pushed away' yolk in its immediate vicinity, starting with the ventral horn of medium yolk (Figs 3, 4). Medium yolk closely surrounded the pigmented sperm trail. It was in its turn surrounded by fine yolk into which the newly formed aster rays extended. Immediately in front of this region were loosely packed medium and fine yolk granules with short blue-staining fibrillar structures in between (Fig. 5). Still further dorsally and animaly from the equatorial region the yolk pattern was progressively more like that of the newly fertilized egg.

Thus during growth of the spermaster the original axially symmetrical pattern of yolk changed progressively, from the SEP towards the opposite side (Figs 3, 4). During the entire period aster rays, also in the equatorial region, were present among the medium and fine but never among the coarse yolk granules.

As a result of yolk shifts the CC was found progressively more excentrically from t₀.₃ onwards (Fig. 3), forming the DC. Fibrils inside or connected with the DC bent towards the pronuclei. The gradually developing yolk asymmetry became suddenly much more pronounced after fusion of the pronuclei in the t₀.₇—₀.₈ period. This moment was only caught in a few cases. In those eggs in which the pronuclei had fused and mitosis had not yet begun, a narrow subcortical ridge of coarse and medium yolk granules was located slightly above the equator on the dorsal side, which we assume to represent Pasteels' (1964) vitelline wall.

After extrusion of the second polar body (≤₀.₂₅) the female nucleus moved centripetally, and simultaneously with the male nucleus progressively increased in size. Fibrillar structures occasionally appeared between the female nucleus and the CC, when the former was localized about halfway the distance between the animal pole and the upper layer of large yolk granules. Between t₀.₄ and t₀.₅ the two pronuclei met in the centre (Figs 2, 3), but they only fused between t₀.₇ and t₀.₈; the first mitosis then soon followed.

b) The post fertilization wave reflects spermaster growth (cf. Figs 2, 3). Video time-lapse observation showed the Activation Wave (AW), which started between t₀.₀₆ and t₀.₀₈ from the Sperm Entrance Point (SEP) and moved with a speed of 10μm/sec towards the opposite side; the animal pigment cap then contracted towards the animal pole region, followed by the gravitationally directed 'rotation of orientation', while by redispersion of the pigment the SEP became clearly visible around t₀.₁₆. Then again a perceptible change in reflectivity proceeded (cf. Hara et al. 1977) from the SEP to the opposite side, but now
Fig. 4. Spermaster development in artificially fertilized oocyte at relative time to. In the cortical region pigment granules are arranged along the blue-staining aster fibrils (A, B). cf: male pronucleus. Bouin d'Hollandé fixative; 6 µm, para-median, Histowax section. Bar = 50 µm in all figures. 5 refers to Fig. 5.
Fig. 5. Yolk distribution during spermatodesm growth, same sections as in Fig. 4. Zone 1: fine yolk with aster rays; zone 2: loosely packed medium and fine yolk. Bar = 50 μm.

Fig. 6. 6 μm section of egg at relative time 46. Spermatodesm rays (↓↓) reach dorsal cortical region (↓↓ to the right). V: ventral; D: dorsal. Bouin d’Hollande; azofuchsine-anilin blue stain. Bar = 50 μm.
Determination of dorsoventral axis in Xenopus egg

with a speed of 1 µm/sec. This first PFW was occasionally followed by a second one. Our cytological analysis showed that in para-median sections the extension of the growing spermaster is well correlated with the level of the first PFW on the egg surface, as registered by video time-lapse: *no aster rays reach the cortex beyond the level of the PFW* (Fig. 3).

When viewed from the animal side the PFW was no longer visible around to-0.5. Between to-0.45 and to-0.65 the aster rays extended dorsally only slightly further and the ventral rays started to break down at to-0.53. However, between to-0.65 and to-0.8, shortly before fusion of the pronuclei, aster rays in the equatorial region resumed growth in the dorsal direction, and reached the dorsal cortex (Figs 6A, B). Meanwhile, aster rays in the cytoplasm closely surrounding the pronuclei started to disintegrate rapidly shortly before first cleavage, thus causing the cytoplasm in this region to become very loose (Fig. 3).

c) *Fertilization produces a grey crescent in a non-random position.* The visibility of the grey crescent (GC) (Fig. 7) in *Xenopus laevis* varied with the pigmentation of the eggs, which differed among egg batches from different females, and occasionally also between eggs within the same batch. Diffuseness of the GC prevented its exact localization. In well-pigmented eggs we recorded the position of the centre of the GC in relation to the SEP by marking eggs in an equatorial position, either closest to the SEP, or at the meridian 180 degrees from the SEP (Table 2) as soon as the SEP was visible; the position of the GC was recorded around to-0.5. About 12 h later, we recorded the position of the blastopore in the

Table 2. Localization of SEP, first cleavage plane and dorsal blastoporal lip

<table>
<thead>
<tr>
<th>Localized item</th>
<th>180 - 160 - 140 - 120 - 100</th>
<th>Localization of mark</th>
</tr>
</thead>
<tbody>
<tr>
<td>grey crescent centre (n = 118)</td>
<td>89 10 1</td>
<td>180° from SEP</td>
</tr>
<tr>
<td>(14 batches)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>first cleavage plane (n = 131)</td>
<td>78 17 4 1</td>
<td>180° from SEP</td>
</tr>
<tr>
<td>centre dorsal blastoporal dip (n = 77)</td>
<td>65 24 11</td>
<td>180° from SEP</td>
</tr>
<tr>
<td>(n = 83)</td>
<td>71 21 4 4</td>
<td>on SEP</td>
</tr>
<tr>
<td>Ditto (Kirschner et al., 1980)</td>
<td>70 27 3</td>
<td>on SEP</td>
</tr>
</tbody>
</table>
same embryos (Table 2), which in about 70% of eggs was in the sector 160–180° from SEP, in about 27% in the sector 120–160° and in only about 3% at 100–120°. As in other anuran species, in fertilized eggs both the grey crescent and the blastopore form on the meridian opposite that of the SEP. The SEP is

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Fig. 7. Egg at relative time t₀·₆₄, showing clear grey crescent (gc). SEP: sperm entrance point.

Fig. 8. Beginning spermaster. ♂: sperm head. Bar = 50μm.

Fig. 9. 6μm paramedian section of egg 50 min after pricking, locally activated by pricking, shows the central cytoplasm (cc) at the animal pole. The axial symmetry of the egg is unchanged. PrP = position of prick activation. Bar = 50μm.
**Determination of dorsoventral axis in Xenopus egg**

A distinct spot and as a reference point for the future dorsal–ventral axis is more accurate than the less well-circumscribed GC, although when the centre of the GC is identifiable it is located close to the meridian opposite the SEP.

d) **Prick activation produces a grey crescent in a random position and does not cause yolk asymmetry as seen in fertilized eggs.** Well-pigmented eggs, carefully selected for a centrally located maturation spot, were activated by pricking at a point (PrP) between the animal pole and the equator. Of these eggs 5–10% died because of the treatment. The egg reacts upon prick activation by an activation wave and activation contraction, extrusion of the second polar body and formation of a GC (Table 3), the orientation of which appeared to be independent of the site of the PrP. The time interval between GC formation and pricking was similar to that between fertilization and GC formation in fertilized eggs.

In all eggs fixed immediately after pricking the second maturation spindle was found at the animal pole and the various yolk granules showed the normal axially symmetric arrangement. 20 min after pricking the second polar body had been extruded and the CC had shifted from the centre towards a somewhat more eccentric or animal position. At 40 min after pricking the haploid nucleus was still located near the animal pole. Occasionally it had divided once or twice. The original axial yolk symmetry was less apparent. The CC was found either between the centre and the animal pole, on top of a column of medium yolk granules, or in a lower and more eccentric position, in a few cases even at the animal pole (Fig. 9). The latter position was more common when fixation was delayed to 60 or 85 min after pricking. Only exceptionally was the CC found opposite the PrP. It was then connected to the egg nucleus by thin fibrils, vaguely suggesting the formation of a small female monaster through limited activation of the egg centriole.

Similar observations have been made in eggs activated by keeping them in a hypertonic medium (Rzehak & Ubbels, unpubl.). Both groups of artificially activated eggs showed decreased cytoplasmic segregation when compared to fertilized eggs. We conclude that activation in the absence of a sperm strongly interferes with the normal process of cytoplasmic segregation. In the absence of

<table>
<thead>
<tr>
<th>Table 3. Localization of grey crescent in respect to the pricking point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Localization grey crescent centre (n = 37)</td>
</tr>
<tr>
<td>Angle from meridian through pricking point</td>
</tr>
<tr>
<td>9 4 6 3 2 7 2 2 2</td>
</tr>
</tbody>
</table>

Eggs were pricked between central animal pole patch and the equator, and after activation kept in 25% MMR containing 5% Ficoll. In 37 eggs, i.e. about 15% of the pricked eggs, the grey crescent could be clearly determined; 5% of the pricked eggs died.
a sperm the yolk apparently rearranges under the influence of gravity alone.

e) **Asymmetry after fertilization is blocked by microtubule poisons.** In order to test the role of microtubules in the process of symmetrization, eggs were incubated in different solutions of vinblastine (VB), viz. 1·0, 0·2 and 0·04 mg/ml (VB was dissolved in 25 % MMR) for various time intervals, starting at different times after fertilization: t₀-1₄ to t₀-₄₈; t₀-₁₄–t₀-₇₅; t₀-₃₄–t₀-₄₁; t₀-₃₄–t₀-₇₅. Concentrations lower than 0·04 mg VB/ml did not interfere with cleavage.

When the VB incubation (1 mg/ml) started at t₀-₁₄, i.e. before the extrusion of the second polar body (Fig. 2), the egg nucleus stayed in the cortex, the male nucleus either in the cortex or in the subcortical cytoplasm. The CC was in most cases found somewhere between the centre of the egg and the animal pole when such eggs were fixed at t₀-₄₈; however, when fixation was made at t₀-₇₅ the CC was located near the animal pole, as in prick-activated eggs. In these cases the axial yolk symmetry was maintained.

When a short incubation (t₀-₃₄–t₀-₄₁) started after the extrusion of the second polar body even stronger solutions (1·0 or 0·2 mg VB/ml) did not influence the positions of the male and female pronuclei, the spermaster, the CC or the yolk compared to controls. However, after a longer treatment (t₀-₃₄–t₀-₇₅) with 1·0 mg VB/ml the CC was always near the animal pole and the pronuclei never fused. In the lowest VB concentration (0·04 mg/ml) the pronuclei did fuse at the same time as in untreated control eggs, but yolk asymmetry was only observed in some of these eggs.

Fertilized eggs which had been injected with 20 nl of 0·5 mM-colchicine, which blocked cleavage in control eggs, showed phenomena similar to those described for the vinblastine-incubated eggs.

Thus vinblastine incubation and colchicine injection interfere with normal cytoplasmic segregation in fertilized eggs to a degree dependent on VB concentration and duration of treatment. Pronuclear movements are not influenced by the treatment when it starts after extrusion of the second polar body. The pronuclei do not fuse in concentrations higher than 0·04 mg/ml.

f) **Rotation causes reversal of yolk asymmetry and reversal of dorsal–ventral axis.** Newly fertilized eggs in 25 % MMR containing 5 % Ficoll, having their
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SEP in the equatorial region, were rotated by 90° about a horizontal axis and kept at this orientation so that the equatorial position near the SEP was either at the top or at the bottom (cf. Materials and Methods). The treatment started at an appropriate time and lasted for a sufficient period, so that in all embryos not fixed for cytology the blastopore developed in the region which had been uppermost during the treatment. Thus we were reasonably sure that in the fixed eggs gravity had either upset (in eggs having their SEP up) or reinforced (in eggs having their SEP down) normal axis formation as directed by the sperm. After fixation the SEP was marked by a small cortical wound.

In histological sections of such embryos a narrow ridge of coarse yolk granules was closely apposed to the cell membrane on the side along which the yolk mass had moved under gravity after egg rotation, i.e. opposite the SEP in SEP-down eggs and on the SEP side in SEP-up eggs (Figs 10, 11). Medium yolk granules accumulated near the ridge. However, in contrast with the yolk ridge, the
Cortical contraction and yolk asymmetry

Fig. 13. The progressively expanding spermaster causes shifts of animal cytoplasmic materials, while the haploid female pronucleus, occasionally developing a tiny aster, moves centrally after extrusion of the second polar body. The joined pronuclei lie together, while the central cytoplasm progressively shifts dorsally. Only after fusion of the pronuclei does an asymmetrical cortical contraction towards the sperm entrance point, possibly induced by the interaction of aster rays and dorsal cell membrane or cortex, cause yolk shifts in the vegetal half, resulting in asymmetrical yolk distribution and vitelline wall formation.

localization of the DC was no different from that in unrotated controls, viz. the DC was found on the side opposite the SEP in both groups of reversals (Fig. 12), irrespective of their prospective dorsal side.

To find out whether microtubules prevent the DC from moving, eggs were rotated during incubation in MMR containing VB (concentrations interfering with cleavage). In this series the CC was found in the region which was uppermost after egg rotation, independent of whether the equatorial position near the SEP was up or down. In one egg sample VB had apparently penetrated less well, since the spermaster had not been broken down in all eggs. In those eggs the CC was either in its normal position or only slightly eccentrically placed. However, in all cases, a clear extended vitelline wall was present, more so than in rotated eggs not exposed to VB; this suggests that microtubules may constrain the movement of the cytoplasmic content in normal eggs.

**DISCUSSION**

Vital-staining experiments in *Xenopus laevis* eggs show that, as in other anuran species (cf. Gerhart, 1980), the centre of the grey crescent as well as the blastopore (Kirschner et al. 1980) mark the dorsal side of the embryo. Since the grey crescent is not always clearly visible and sometimes rather diffuse we prefer to use the SEP as an early visible and reliable reference point for the prospective dorsal–ventral axis under normal conditions.

The Activation Wave and the Post Fertilisation Wave(s), as visualized by time-lapse cinematography, start from the SEP and thus appear to be early expressions of dorsal–ventral polarity. The question is whether they function in
the process of dorsoventralization or only secondarily reflect some other event(s) concerned with the origin of dorsoventral polarity.

The AW starts between times $t_{0.06}$ and $t_{0.08}$ from the SEP in fertilized eggs (Hara & Tydeman, 1979) and from the pricking point in locally activated eggs (Hara et al. 1980) and moves as a dark zone (speed $10 \mu m/sec$) towards the opposite side. Hara & Tydeman (1979) suggest that it reflects the propagation of the front of the cortical granule breakdown and related phenomena (cf. Grey, Wolf & Hedrick, 1974). Their time-lapse films clearly show that, after passage of the AW, the pigment cap contracts first and then the egg starts to rotate in the vitelline membrane under gravity, while the pigment cap relaxes again (cf. Fig. 1 in Hara & Tydeman, 1979). Stewart-Savage & Grey (1982) assume that the contraction involves the entire cortex and mention that it occurs during the same time span as the wave of cortical granule exocytosis. To our knowledge the work of these authors did not involve time-lapse filming.

The nature of the PFW (we will only consider the first one) differs from that of the AW. In fertilized eggs the sperm centriole initiates the formation of a spermaster. Between $t_{0.16}$ and $t_{0.45}$ the PFW moves over the egg surface more slowly than the AW ($1 \mu m/sec$) from the SEP towards the opposite side, as a perceptible change in reflectivity of the surface (Hara et al. 1977). It coincides with internal cytoplasmic movements, grey crescent formation, and additional rearrangements of the cytoplasm (Ubbels, 1977), which ultimately lead to displacement of the CC to the dorsal side and formation of the vitelline wall. The cytological analysis of 65 uncleaved fertilized eggs fixed after video time-lapse observation of the PFW (cf. Fig. 3), shows that the PFW corresponds to the moving border of aster microtubule extension. The nature of possible interactions of the aster rays with the egg cell membrane and/or cortex remains to be established. The sections show that the cortical pigment migrates inward from $t_{0.17}$ to $t_{0.51}$, but rather abruptly returns to the outer region between $t_{0.62}$ to $t_{0.71}$ (Ubbels & Paleček, unpublished observations) (cf. Table I).

By determining the displacement of cortical pigment granules under mild centrifugation and using this as a measure of cortical rigidity, Kubota (1967) has demonstrated a gradual rise of cortical rigidity between $t_{0.18}$ and $t_{0.50}$. Spermaster enlargement occurs between $t_{0.18}$ and $t_{0.66}$ (cf. Table 1) (for the sake of convenience Kubota's absolute time scale has been converted to our relative time scale; cf. Table I and Gerhart, 1980). Elinson & Manes (1978), Elinson, 1980 describe a wave of shortening microvilli in fertilized eggs of *Rana pipiens* in the same relative time period as that of the PFW in *Xenopus laevis* eggs, also starting from the SEP. They assume that spermaster growth causes an increase in stiffness of the cytoplasm, which influences cortical movements finally resulting in grey crescent formation.

In *Xenopus laevis* the PFW is visible from $t_{0.16}$ to $t_{0.45}$, and spermaster growth occurs between $t_{0.16}$ and $t_{0.65}$ (cf. *Rana nigromaculata*, Kubota, 1967), although from $t_{0.45}$ onwards the dorsally directed aster growth is rather limited and the
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aster even starts to break down ventrally from to-53 onwards. In this period the aster fills about three quarters of the animal hemisphere (Fig. 3). We think that this may explain the egg’s increasing resistance to rotation and centrifugation around to-6 (Gerhart et al. 1981; Scharf & Gerhart, 1980).

No spermaster forms in prick-activated Xenopus eggs which lack the sperm centriole, nor in fertilized eggs incubated in vinblastine which interferes with microtubule assembly. Both experimental treatments interfere with the cytoplasmic segregation which in normal development leads to dorsoventralization of the embryo (Ubbels, 1977), and the original axial symmetry of the egg is maintained. In both cases the central cytoplasm is ultimately found at the animal pole, at least when the interval between the beginning of the treatment and the fixation is long enough (Ubbels & Koster, 1980). In such eggs yolk rearrangements can be directed by gravity alone, the PFW is absent and cortical pigment movements are abnormal. We propose that the presence of the sperm and a microtubule array is essential for normal cytoplasmic segregation: obviously structuring of the cytoskeleton under the influence of the sperm (most probably of its centriole), is essential for the establishment of the dorsoventral axis and for normal development of the embryo upon egg activation (Subtelny & Bradt, 1963; Manes & Barbieri, 1977; Raff, 1979 and literature cited there). We think that this view is supported by the results of recent experiments by Scharf & Gerhart (pers. comm.). They treated Xenopus eggs by hydrostatic pressure or cold, both treatments known to break down microtubules, and found that the eggs are most sensitive to both treatments around to-6. The embryos become radialized by such a treatment when a high dose is applied. Our material shows that around this time the spermaster is maximally developed and we assume that this is the target broken down by these treatments.

In fertilized eggs the centre of the grey crescent is found opposite the sperm entrance point in 89% of the eggs. However, as in Bufo arenarum (Manes & Barbieri, 1977) and Rana fusca (Brachet, 1911), although after activation both the second polar body and the grey crescent appear at the usual time, we find no relation between the position of the centre of the grey crescent and that of the prickling point.

It has been suggested that in fertilized eggs the grey crescent may form as a result of an asymmetrical cortical contraction (Løvtrup, Landstrøm & Løvtrup-Rein, 1978). This contraction may draw pigment in the animal hemisphere towards the sperm entry site and may also perturb deeper cytoplasmic materials and thereby establish regional differences along the prospective dorsoventral axis (Gerhart et al. 1981). In sections pigment granules are localized along cortical fibrillar structures. We assume that pigment movements concerned with grey crescent formation reflect rearrangements of the cytoskeleton of the egg involved in determinative localization of cytoplasmic components, and that cortical contraction occurs at a particular phase of the cell cycle initiated by egg activation. However, the presence of a centriole is essential for the orientation of the
cortical contraction (cf. Manes & Barbieri, 1977; Raff, 1979, and literature cited there).

In fertilized eggs the equatorial aster rays resume growth at t0.65, reach the dorsal egg cortex between t0.67 and t0.8, and are occasionally seen to curve towards the animal pole (cf. Fig. 3). This might be a direct morphological expression of the asymmetrical cortical contraction which we suppose causes further rearrangements in the animal and vegetal hemisphere (Gerhart et al. 1981). It may also tentatively explain why eggs which only gradually (t0.2–t0.45; Ubbels, 1977; Paleček et al. 1978) develop a distinct grey crescent show a rather constant pigment pattern in the t0.45–t0.65 period, and a slight accentuation of the grey crescent for a short period between t0.65 and t0.8. Such an accentuation of the grey crescent in *Xenopus laevis* would be consistent with observations in *Rana fusca* and *Rana pipiens* (Ancel & Calame, 1959; Manes, Elinson & Barbieri, 1978; Manes & Elinson, 1980). It seems possible that the interaction of the aster rays with the dorsal equatorial cortex initiates this second phase of the cortical contraction.

In eggs immobilized and rotated 90° off the vertical axis for a short period (Kirschner et al. 1980) the blastopore forms in the region which was uppermost during the treatment. The dorsal side later forms on that side where the heavy yolk moved down beneath the egg cortex under the influence of gravity, leaving a thin layer of coarse yolk on the new dorsal side (cf. Figs 10, 11; Ubbels et al. 1979). This is tentatively considered as a ‘mur vitellin’ (Pasteels, 1964) and in some way it creates or reinforces conditions for dorsal development (Kirschner et al. 1980, 1981; Gerhart et al. 1981). The yolk patterns of eggs rotated with their SEP either up or down mirror each other (Fig. 12). The dorsal cytoplasm and the pronuclei do not alter their positions, however, the dorsal cytoplasm together with the pronuclei and the internal egg cytoplasm apparently form an entity anchored in the egg by a central web of microtubules, including the spermaster and additional cytoskeletal and cytomuscular elements (Fig. 13). We assume that the yolk rearrangements in the unrotated fertilized egg are caused by cortical contraction oriented by the sperm centriole and that the yolk rearrangements in the rotated eggs are caused by gravity. The PFW’s do not directly function in the process of dorsoventralization but externally reflect internal events concerned with the origin of dorsoventral polarity.

In *Xenopus laevis* the germinal vesicle content stains intensely blue with azofuchsin-anilin blue. An amount of blue-stained fibrillar cytoplasm progressively increasing with time in artificially matured oocytes, is present at and beneath the inner pole of the dissolving germinal vesicle. This material is still found on top of the mass of coarse yolk in the centre of unfertilized mature oocytes and newly fertilized eggs as the Central Cytoplasm (CC) (Ubbels et al. in prep.). During the first cleavage period the central cytoplasm moves to the dorsal side and becomes known as the dorsal cytoplasm.

The rotation experiments show that, just as the grey crescent cortex, the dorsal
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cytoplasm does not contain irreplaceable, long-lasting dorsal determinants. We assume that both structures secondarily reflect rearrangements in the cytoskeleton which in normal development appear to be guided by the sperm centriole. This paper provides experimental support for the view that the shift of the cytoplasmic materials, as well as their interaction with the spermaster rays, may influence the egg cortex and cue the asymmetrical cortical contraction. This finally leads to the formation of the vitelline wall and the 'vegetal dorsalizing centre', which is thought to be responsible for dorsoventral polarity in the process of mesoderm induction (Kirschner et al. 1981). It should be remembered that the AW reflects the extrusion of the cortical granules. Whether fusion of the cortical granules with the egg membrane affects the cortical structure and contributes to the asymmetry of cortical contraction(s) in subsequent development is unknown.

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