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Deep cytoplasmic rearrangements during early development in *Xenopus laevis*

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Summary

The egg of the frog *Xenopus* is cylindrically symmetrical about its animal–vegetal axis before fertilization. Midway through the first cell cycle, the yolky subcortical cytoplasm rotates 30° relative to the cortex and plasma membrane, usually toward the side of the sperm entry point. Dorsal embryonic structures always develop on the side away from which the cytoplasm moves. Details of the deep cytoplasmic movements associated with the cortical rotation were studied in eggs vitally stained during oogenesis with a yolk platelet-specific fluorescent dye. During the first cell cycle, eggs labelled in this way develop a complicated swirl of cytoplasm in the animal hemisphere. This pattern is most prominent on the side away from which the vegetal yolk moves, and thus correlates in position with the prospective dorsal side of the embryo. Although the pattern is initially most evident near the egg's equator or marginal zone, extensive rearrangements associated with cleavage furrowing (cytoplasmic ingression) relocate portions of the swirl to vegetal blastomeres on the prospective dorsal side.

Key words: *Xenopus*, cleavage, cytoplasmic localization, dorsal–ventral axis, first cell cycle, ingression.

Introduction

The polarized organization of the *Xenopus* egg's contents relates directly to the region-specific development of the embryonic germ layers (reviewed in Gerhart, 1980; Gerhart *et al.* 1989). This regular correspondence between egg and embryo organizations suggests that there are specific molecules, activities or structures in the unfertilized egg which become localized and function to direct the various developmental pathways that tissues take in order to differentiate. The steps in this localization process have not been completely elucidated.

In *Xenopus*, the point of sperm entry (SEP) defines the orientation of the embryonic dorsal–ventral axis, with the dorsal side normally forming opposite the SEP (Ancel and Vintemberger, 1948; Elinson, 1975, 1980). During the first cell cycle, the vegetal yolky cytoplasm – the vegetal yolk mass – rotates relative to the plasma membrane and cortex about 30° *toward* the SEP, that is, *away* from the prospective dorsal side of the embryo (Vincent *et al.* 1986; Vincent and Gerhart, 1987). This movement seems to depend on an array of parallel microtubules which appear transiently in the vegetal cortex during the first cell cycle (Elinson and Rowning, 1988).

The vegetal cortical-cytoplasmic rotation is thought to be the earliest determining event in the specification of the embryo's dorsal–ventral axis, as suggested by a number of experiments in which redirecting or halting the vegetal cortex movement alters the orientation or degree of development of dorsal structures (Grant and Wacaster, 1972; Malacinski *et al.* 1977; Manes and Elinson, 1980; Scharf and Gerhart, 1980, 1983; Kirschner and Gerhart, 1981; Gerhart *et al.* 1981; Black and Gerhart, 1985). The process by which this cortical-cytoplasmic rotation specifies the dorsal side is not understood, but it is clear that, before fertilization, the potential for producing dorsal structures exists at every point around the animal–vegetal axis. It is unlikely that the specified dorsal information actually resides in the cortex itself: first, because the embryonic fate map can be reversed relative to the cortex by centrifugation during first cell cycle (Malacinski, 1984; Cleine and Dixon, 1985; Black and Gerhart, 1986), and second, because a dorsal-determining target of UV that is distinct from the rotation machinery exists in premeiotic oocytes (Elinson and Pasceri, 1989). Thus, the rotation's function is presumably to redistribute or activate components necessary for dorsal–ventral specification in the egg.

The rotation machinery is located in the vegetal cortex, but its physical relationship to the dorsal specification process is not known. Information necessary for normal dorsal development is known to reside in the vegetal tiers of blastomeres at the 32-cell stage

(Gimlich and Gerhart, 1984; Gimlich, 1986), suggesting a proximity to the rotation machinery. However, a number of recent experiments suggest that blastomeres in the animal hemisphere have specialized in some way as well (Kageura and Yamana, 1984; Cardellini, 1988; London *et al.* 1988; Takasaki and Konishi, 1989). How the cortical-cytoplasmic rotation would transmit information to the animal hemisphere remains enigmatic (see Wakahara, 1989).

Rearrangements of the deep cytoplasm that might accompany cortical-cytoplasmic rotation have not been documented because it is difficult to label selectively regions of egg cytoplasm before cleavage. In *Xenopus* eggs, the only reliable cytoplasmic marker has been the yolk platelet gradient, which has been used to indicate movement of various cytoplasmic regions during early development (Nakatzuji, 1975; Phillips, 1985; Ubbels *et al.* 1983; Neff *et al.* 1984; Black and Gerhart, 1986). We have sought to improve this situation by selectively marking peripheral and animal hemisphere yolk platelets during oogenesis with the fluorescent dye trypan blue (TB). Epifluorescence microscopy of histological sections and confocal scanning laser microscopy of whole-mount specimens have enabled us to obtain detailed information about cytoplasmic movements of the first cell cycle and cleavage period in normal development in *Xenopus*. In subsequent reports, we will examine the roles of these rearrangements in establishing body pattern in the early embryo.

Materials and methods

Oocytes, eggs and embryos

In vitro maturation of manually defolliculated oocytes was accomplished by adding progesterone ($4\,\mu g\,ml^{-1}$) to full-strength MMR. Eggs of *Xenopus laevis* were fertilized, dejellied and cultured as previously described (Vincent *et al.* 1986). Dejellied eggs were immobilized on plastic dishes by immersion in 6% Ficoll, arranged with sperm entry points (SEPs) in a known orientation, and cultured at 18–22°C to appropriate stages. Before fixation in Bouin's fixative, eggs were gently pricked with a glass needle at the SEP to produce a tiny reference scar, since vital dyes such as Nile Blue Sulfate disappear during fixation. The average orientation of the definitive dorsal–ventral axis relative to the SEP was determined by scoring a group of control embryos at stage 14. Developmental times in the first cell cycle are normalized to a scale of 0 to 1.0, referring to fertilization and appearance of the first cleavage furrow, respectively.

Fluorescent marking of egg cytoplasm

Eggs with labelled yolk platelets were obtained by methods previously developed to study vitellogenesis in *Xenopus* (Danilchik and Gerhart, 1984, 1987; Callen, 1986). 7 to 10 days after gonadotropin-induced spawning, female frogs were injected in the dorsal lymph sac with 100 to 400 μl of $10\,mg\,ml^{-1}$ TB (Polysciences or Sigma), which had been dialyzed extensively against distilled water (MWCO 15000), lyophilized, and dissolved at $10\,mg\,ml^{-1}$ in MMR. 10 to 14 days after injection of label, frogs were primed by injection of 50 i.u. of pregnant mare serum gonadotropin (Calbiochem), and on the following day again induced to spawn by injection of 800 i.u. of human chorionic gonadotropin (Sigma).

Time-lapse recording of vegetal yolk mass rotation

The direction of cortical-cytoplasmic rotation was determined by video time-lapse recording of the vegetal surface of TB-labelled eggs during the first cell cycle. The time-lapse recorder consisted of a BioRad MRC-500 scanning laser confocal imaging system connected to a Nikon Diaphot inverted microscope. Images were accumulated from three to five sequentially scanned frames and then contrast-enhanced. The resulting video output was then recorded on a JVC BR-9000U time-lapse VCR. Successive video images were collected every 15 to 20 s. The direction of vegetal yolk mass rotation was easily determined with this system in TB-labelled eggs because individual fluorescent yolk platelets could be tracked across the entire vegetal surface. Up to 6 eggs could be viewed simultaneously by using a 4×/0.2 N.A. objective. At the end of the first cell cycle, the direction of rotation was determined by playing back the tape. Eggs were then gently pricked with a glass needle to make an unambiguous mark of the rotation direction, and fixed in Bouin's one minute later.

Histology and microscopy

Eggs and embryos were fixed and sectioned as described previously (Danilchik and Black, 1988). Eggs were embedded in JB-4 (Polysciences) with known orientation relative to the SEP or other reference mark, and sectioned either sagittally (parallel to the plane defined by the animal–vegetal axis and either the SEP meridian or direction of cortical-cytoplasmic rotation), or horizontally (perpendicular to the animal–vegetal axis). Sections were examined by fluorescence microscopy (rhodamine excitation).

For confocal microscopy, eggs were fixed with Bouin's fixative and washed with 5 mM NH_4OH in 50% ethanol. Pigmented eggs were then bleached for 2.5 h with 15% hydrogen peroxide (Baker) and 50% ethanol. Bleaching was promoted by illuminating the eggs in their glass shell vials on a standard white fluorescent light box. This treatment did not detectably affect the TB labelling. Samples were then washed in 50% ethanol and dehydrated and cleared as in Dent and Klymkowsky (1989).

Results

Labelling of cytoplasm with fluorescent yolk platelets

Previous work with fluorescent and radioactive analogues of vitellogenin indicated that uptake of vitellogenin by endocytosis is nearly uniform over the *Xenopus* oocyte surface (Danilchik and Gerhart, 1987). Oocytes readily take up TB from the maternal circulation (Dumont, 1972), and deposit it into yolk platelets in a pattern similar to that of vitellogenin (Danilchik and Gerhart, 1984, 1987; Callen, 1986). The dye is fixable *in situ* with Bouin's fixative (Telfer and Anderson, 1968). In endosomes and yolk platelets, it is highly fluorescent, with a broad emission spectrum similar to that of rhodamine. Thus, even invisibly small amounts of the dye, as in individual yolk platelets, are readily detectable by epifluorescence microscopy (Fig. 1). Large numbers of fertilizable, developmentally normal eggs with nearly identical labelling patterns are obtained by injecting TB into frogs one to two weeks after hCG-induced spawning, and then inducing a second spawning within two weeks. Although TB has powerful teratogenic effects when present in the

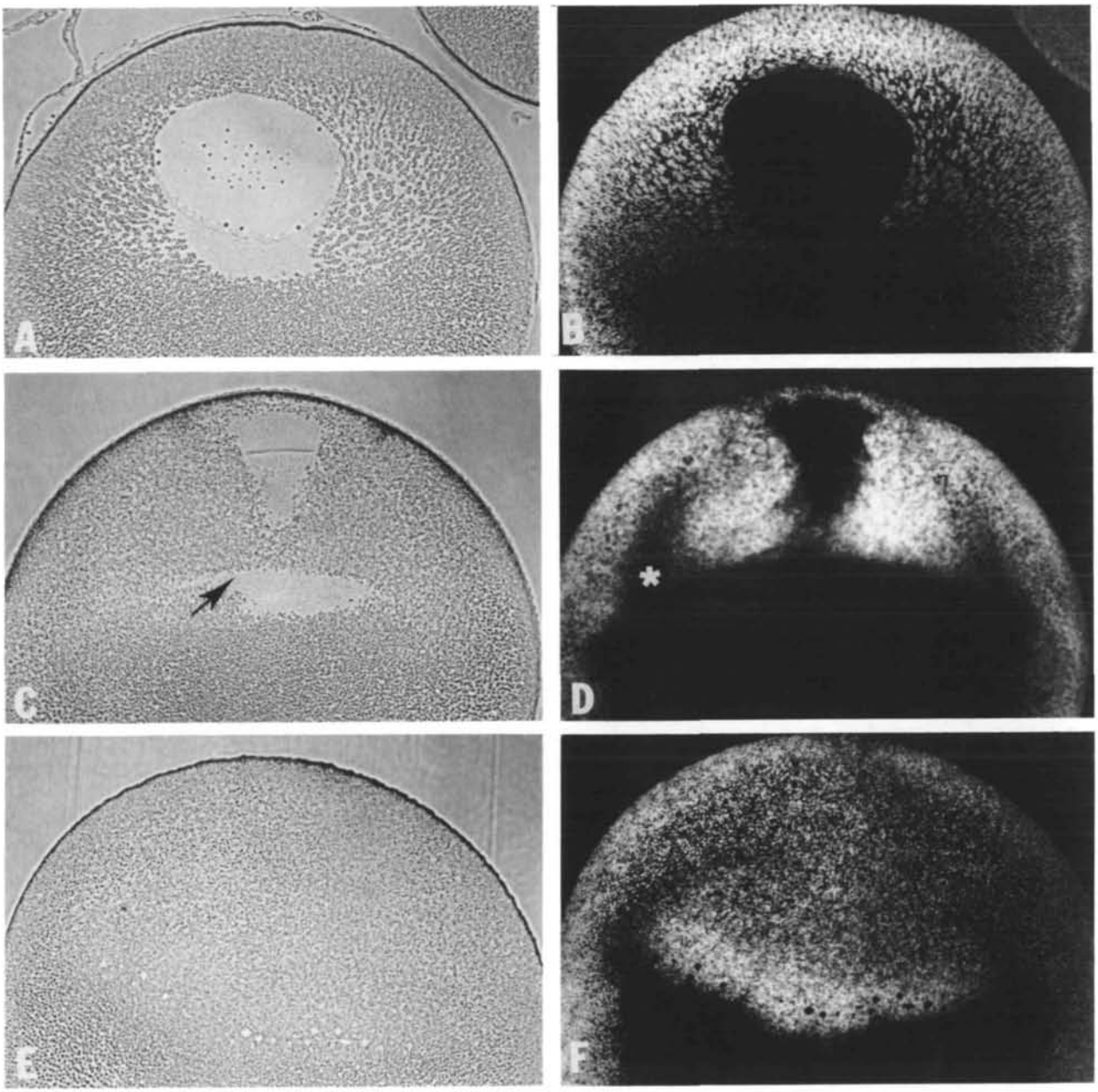

Fig. 1. Oocyte contents undergo significant rearrangements during germinal vesicle breakdown. (A,B) Oocyte, fixed 6 days after injection of TB into female frog, was sectioned at 6 μm, as described in *Methods*. (C,D) Stage V–VI oocyte 3 h after progesterone treatment, with orientation as above. (E,F) Unfertilized egg spawned from same female from which oocytes in previous panels had been obtained 3 days earlier. All sections are through the animal–vegetal axis, animal pole up. Left panels: phase-contrast. Right panels: epifluorescence (rhodamine filter set). Magnification: 15.5×.

blastocoel before gastrulation (Waddington and Perry, 1956; Greenhouse and Hamburgh, 1968; Gerhart *et al.* 1984; Danilchik, 1986), the dye has no teratogenic activity when confined to yolk platelets, as evidenced by normal development at least through metamorphosis of tadpoles derived from intensely labelled eggs (not shown).

Cytoplasmic rearrangements during maturation

Oocytes removed from a frog injected with TB 6 days earlier reveal a pattern of dye incorporation similar to that previously described for Lucifer Yellow-conjugated vitellogenin (cf. Fig. 6A, Danilchik and Gerhart, 1987). In the animal hemisphere, the small yolk platelets are in radial columns (Fig. 1A, phase-contrast) centering on the large yolk-free region at the vegetal base of the germinal vesicle (GV). Nearly all are heavily labelled with TB (Fig. 1B, fluorescence), indicating that they were undergoing growth at the time of labelling and probably originated during the terminal stages of oogenesis. In contrast, in the vegetal hemisphere, labelling is confined to a thick (100 to 150 μm)

peripheral layer composed of platelets ranging in diameter from 1 to 14 μm. In the deep interior of the vegetal hemisphere, yolk platelets are unlabelled. The thickness of the deposition layer in the vegetal hemisphere depends on the period of exposure to TB during a particular oocyte's growth.

During progesterone-induced *in vitro* maturation, the GV rises to the animal pole and the nuclear envelope breaks down, starting from the vegetal side of the GV (Fig. 1C). Concomitant with the GV's poleward movement, a large volume of fluorescent yolk platelets is displaced inward from the animal pole surface (Fig. 1D), producing a roughly conical arrangement of brightly fluorescent platelets around the nonfluorescent central column of released GV sap. This cone-shaped fluorescent region is flanked laterally by blunt, upturned extensions of the underlying central cytoplasm (Fig. 1D, asterisk). These extensions contain unlabelled yolk platelets that are usually somewhat larger than those found nearby, suggesting that they were displaced there from deeper in the vegetal interior. The yolk platelets in the animal hemisphere are no longer arranged in radial groups as they were before maturation. Rather, individual yolk platelets have freely intermingled with the released GV contents and the surrounding cytoplasm. During maturation, the sub-GV yolk-free region flattens, and appears to limit the extent of inward movement by the labelled animal hemisphere yolk platelets (Fig. 1C; arrow).

Eggs spawned between 10 and 20 days following TB injection yield a consistent labelling pattern: the animal hemisphere cytoplasm labelled throughout with small, fluorescent yolk platelets, and the vegetal hemisphere cytoplasm only at its periphery (Fig. 1E,F). In the animal hemisphere, the small yolk platelets have become more or less evenly dispersed (Fig. 1E), and form a large fluorescent cytoplasmic mass near the center of the egg (Fig. 1F). This central mass of small fluorescent platelets is delimited near the equatorial plane by an abrupt transition to larger, unlabelled platelets. The position of this boundary varies slightly with the length of time between TB injection and spawning, as might be expected from the progressive vegetad movement of labelled animal hemisphere yolk platelets during oogenesis (Callen, 1986; Danilchik and Gerhart, 1987). The upturned extensions of vegetal unlabelled cytoplasm that developed during maturation are still visible at the lateral edges of the central mass (see Fig. 1D). A number of 30–50 μm diameter yolk-free cytoplasmic bodies, possibly remnants of the oocyte's yolk-free region, are frequently found in a plane near the transition between labelled and unlabelled yolk. Additional small, yolk-free areas are occasionally seen throughout the animal hemisphere cytoplasm.

The label distribution in the vegetal hemisphere appears to remain largely undisturbed by the cytoplasmic movements of oocyte maturation.

Cytoplasmic rearrangements during the first cell cycle

To study rearrangements during the first cell cycle, TB-labelled eggs were fixed at various times following fertilization, and sectioned in known orientation relative to the SEP. During the middle third of the first cell cycle, the central animal hemisphere cytoplasmic mass shifts progressively toward the prospective dorsal side. This movement produces a complicated swirl, consisting of alternating labelled and unlabelled cytoplasmic regions in the equatorial zone. The development of this swirl is shown in Fig. 2. Minimal rearrangements of the original egg organization are apparent at 0.3 of the cell cycle (Fig. 2A), consistent with the expectation that the vegetal yolk mass only begins to shift toward the sperm entry point at this time (Vincent *et al.* 1986). The sperm trail is visible as a horizontal dark line of yolk-free cytoplasm penetrating the animal hemisphere from the left side of the egg (S). The upward extensions of unlabelled vegetal cytoplasm that flank the labelled central yolk have begun to lose their earlier symmetry (cf. Fig. 1F): on the SEP side of the egg (left in Fig. 2), the extension forms a more acute angle with the underlying unlabelled cytoplasm, while that on the right has begun to foreshorten and is now nearly perpendicular to the equatorial plane.

By 0.5 of the cell cycle, when the vegetal yolk mass rotation is well under way, the rearrangements of the egg's original cytoplasmic organization are more pronounced (Fig. 2B). The central labelled mass has shifted considerably toward the future dorsal equatorial zone. The upward extensions of unlabelled material flanking it have become even more asymmetric: on the SEP side it has lengthened and flattened, while on the opposite side, it has begun recurving toward the animal pole. The egg itself has flattened under the influence of gravity.

At the time of first cleavage furrow formation (90 min post-fertilization), an extensive cytoplasmic rearrangement (Fig. 2C) with alternating fluorescent and nonfluorescent layers has developed in the prospective dorsal equatorial zone and animal hemisphere. Although the pattern varies in minor details from egg to egg and from batch to batch, common features consistently seen in sagittal section are: (1) an asymmetric, unlabelled vegetal yolk mass, the upper limit of which is slightly higher on the SEP's equatorial zone, presumably as a consequence of its SEP-ward rotation; (2) a long, comma-shaped fluorescent mass, derived from the labelled central cytoplasm, the head of which is always found in the equatorial zone on the side opposite the SEP; and (3) a thick layer of unlabelled cytoplasm, about 150 μm thick, tapering toward the animal pole from the prospective dorsal side of the vegetal yolk mass, presumably deriving from the now greatly lengthened unlabelled extension described above. At its base near the egg's equator, this layer contains slightly larger unlabelled yolk platelets brought up from the deep equatorial region. The prospective dorsal side thus inherits a triple-layered labelling pattern, which we will henceforth refer to as 'the swirl': (1) a deep zone of labelled, formerly central, cytoplasm, internal to (2) a layer of unlabelled cytoplasm derived from the equatorial deep yolk, which is itself internal to (3) the

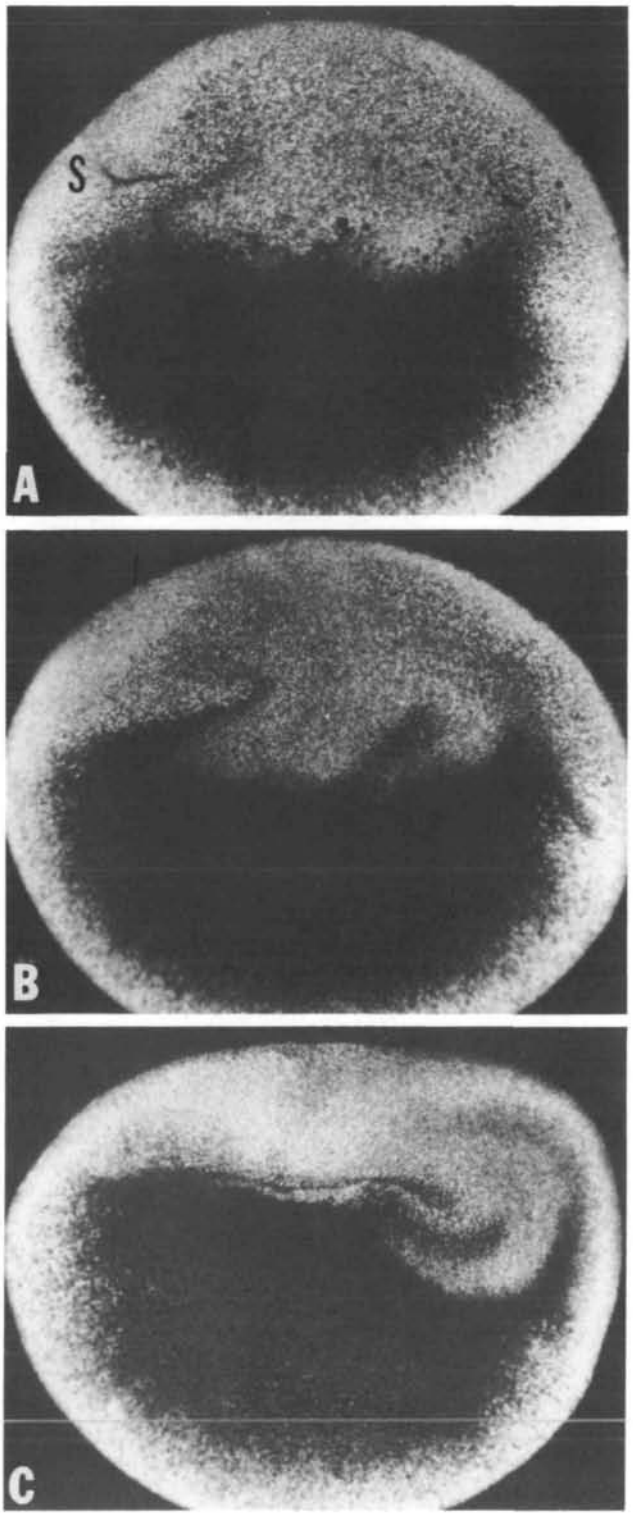

Fig. 2. Cytoplasmic redistributions during first cell cycle. Frog was injected with TB 10 days before spawning, as described in *Methods*. Eggs were fertilized, dejellied and fixed at various times during the first cell cycle, and then sectioned parallel to the SEP meridian and the animal–vegetal axis. Eggs shown here are oriented with animal pole up, SEP on the left side. (A) 30 min post-fertilization (0.3 of first cell cycle). S=sperm trail. (B) 45 min post-fertilization (0.5 of first cell cycle). Fluorescent animal cytoplasm begins shifting toward side opposite SEP. (C) 90 min post-fertilization (1.0 of first cell cycle).

labelled peripheral cytoplasm. In contrast to this dramatic pattern, the equatorial cytoplasm on the SEP side is more uniformly labelled.

The great depth of the unlabelled layer of the swirl (typically more than 120 μm) and its composition (medium-sized, unlabelled yolk platelets from the central equatorial plane) distinguish it from the 'vitelline wall', classically described as a near-surface layer containing large yolk platelets (Pasteels, 1964; Ubbels *et al.* 1983; Phillips, 1985).

The eggs shown in Fig. 2 were obtained from a spawning in which the SEP was a particularly good indicator of the future dorsal side (average angle of SEP to neural groove of 27 voucher stage 18 embryos from the same batch was 149°). Each section represents essentially identical patterns in 4 or more embryos at each time point examined. No significant variations from this pattern were seen in this experiment. In each specimen, a prominent swirl developed on the side opposite the SEP. Confocal scanning laser microscopic examination of 18 embryos (see below) obtained from several spawnings in which the direction of cortical-cytoplasmic rotation was recorded confirms that, in unperturbed eggs, the swirl only develops on the side away from which the vegetal yolk mass rotates. We thus conclude that development of the swirl always accompanies dorsal axis specification in normal *Xenopus* eggs.

The swirl is present not only at the midsagittal plane, but also around a wide sweep of the equatorial zone on the prospective dorsal side. Confocal scanning laser microscopy was used to examine individual intact specimens in multiple planes of section. Fig. 3A is a sagittal optical section of a fertilized egg which has developed a prominent swirl on the side opposite the direction of cortical-cytoplasmic rotation at the end of the first cell cycle. An equatorial section of the same egg (Fig. 3B) indicates that most of the dorsal half of the egg contains elements of the swirl, most notably the alternating layers of labelled and unlabelled cytoplasm.

Cytoplasmic rearrangements in activated eggs

To what extent does the swirl's development depend on expansion of the sperm aster in the animal hemisphere? Eggs activated by electric shock or pricking undergo a cortical-cytoplasmic rotation (Ancel and Vintemberger, 1948), but have no sperm aster. Moreover, activated eggs rescued for normal development by delayed nuclear transplantation, like normal fertilized eggs, develop dorsal structures on the side away from the cortical-cytoplasmic rotation (Gerhart *et al.* 1986*a*,*b*). This result suggests that the rotation itself, or a process closely coupled to it, but not the sperm nucleus or sperm centriole, is required for dorsal–ventral axis specification.

To determine whether the cortical-cytoplasmic rotation alone would produce a swirl in the animal

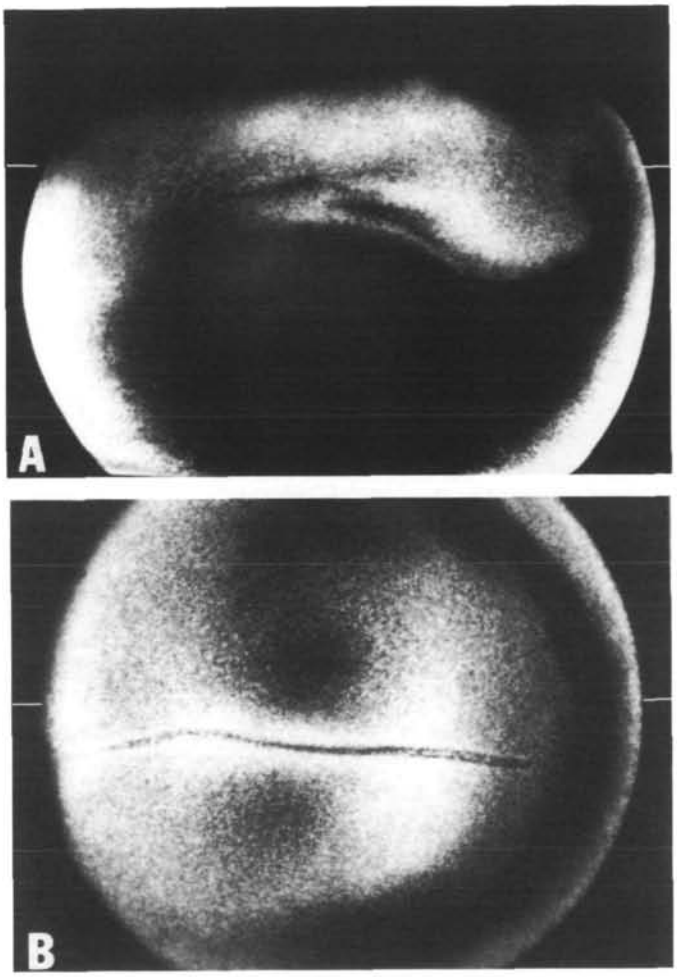

Fig. 3. Confocal laser scanning images of egg near end of first cell cycle. Egg is oriented so that direction of cortical-cytoplasmic rotation was to the left. (A) Mid-sagittal section, with prospective dorsal side to right. (B) Horizontal section of same egg, approximately 300 μm from the animal pole. White bars in margins of each panel indicate plane of section in the other panel.

hemisphere cytoplasm, we activated eggs electrically and then followed the direction of cortical-cytoplasmic rotation by observing pigment granule movement at the vegetal surface *via* video time-lapse recording. Eggs were then fixed and sectioned parallel to the direction of cortical-cytoplasmic rotation. As shown in Fig. 4, the animal hemisphere cytoplasmic flow resembles that of fertilized eggs, with the labelled central mass shifting to the side opposite the direction of vegetal yolk mass rotation. This result demonstrates that the swirl develops in concert with the vegetal yolk mass rotation, and does not require a sperm aster or mitotic spindle. Presumably the swirl develops because the animal hemisphere cytoplasm is somehow displaced by the coherent yolk mass rotating against the vegetal cortex. This possibility is examined in the next section.

Physical model for cytoplasmic flow during cortical-cytoplasmic rotation

Are the 30° rotation of the vegetal yolk mass and the

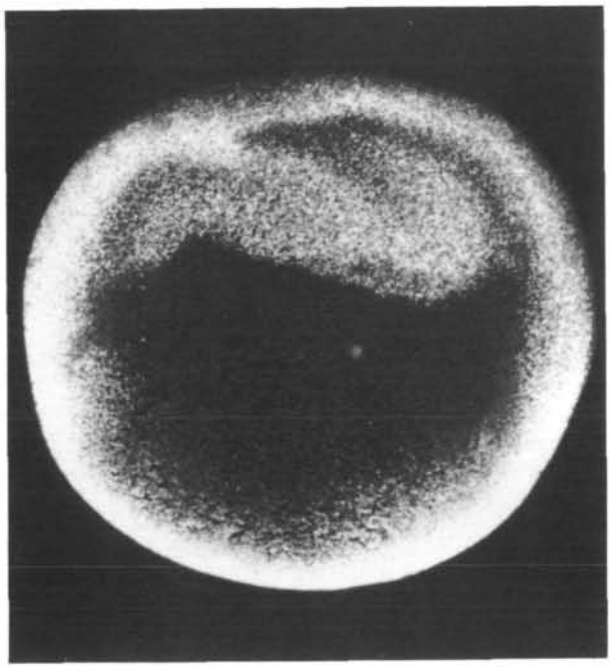

Fig. 4. Swirl in activated egg resembles that of fertilized egg. Egg was dejellied with cysteine in full-strength MMR and activated electrically (Vincent and Gerhart, 1987). Direction of cortical-cytoplasmic rotation was determined by video time-lapse microscopy of the egg's vegetal surface. At 85 min (0.94 of the cell cycle), egg was fixed and sectioned parallel to the recorded direction of cortical-cytoplasmic rotation.

complex swirl pattern mechanically related? To answer this question, we constructed a large-scale model to investigate the flow patterns that would be generated by a 30° rotation of a solid, hemispherical mass within a spherical fluid volume. Two pieces, a block (E) with a hemispherical cavity of radius 1″, and a complementary solid quarter sphere (V), were machined from Teflon to represent half an egg and its vegetal yolk mass as though cut through the animal–vegetal pole (Fig. 5A). The close fit between the two pieces (0.001″ clearance), a circular flange on V and a complementary track in E, ensured that relative rotation would be strictly concentric. The empty volume of the cavity in E was filled with liquid to represent fluid cytoplasm in the animal hemisphere. Flow patterns were studied by following the motion of small drops of dye applied to various positions and depths in the liquid.

An important consideration in modelling flow patterns is to ensure that the Reynolds number (*Re*) – a parameter relating scale, viscosity, and velocity of flow – is of similar magnitude in the systems being compared. For the *Xenopus* egg vegetal yolk mass rotation, we estimated a *Re* in the range of 10^{-7} – using 1200 μm for egg diameter, the kinematic viscosity of glycerol at 20°C ($1.2\times10^{-3}\,m^2\,s^{-1}$) as the value for animal hemisphere cytoplasm, and 8 $\mu m\,min^{-1}$ for the velocity of the vegetal subcortex relative to the surface (Vincent *et al.* 1986). Because the model diameter is 42× greater than that of the *Xenopus* egg, we altered

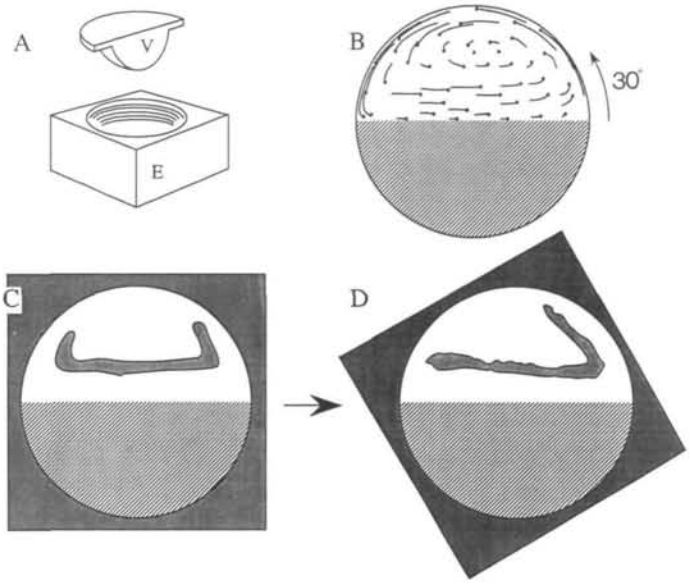

Fig. 5. Teflon model of cytoplasmic flow in frog egg. (A) Diagram showing hollowed out Teflon block (E) to represent spherical egg interior, and quarter sphere (V) to represent vegetal yolk mass. (B) Pattern of flow in glycerol-filled 'animal hemisphere' generated by 30° counterclockwise rotation of block E relative to V. Lines indicate position of dye marks at beginning (no dot) and end (dot) of rotation. (C) Dye mark before rotation. (D) Same dye mark after 30° rotation of outer piece.

fluid viscosity and rotation rates to obtain a *Re* in the same range as that estimated for the egg. By cooling glycerol to −25°C (increasing its kinematic viscosity by almost 200×) and rotating V relative to E at $1°\,min^{-1}$, we achieved a *Re* of about 10^{-6}. At values $\ll 1.0$, tenfold differences in *Re* are not significant, and flow patterns should be identical (Vogel, 1981). In fact, we found that flow patterns were nearly identical with *Re* as high as 10^{-2}. Thus, it is reasonable to expect that flow patterns generated by the model resemble those occurring in a similar system the size of the *Xenopus* egg.

To extend these results to real *Xenopus* eggs, some assumptions must be made about the egg's interior, for which little information is available: (a) that the vegetal yolk mass is indeed hemispherical and solid relative to the animal hemisphere cytoplasm (see Neff *et al.* 1984), (b) that the animal hemisphere cytoplasm is a uniform, Newtonian fluid (see Elinson, 1983), and (c) that there exists friction, i.e. no distinct shear zone, between cortex and subjacent cytoplasm in the animal hemisphere.

Simulations carried out with the model indicate that the volumetric displacement of animal hemisphere cytoplasm by 30° rotation of the solid vegetal yolk mass is indeed sufficient to account for many features of the cytoplasmic swirl in labelled *Xenopus* eggs. Fig. 5B shows the overall flow pattern at the midsagittal plane of the fluid hemisphere generated by a 30° counterclockwise rotation of E relative to V. Flow is circular and counterclockwise, maximal near the surface of E, and diminishing inward to an almost stationary zone about one-third radius from the surface. The counterclockwise flow is deflected inward along the planar surface of V where the two pieces meet, but friction slows the flow near this stationary surface. The fastest interior flow thus occurs about halfway between this surface and the stationary zone.

It is important to note that the pattern of flow depends only on the relative motion of the two pieces, regardless of which is actually moving relative to an outside observer. In other words, identical patterns are generated whether the mass is rotated within the hemisphere, or the spherical surface is rotated around the stationary mass. This observation suggests that, in the *Xenopus* egg, vegetal yolk mass rotation will accomplish essentially the same rearrangement whether the egg surface is fixed to the substratum (as in a Ficoll-filled plastic dish) or free to rotate within its fertilization envelope.

The simulation shown in Fig. 5C and D illustrates how this pattern of flow might generate the swirl seen in labelled *Xenopus* eggs. A dye mark was positioned to represent features of the fluorescent pattern in an unfertilized, TB-labelled egg (Fig. 5C), in particular the boundary between fluorescent and nonfluorescent cytoplasmic zones and the lateral extensions of nonfluorescent cytoplasm protruding into the animal hemisphere (cf. Fig. 1F). Following a 30° rotation, this pattern distorts into a markedly asymmetric swirl (Fig. 5D). This result suggests that, in the *Xenopus* egg, a 30° vegetal yolk mass rotation should be sufficient to produce a substantial, asymmetric swirl on the side away from which the vegetal yolk mass is moving, i.e. the prospective dorsal side.

Cytoplasmic rearrangements during the early cleavage period

The swirl pattern described above persists through most of the early cleavage period. However, an extensive alteration of this organization, brought about by the action of cleavage itself, becomes superimposed on the initial pattern during early development. This process completely reorganizes the contents of the egg during early development.

The section in Fig. 6A shows that many features of the swirl are still present at the beginning of second cleavage, principally the prominent, upturned layer of unlabelled cytoplasm extending from the equatorial zone on the side opposite the SEP. Fig. 6B shows a substantial subduction of peripheral cytoplasm running along the completed first cleavage furrow. A similar subduction appears to accompany the just-begun second furrow. This subduction will be referred to as 'cytoplasmic ingression', following classical terminology although, as will be shown, this ingression is neither unipolar (Schechtman, 1934, 1937) nor cortical (Ballard, 1955). A layer of peripheral cytoplasm, nearly 20% of the egg's radius in thickness, is brought inward along the plane dividing sister blastomeres. This rearrangement is most evident on the right side of Fig. 6B, where the alternate layering of labelled and

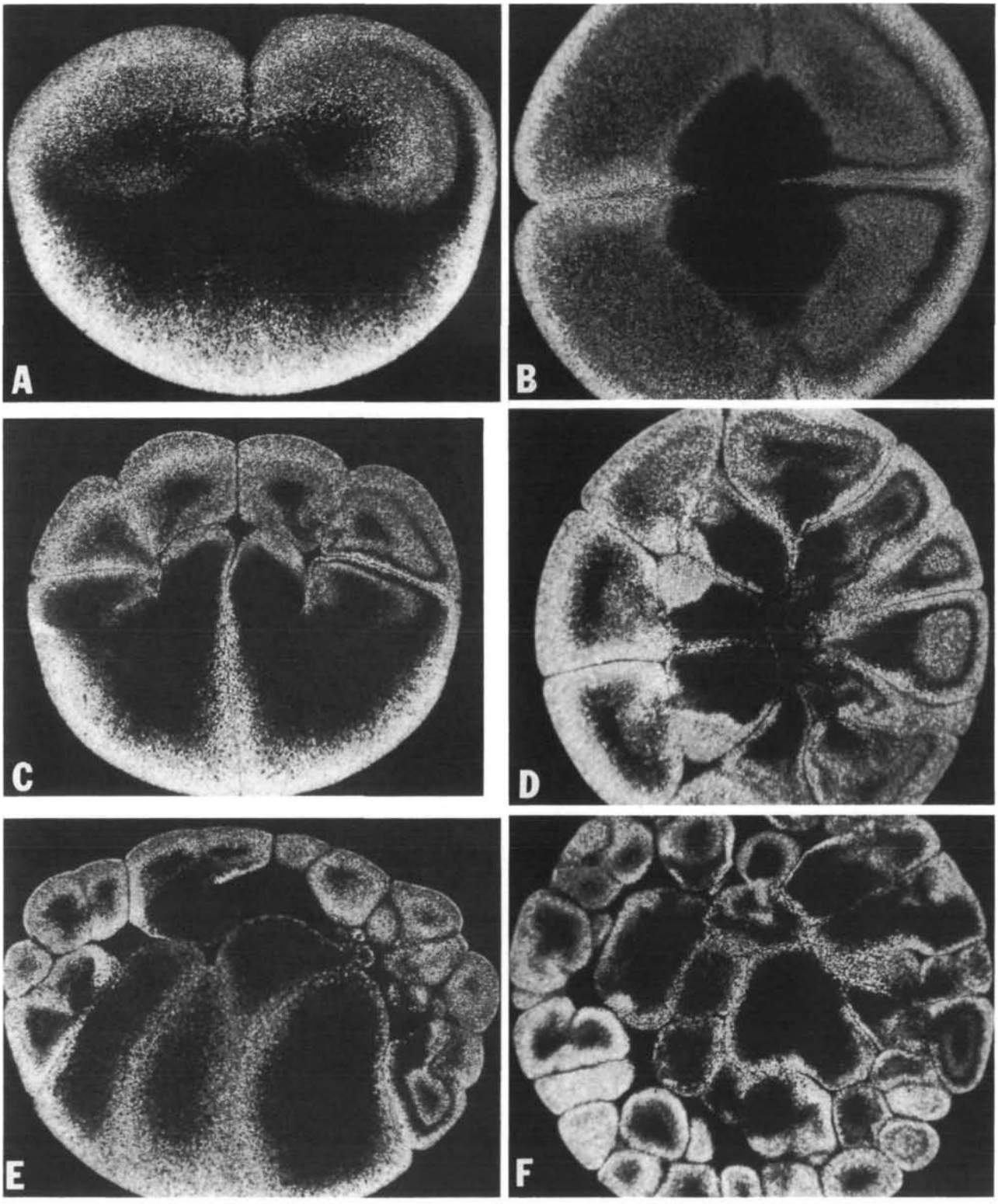

Fig. 6. Cleavage stages. (A,B) Beginning of second cleavage, in sagittal (A) and equatorial section (B). In B, the sagittal first cleavage plane runs left to right in the figure. (C,D) Beginning of fourth cleavage, in sagittal (C) and equatorial (D) section. (E,F) 64-cell stage, in sagittal (E) and equatorial section (F). SEP is oriented to left in all panels. The swirled pattern persists on the prospective dorsal side, in the form of alternating fluorescent and nonfluorescent layers.

unlabelled cytoplasm – the dorsal swirl – is preserved along the completed first cleavage plane in each blastomere. Each blastomere thus inherits the egg's original concentric organization, and the new membrane inserted at the cleavage plane is prevented from coming into contact with deep cytoplasm by a thick peripheral layer.

Fig. 6C and D show sagittal and equatorial sections of labelled eggs after third cleavage. Features of the swirl are still evident on the prospective dorsal side. However, ingression has redoubled the alternating labelled and unlabelled layers of the swirl along each surface of the horizontal third cleavage plane. Thus, portions of the swirl are inherited by both animal and vegetal blastomeres. Ingression along the completed second cleavage furrow (perpendicular to the plane of section in Fig. 6C) has brought a thick layer of large labelled yolk platelets from the vegetal pole deep into the interior of the egg. Near the egg's center, these large platelets come into direct contact with small labelled yolk platelets that originated in the animal hemisphere. A horizontal section of an 8- to 16-cell embryo (Fig. 6D) shows that layers of the swirl have been distributed among all the blastomeres on the prospective dorsal side.

The alternating fluorescent and nonfluorescent layers of the swirl ultimately lie in the second and third tiers (Fig. 6E). The swirl is apparently brought to this region by the combined action of cytoplasmic ingression during each cleavage and the geometry of the fifth cleavage plane. Thus, the cytoplasm that in the precleavage egg was found above the equator is later found surprisingly far into the vegetal, or 'organizer region' – a region that will give rise to much of the dorsal axial tissue (reviewed in Elinson and Kao, 1989; Wakahara, 1989).

Both the depth and the thickness of the swirl's unlabelled layer diminish at each successive cell division (compare in panels 6B, D and F), suggesting that the volume of the peripheral layer is conserved and thins to accommodate the increase in blastomere surface area during each cleavage.

Organization of blastula and gastrula

Cytoplasmic ingression is not only a property of early cleavages, but evidently occurs at each cell division through at least the blastula (Fig. 7A) and gastrula stages (Fig. 7B). Nearly every cell contains a thin layer of labelled yolk platelets around an unlabelled central region. The result is that deep and peripheral cytoplasmic regions have been brought into close contact by the onset of gastrulation. This rearrangement completely obscures the original concentric organization of the egg.

Discussion

Rearrangements during the first cell cycle

A mass of deep cytoplasm flows progressively *toward* the prospective dorsal side of the fertilized or activated *Xenopus* egg during the middle part of the first cell cycle

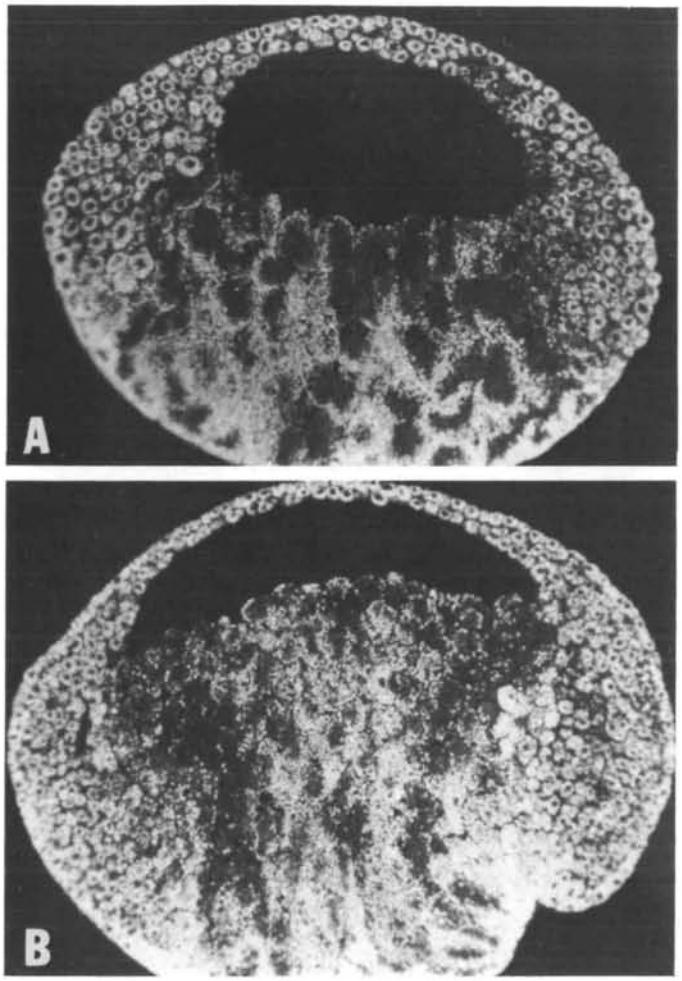

Fig. 7. Stage 8 blastula (A) and stage 10+ gastrula (B). Late-stage TB-labelled embryos were fixed and sectioned relative to the SEP as described above.

(Fig. 2). By the time of first cleavage furrow formation (90 min post-fertilization), a complicated swirl develops in the equatorial zone opposite the SEP (Fig. 2C).

Because the swirl also appears in activated eggs, it clearly does not depend on the presence of an expanding sperm aster. Rather, the swirl appears to develop by reciprocation with the yolk mass movement that occurs during cortical rotation. Using a mechanical model, we determined that rotating a solid hemispherical mass 30° within a spherical, fluid-filled space would be sufficient to generate many of the salient asymmetric features of this swirl.

This complicated pattern of cytoplasmic flow is intimately associated with dorsal–ventral axis formation: it develops during the dorsal specification period of the first cell cycle (Scharf and Gerhart, 1980), and uniquely marks the equatorial zone on the future dorsal side of the embryo. The swirl occupies a broad angular region on both sides of the prospective dorsal midline, and is particularly prominent at the embryonic midline, as might be expected of a precursor of the organizer (Stewart and Gerhart, 1990). Finally, the swirl corre-

lates well with the position of the prospective dorsal side in embryos tipped off-axis, and its appearance is sensitive to UV irradiation of the vegetal pole (in preparation). This latter treatment inhibits the development of dorsal axial structures (Grant and Wacaster, 1972).

It is necessary to follow the swirl through the early cleavage period to understand its potential developmental significance. A number of experimenters, e.g. Gurdon *et al.* (1985), Gimlich and Gerhart (1984), and Nieuwkoop (1977) point to the fact that information required for dorsal body axis specification is primarily in the vegetal hemisphere (the third and fourth tiers at the 32-cell stage). On this basis, one could argue that the equatorial swirl, as seen in Figs 2C, 3 and 4, is not involved in dorsal axis specification. However, experiments by Wakahara (1986) and Yuge *et al.* (1990) strongly suggest that dorsal 'activity' is indeed near the equator, and probably in the deep cytoplasm, as opposed to the cortex. As we have shown, the cytoplasmic swirl persists through the early cleavage period (Fig. 3), and ultimately portions of this distinctive region come to lie in the third tier at the 32- to 64-cell stage (Fig. 6E). Thus, cytoplasm originally found high in the dorsal equatorial zone of the precleavage egg is later found in the dorsal organizer region. This localization apparently is brought about by the action of cytoplasmic ingression and the oblique geometry of the 'horizontal' fifth cleavage plane. The swirl therefore has many of the hallmarks of a localization that would be involved in dorsal–ventral axis specification in *Xenopus*.

Localization of maternally derived dorsal determinants, the presence of which would cause a cell population to develop along a dorsal pathway, is only one potential mechanism whereby rotation might induce a dorsal body axis in *Xenopus*. A second mechanism was suggested by Black and Gerhart (1986) to explain their centrifugation-generated twinning results. They proposed a recording system whereby the cytoplasmic rearrangement produced by cortical-cytoplasmic rotation or off-axis centrifugation would cue one region of the uniformly responsive equatorial periphery to develop along a dorsal pathway. This second model was preferred by Black and Gerhart because it accounted more easily for the observation that relocalizing the vegetal yolk mass twice could generate two dorsal organizing centers and lead to conjoined twins. The critical physical requirement for axis cueing was a minimum 10 min contact of the vegetal yolk mass with equatorial cortex, and the subsequent sliding away of the yolk mass from the equator.

These two models are not distinguishable by our results. The swirl reflects a sizable rearrangement of cytoplasm which could represent (1) a localization of a long-lived maternal determinant, (2) a source for a small or easily diffusible substance required for cueing the receptive periphery, or (3) some mechanical way of inducing a cell signalling event in a receptive region of the egg. In any case, dorsal determinants, or components of a cueing system, would be located symmetrically in the equatorial deep cytoplasm before fertilization and then displaced toward the dorsal equatorial zone during the first cell cycle.

Cleavage-related cytoplasmic ingression

Cytoplasmic ingression was first discovered in vital dye marking experiments (Schechtman, 1934, 1937). Nile Blue marks placed on the vegetal surface of eggs before cleavage were later found deep in the interior of the embryo, as though the cortex had been drawn inward along the first cleavage furrow. Ballard (1955) later showed that this ingression occurred along many early cleavage furrows, and called the phenomenon 'multipolar cortical ingression.' As we have shown here, cytoplasmic ingression is indeed multipolar, in that it occurs at each cleavage. However, the rearrangement clearly involves more than just the cortex: a thick, subcortical layer of cytoplasm advances inward along each cleavage plane during furrow formation.

The most extensive rearrangements occur along the earliest cleavage furrows (Schechtman, 1937), probably accounting for the cross-shaped upwelling of large vegetal yolk platelets into the equatorial zone observed by Harris (1964) and Phillips (1985). Despite the progressive thinning of the peripheral layer during successive cleavages (Fig. 6), diffusion of yolk platelets between peripheral and interior regions of blastomeres does not occur to a great extent during the pregastrular cleavage period. Even as late as the blastula stage, distinct peripheral and interior domains can be found in nearly every cell (Fig. 7A). Apparently, individual cells actively maintain in some way the original concentric organization of the uncleaved egg.

Why does the egg go to the trouble of rearranging its components so extensively? The developmental function of cytoplasmic ingression is not known, but it is difficult to imagine that such major movements have no biological role. Perhaps all cells carry out extensive subcortical ingression in the course of cytokinesis. In terms of a purely developmental role, one function of cytoplasmic ingression might be to cause the apposition of previously separate cytoplasmic regions. For example, the blastopore lip always appears where cells containing large yolk platelets confront those containing small yolk platelets (Pasteels, 1964; Nakatzuji, 1975). The blastopore lip normally appears at or near the fifth cleavage plane (Dale and Slack, 1987). The abrupt boundary in platelet size is created there *via* the ingression of intermediate-sized yolk platelets during cleavage (Fig. 7E). The egg's original yolk gradient thus becomes interrupted near the surface, and smaller and larger platelets come to confront each other across the cleavage plane at the embryo surface. Whether or not an egg cleaves in a regular, stereotypical pattern is probably irrelevant, since the same major discontinuity in yolk platelet size could still be produced (although to a lesser degree) by a later horizontal cleavage in the same region. This interruption of the preexisting yolk gradient represents a potential patterning mechanism that could affect gradients of other developmentally significant maternal components, particularly those initially localized in the egg cortex or periphery.

To summarize, our observations reveal two large-scale cytoplasmic rearrangements of the deep cytoplasm that completely reorganize the original cytoplasmic organization of the *Xenopus* egg. These major rearrangements serve to bring previously distant cytoplasmic regions in the egg into close contact. Such new associations may be important for normal patterning of the embryo.

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