Blastopore Formation in the Animal Hemisphere: Functional Inversion of Gastrulation by Centrifugation of *Xenopus laevis* Eggs

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**ABSTRACT**

Eggs of *Xenopus laevis* and many other amphibians contain a gradient of yolk platelets along the animal-vegetal axis. Small platelets predominate in the animal hemisphere, and a boundary between medium and large yolk platelets exists near the equator. The blastopore forms at this boundary at the beginning of gastrulation, in the vegetal hemisphere. Does this boundary have a role in determination of the position of the blastopore, or does cortical information predominate? Past experiments using 1g to invert the egg showed a distinct tendency to form the blastopore in the original vegetal hemisphere. Our experiments, however, have used 20g centrifugation to achieve a more complete inversion of the yolk gradient. The blastopore formed in the original animal hemisphere in >95% of surviving gastrulae, if centrifugation was begun at normalized time 0.20–0.25 of the first cell cycle. Nearly normal larvae (DAI grades 4 and 5) form in 64% of cases (ave. DAI = 3.2). These data support the idea that the position of the blastopore depends on the position of the egg’s internal contents rather than cortical determinants.

**INTRODUCTION**

Medial ectodermal eggs of amphibians such as *Xenopus laevis* contain a gradient of yolk platelets along the animal-vegetal axis. This gradient is set up during oogenesis and persists during early cleavage (Danilchik and Gerhart, 1987; Imoh, 1995). Small platelets (<6μm) predominate in the animal hemisphere, medium-sized platelets (6–8μm) are found mostly near the equator, and large platelets (>8μm) are found mostly in the deeper vegetal hemisphere (Imoh, 1995). The horizontal fifth cleavage plane accentuates the interface between medium and large platelets, by drawing medium and large platelets deeper into the embryo via cytoplasmic ingestion (Danilchik and Denegre, 1991; Imoh, 1995). At the beginning of gastrulation in *X. laevis*, epiboly has moved this interface farther into the vegetal hemisphere, and the blastopore lip forms quite near this boundary (Nakatsuji, 1975; Imoh, 1995).

Evidence is accumulating that there are cytoplasmic determinants involved in the establishment of the embryonic axis, and that these molecules are associated with certain egg regions. Fujisue et al. (1993) have shown that a transplantable axis-inducing activity is present in vegetal pole cytoplasm + cortex early in the first cell cycle. This activity virtually disappears by the 16-cell stage. Conversely, dorsal equatorial cytoplasm + cortex has virtually no inducing activity early, but gains activity by the 16-cell stage. In confirmation of the trend with vegetal pole material, Sakai (1996) and Kikkawa et al. (1996) have shown that deletion of vegetal cytoplasm + cortex early in the first cell cycle severely inhibits dorsal axis formation, but that removal near the end of the first cell cycle has little effect. Interestingly, removal of vegetal cytoplasm without associated cortex has essentially no effect on axis formation (Kikkawa et al., 1996). These data support the view that determinant molecules required for embryonic axis formation exist in the *Xenopus* egg, are associated with the vegetal cortex early, and become translocated to a more equatorial region later. Based on cell transplantation and deletion experiments, it is possible that the region in the egg to which the determinants become localized is the area where the medium and large yolk platelets interface (Gimlich, 1986; Kageura, 1995).

Is there evidence that the medium-large yolk platelet boundary has a role in determination of the position of the blastopore? One approach to answering this question has been to invert amphibian eggs at 1g, with the aim of creating new boundaries in ectopic locations. In experiments done before 1940, eggs were pressed between glass plates and inverted. This typically caused the heaviest large yolk platelets to be displaced unevenly into the animal hemisphere. Then, to a highly variable extent, a secondary blastopore formed near one of the new boundaries in the animal hemisphere. A ‘primary’ blastopore also formed in the vegetal hemisphere (see Table 1 for summary). Working with inverted *Ambystoma mexicanum* embryos, Pasteels (1946), Malacinski and Chung (1981), and Chung and Malacinski (1982) reported high frequencies of single blastopore formation in the animal hemisphere. As with the earlier studies, no sections of gastrulae were shown, and there is the additional problem that the inverted embryos did not reach the neurula stage. This suggests that the animal-hemisphere lips were abnormal in some fundamental way. Inversion after fertilization of *Rana pipiens* and *X. laevis* eggs was ineffective: the embryos formed the blastopore in the vegetal hemisphere (Malacinski and Chung, 1981; Chung and Malacinski, 1982).

Neff et al. (1983) achieved very early inversion in *X. laevis* eggs by preventing hydration of the perivitelline space upon fertilization. Eleven percent of inverted embryos formed a single blastopore in the animal hemisphere. No histology at the gastrula stage or explicit description of gastrulation was given, as these experiments did not aim to examine blastopore formation in detail. In summary, 1g inversion experiments have not succeeded in generating fully functional blastopores in the animal hemisphere. Gastrulation was abnormal even in embryos of *A. mexicanum*, in which a blastopore formed in the animal hemisphere at high frequency.

Pasteels (1941) succeeded in producing fully functional blastopores in the animal hemisphere by centrifuging inverted *R. temporaria* eggs. Over 95% of inverted eggs formed a single blastopore in the animal hemisphere, and many of these embryos completed gastrulation and formed normal larvae. ‘Microscopic examination’ of inverted embryos is referred to in the text.
TABLE 1. Inversion experiments with amphibian eggs.

<table>
<thead>
<tr>
<th>Species</th>
<th>Stage treated</th>
<th>Force (g)</th>
<th>Blastopore in animal hemisphere? (%) incidence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rana temporaria</em></td>
<td>1-cell</td>
<td>1</td>
<td>secondary (3–73)*</td>
<td>Schultze, 1894</td>
</tr>
<tr>
<td><em>Rana temporaria</em></td>
<td>1–8-cell</td>
<td>1</td>
<td>secondary (6–28)*</td>
<td>Penners &amp; Schlep, 1928</td>
</tr>
<tr>
<td><em>Rana temporaria</em></td>
<td>1-cell</td>
<td>1</td>
<td>secondary (0–100)*</td>
<td>Penners, 1929</td>
</tr>
<tr>
<td><em>Hydnobius lichenatus</em></td>
<td>1–2-cell</td>
<td>1</td>
<td>no</td>
<td>Motomura, 1935</td>
</tr>
<tr>
<td><em>Bufo vulgaris</em></td>
<td>1-cell</td>
<td>150*</td>
<td>secondary (?)**</td>
<td>Motomura, 1935</td>
</tr>
<tr>
<td><em>Rana temporaria</em></td>
<td>2-cell</td>
<td>1</td>
<td>secondary (2)*</td>
<td>Penners, 1936</td>
</tr>
<tr>
<td><em>Rana temporaria</em></td>
<td>2-cell</td>
<td>1</td>
<td>secondary (19–24)*</td>
<td>Pasteels, 1938, 39</td>
</tr>
<tr>
<td><em>Rana esculenta</em></td>
<td>1-cell</td>
<td>460</td>
<td>yes (&gt;95)</td>
<td>Pasteels, 1941</td>
</tr>
<tr>
<td><em>Ambystoma mexicanum</em></td>
<td>1-cell</td>
<td>1</td>
<td>yes (35†)</td>
<td>Pasteels, 1946</td>
</tr>
<tr>
<td><em>Ambystoma mexicanum</em></td>
<td>1-cell</td>
<td>1</td>
<td>yes (&gt;90§)</td>
<td>Malacinski &amp; Chung, 1981</td>
</tr>
<tr>
<td><em>Rana pipiens</em></td>
<td>1-cell</td>
<td>1</td>
<td>no</td>
<td>Malacinski &amp; Chung, 1981</td>
</tr>
<tr>
<td><em>Ambystoma mexicanum</em></td>
<td>1-cell</td>
<td>1</td>
<td>yes (100§)</td>
<td>Chung &amp; Malacinski, 1982</td>
</tr>
<tr>
<td><em>Rana pipiens</em></td>
<td>1-cell</td>
<td>1</td>
<td>no</td>
<td></td>
</tr>
<tr>
<td><em>Xenopus laevis</em></td>
<td>1-cell</td>
<td>1</td>
<td>yes (11†)</td>
<td>Neff et al., 1983</td>
</tr>
<tr>
<td><em>Xenopus laevis</em></td>
<td>1-cell</td>
<td>1</td>
<td>no</td>
<td>Neff et al., 1984</td>
</tr>
<tr>
<td><em>Xenopus laevis</em></td>
<td>1-cell</td>
<td>1</td>
<td>no</td>
<td>Black &amp; Gerhart, 1985</td>
</tr>
</tbody>
</table>

*Embryos formed a blastopore in the vegetal hemisphere and a second blastopore in the animal hemisphere. Ranges indicate range of response by different clutches of eggs.

**Eggs centrifuged at 0 °C. No numerical data.

§Embryos failed to neurulate.

†It is not clear if these embryos neurulated.

but only idealized line drawings are shown. Thus, although Pasteels succeeded in experimentally provoking functional inversion of gastrulation, no conclusions may be drawn as to the association of the medium-large yolk platelet boundary and the blastopore.

To clarify whether or not a fully functional blastopore can form in the animal hemisphere of *X. laevis*, and to test if the yolk platelet border associated with normal blastopore position continues to operate at the novel blastopore position, we have devised a centrifugation method that inverts animal-vegetal polarity at high frequency. A single blastopore lip forms in the animal hemisphere, epiboly proceeds in a vegetal-to-animal direction, and nearly normal larvae (DAI grades 4 and 5) form in 64% of cases.

MATERIALS AND METHODS

Eggs. *X. laevis* eggs were obtained, fertilized, and dejellied by standard methods. Embryos were embedded in 8% gelatin (175 Bloom, Sigma) in 30% modified Ringer's (MR contains 100 mM NaCl, 1.5 mM KCl, 0.18 mM MgCl₂, 0.75 mM CaCl₂, 10μM ZnCl₂, and 5 mM Na HEPES, pH 7.4), made as described in Black and Gerhart (1985). Dishes were filled with molten gelatin held at 29 °C. Once the temperature had dropped to 27 °C, embryos were pipetted into a dish with a minimum of fluid. Embryos were oriented animal pole up and the dish cooled to solidify the gelatin.

Centrifugation. Eggs were centrifuged in a swinging-bucket rotor mounted on a clinical centrifuge. Speed was monitored by a stroboscope. Eggs were spun with the animal-vegetal axis aligned with the centrifugal force vector, either with the animal pole or vegetal pole uppermost in the centrifugal field. The orientation of the animal-vegetal axis with respect to centrifugal force is expressed as either 0 °C (animal pole uppermost) or 180 °C (vegetal pole uppermost). See Fig. 1. For inverted centrifugation, dishes were covered with plastic wrap and inverted just before centrifugation. After centrifugation, the plastic wrap was removed and the dishes were incubated in the inverted position in a 15 °C incubator.

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![Diagram showing the process of experimental procedure](image)

Figure 1. Outline of Experimental Procedure. Eggs were fertilized, embedded in gelatin, and centrifuged either with the animal pole (AP) or vegetal pole (VP) uppermost in the centrifugal field. Embryos were then incubated in the same position.

Normalized time scale. Times before first cleavage are expressed as decimal fractions of the first cleavage period. Zero is the time of insemination and 1.0 is when the first cleavage furrow forms.

RESULTS AND DISCUSSION

Eggs embedded in gelatin were held firmly in place, even during inverted centrifugation. Centrifugation of inverted eggs at 20g for 1-6 min did not disturb the position of the pigment granules of the animal hemisphere making it easy to identify animal and vegetal hemispheres, and their positions after centrifugation. Confirmation that eggs did not rotate comes from experiments using fluorescein-conjugated potato lectin (experiments courtesy of Dr. J.-P. Vincent). Spots of fluoresceinated lectin were applied to the vegetal surface of some eggs, following the procedure of Vincent et al (1986), and labelled eggs were inverted and centrifuged. The surface spots were not displaced despite the entire yolk mass being displaced into the animal hemisphere (data not shown).

Survival of inverted, centrifuged eggs to the gastrula and neurula stages dropped as the first cell cycle progressed. Fig. 2 shows that survival at normalized time 0.2 was 65% and 46%, respectively; by 0.55 it was virtually zero. Eggs centrifuged at an inclination of 0° showed a similar trend, but survival was higher at all times, declining from ≈85% gastrulae in the first half of the cell cycle to ≈50% in the second half (data not shown).

Inverted eggs formed the blastopore in the original animal hemisphere at very high frequency (>95%) if centrifuged at 0.20-0.25 (N=313 centrifuged eggs surviving to the gastrula stage)(Fig. 3). Latitude of blastopore lip formation was measured to document precisely extent of inversion. Fig. 4 compares centrifuged controls and

![Graph showing survival to gastrula and neurula stages](image)

Figure 2. Survival to Gastrula and Neurula Stages of Inverted, Centrifuged Eggs. Times indicate when centrifugation began in first cell cycle. Filled circles, average survival to midgastrula. Open, average survival to early neurula. Average at 0.2 represents 95 centrifuged eggs from 4 females. Averages at 0.25-0.45 represent 140 eggs from 7 females; averages at ≥0.50 represent ≥31 centrifuged eggs from at least 2 females.
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Figure 3. Formation of the Blastopore in the Original Animal Hemisphere Occurs Reliably only if Inverted Eggs are Centrifuged Very Early in the First Cell Cycle. Times indicate when centrifugation was begun. For times 0.20 and 0.25, averages represent N = 205 and N = 108 surviving gastrulae, respectively. From 0.30–0.45, averages represent at least 23 gastrulae; from 0.50 onward, few eggs survived to the gastrula stage, and averages represent from 0–15 gastrulae.

Figure 4. Latitude of Blastopore Formation in Control and Inverted Eggs. Gastrulae were fixed and sectioned; a mid-sagittal section was digitized and the angle separating the latitude of the blastopore and the original vegetal pole was measured using NIH Image software. Average angle, 1g, controls = 42°. Average angle, inverted embryos = 152°. N = 33.

inverted, centrifuged eggs: the controls formed the blastopore an average of 42° from the vegetal pole, which agrees well with published values for normal development (±45°) (Keller, 1975; Souza et al. 1995). Inverted, centrifuged embryos formed the lip an average of 152° from the vegetal pole, because the lip formed in the animal hemisphere. Sections of embryos at this stage of gastrulation are shown in Fig. 5. The blastopore lip in both controls and inverted embryos comprises typical bottle cells. Bottle cells are apically constricted in both cases, but because the inverted lip is composed of pigmented animal hemisphere cells, it appears darker than the control lip. Darkfield views of the same sections show the yolk platelets very clearly. The blastopore lip has formed in both cases just above the medium-large yolk platelet boundary (Fig. 5). That the position of the boundary and lip continue to correlate in embryos functionally inverted by centrifugation confirms the importance of the boundary to blastopore formation. Moreover, that inverted embryos formed a functional blastopore entirely within the animal hemisphere shows that the vegetal hemisphere cortex is not uniquely capable of contributing to a blastopore. A quantitative definition of the boundary and analysis of its position relative to normal and inverted blastopores is in progress.

Epiboly operates in the reverse direction from normal in inverted gastrulae; superficial cells move toward the blastopore in the vegetal-to-animal direction. External views of an uncentrifuged control and an inverted gastrula at the yolk-plug stage are shown in Fig. 6. Note that the yolk plug of the inverted embryo is pigmented, indicating that it comprises cells cleaved from the animal hemisphere. At this stage the animal hemisphere blastopore appears somewhat abnormal, with an unusually wide zone of cells at the blastopore lip. However, the internal view at this stage shows a nearly normal archenteron, indicating that involution is proceeding nearly normally.

That gastrulation proceeded relatively normally despite the lip forming in the animal hemisphere is indicated by the extent of subsequent dorso-anterior development. Tadpoles reared from inverted gastrulae generally developed with the full suite of dorso-anterior structures, as quantified by the dorso-anterior index (DAI) of Kao and Elinson (1988). Sixty-four percent of tadpoles had a DAI of 4 (reduced forehead, but with two eyes) or 5 (normal), although a substantial proportion developed with reduced dorsal structures. See Fig. 7. Some grade 4 and 5 tadpoles were reared through metamorphosis, and developed into frogs with gametes. Inverted embryos may provide a convenient means of testing the importance of the germinal plasm in germ cell development.

What is the effect of centrifugation on the topographic relationship between the sperm entry point (SEP) and embryonic axis? In normal development, the dorsal structures typically form on the side of the egg opposite the side of sperm entry (Pflüger, 1883; Ancel and Vintemberger, 1948; Vincent et al., 1986). This topography can be overridden if the internal contents are displaced laterally, as is the case with 90° centrifugation (Black and Gerhart, 1985). In these eggs, the embryonic axis forms from the side of the egg away from which the yolk is displaced by centrifugation. This is similar to the situation in control gelatin-embedded eggs, in which
Figure 5. Sections of Blastopore Lips in Control, Uncentrifuged Embryos (A, brightfield), (C, darkfield) and Inverted Embryos (B, brightfield), (D, darkfield). Original animal pole is uppermost in all panels; arrows point to blastopore. In both cases, the blastopore forms near the interface of medium and large yolk platelets.

Subsurface yolk platelets are seen to move away from the prospective dorsal side (Vincent et al., 1986). The lateral displacements elicited by centrifugation are believed to act by substituting in some manner for the egg’s own lateral displacement of subcortical cytoplasm relative to the surface. Pasteels (1938, 1946) observed that inverted embryos tended to form the secondary blastopore opposite the side of sperm entry. Does this topography hold for inverted X. laevis eggs? Fig. 8 shows that it does not; to the contrary, the SEP-neural plate topography is reversed from normal. The definitive dorsal structure, the neural plate, forms on the same side as the SEP meridian. Centrifugation at an inclination of 0° does not alter this relationship very much. [Interestingly, if the centrifugal force is increased to 30g, the effect is to randomize the SEP-neural plate relationship during the period 0.30-0.60 (Black and Gerhart).] It is surprising that 180° centrifugation acts to reverse SEP-neural plate polarity, because there is no apparent lateral component to the force vector. The explanation may lie in the way the yolk mass is displaced to the animal hemisphere. Analysis of internal displacements in eggs centrifuged at 90° suggest that the yolk mass tends to move as a whole (Black and Gerhart, 1985). It is possible that the forming sperm aster rigidifies the SEP side of the egg enough so that the yolk mass moves in the opposite direction, in the path of least resistance. In this scenario, the side away from which the yolk moves would be the SEP side and would therefore mark the SEP side as dorsal.

Acknowledgement

We thank Jason A. Oakes for cheerfully doing the histology.

REFERENCES

Figure 6. The Blastopore Formed in the Vegetal Hemisphere in Uncentrifuged Controls (left). In inverted embryos, the blastopore formed in the animal hemisphere (right).

Figure 7. Range of Dorso-anterior Development of Tadpoles Developed from Inverted, Centrifuged Eggs. A value of 0 indicates no dorsal development; a value of 5 is normal. Average DAI = 3.2. N = 45. Dorso-anterior index of Kao and Elinson (1988).


Figure 8. Inverted Eggs Develop with Reversed Sperm Entry Point-neural Plate Topography. Times indicate when centrifugation began. Filled circles, average SEP-neural plate angles for inverted eggs. Open circles, average angles for eggs in normal orientation. N = 452. 1g, 0° control data, for comparison (Black and Gerhart, 1985).


