Localization of the factors producing the periodic activities responsible for synchronous cleavage in *Xenopus* embryos

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SUMMARY

This paper investigates the localization within the *Xenopus* egg of the factors responsible for the periodic activities such as the cyclic rounding-up and flattening, related to the cleavage cycle. Denuded eggs were bisected along the boundary line between the animal and the vegetal hemispheres immediately after being rotated through 90° off the vertical axis (Early Bisection). The resulting animal halves, though prevented from cell division by colchicine, showed typical periodic rounding-up as previously observed in enucleated egg fragments, whereas the vegetal halves did not. This result indicates that the factors inducing the periodic rounding-up are not distributed uniformly throughout the egg but localized mostly in the animal hemisphere. Furthermore, the distribution of these factors between the cortex and endoplasm of the animal hemisphere was investigated. Eggs were separated into animal and vegetal halves following incubation for 30 min after the 90°-off axis rotation (Late Bisection). During this incubation the endoplasmic components become relocated in the rotated egg under the force of gravity. After the rotation, the Late-Bisected vegetal halves showed typical cyclic rounding-up in contrast to those formed by Early Bisection. These results suggest that the factors inducing the periodic rounding-up (and probably also many other cyclic activities, closely linked with the rounding-up movement) are localized in endoplasmic components which can be displaced by gravity from the animal to the vegetal hemisphere of the *Xenopus* egg.

INTRODUCTION

Early cleavage of animal embryos can be distinguished from the usual cell division in somatic cells not only because each cell cycle in this stage consists mostly of S- and M-phases but also because during this period embryos show no synthesis of mRNA, and no increase in cell mass (Flickinger, Lauth & Stambrook, 1970). Moreover, during this period each blastomere cleaves synchronously at a regular interval characteristic of the species, 30 min at 21°C in *Xenopus* embryos for example (Boterenbrood, Narraway & Hara, 1983). It has, therefore, been considered that early embryos might have a particular mechanism which regulates

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the cycles of early cleavage. Study of the specific timing mechanisms in early cleavage is thus important for an understanding of early development.

Recently it has been reported that anucleate egg fragments formed by bisecting uncleaved eggs show cyclic changes similar to those seen in normally cleaving embryos. These include cyclic change in stiffness (Yoneda, Ikeda & Washitani, 1978), cyclic rounding-up and flattening, and the cyclic appearance of surface contraction waves (SCWs; Sawai, 1979; Hara, Tydeman & Kirschner, 1980; Sakai & Kubota, 1981; Sakai & Shinagawa, 1983; Shinagawa, 1983). Furthermore, it has also been reported that those eggs in which the nucleus is prevented from mitosis by antimitotic agents persist likewise in the cyclic changes. The periodicity of the cyclic changes in either enucleated, or colchicine-injected eggs is comparable to that of the cleavage cycle in normal embryos. These results consequently suggest that the cyclic divisions during early development might be regulated by certain factors present in the cytoplasm and/or the cortex, independent of the nucleus.

Kirschner, Gerhart, Hara & Ubbels (1980) consider the factors to constitute a master oscillator of early cleavage, which could regulate the cyclic change of various activities such as DNA synthesis, duplication of centrioles, mitosis, and surface contraction waves. Even nuclei inserted into the cytoplasm of another egg are entrained by the surrounding cytoplasm so as to be in harmony with the phase of the cytoplasmic cycle quite independently of the original phase of the nuclei (Graham, Arms & Gurdon, 1966; Sakai & Shinagawa, 1983). This may suggest a relationship between the cytoplasmic factors mentioned above and the maturation promoting factor or the cytostatic factor, known to be likewise in the cytoplasm and to control the morphology of the nucleus (Wasserman & Smith, 1978; Lye, Newport & Kirschner, 1983).

The localization within the egg of the factors producing these various periodicities is, however, not known. The present study was undertaken in an attempt to answer this question. Most of the phenomena apparent on the surface of amphibian eggs originate at the animal pole, and propagate toward the vegetal hemisphere; for example the SCWs (Yoneda, Kobayakawa, Kubota & Sakai, 1982), the waves of stiffness in the surface (Sawai & Yoneda, 1974; Yoneda et al. 1982), and the waves of activity of furrow-inducing components (Sawai, 1972). This raises the possibility that the factors inducing the periodic activities of the egg are not located uniformly throughout the egg but are restricted to the animal hemisphere.

In order to test this possibility, the cyclic changes in animal and vegetal halves, formed by bisecting uncleaved *Xenopus* eggs, were compared with each other. In addition, to ascertain whether the cytoplasmic factors are localized in the endoplasm or in the cortex of an egg, the same comparison was carried out between those halves after the constitution of the endoplasmic components, but not of the cortical components, had been altered.
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**MATERIALS AND METHODS**

**Egg preparation**

Eggs of *Xenopus laevis* were obtained by natural mating following injection with 200 i.u. and 300 i.u. of human gonadotrophic hormone (Gonatropin, Teikoku-zoki Co. Ltd, Tokyo) into a male and a female respectively. Jelly was removed by carefully stirring eggs in 10% modified Steinberg's saline containing 1% sodium thioglycolate (Wako Pure Chemical Industries Ltd, Osaka, Japan) at pH 9-10 for 2 min. Dejellied eggs were treated with 0.5% pronase in modified Steinberg's saline (Sakai & Kubota, 1981; 58.2 mM-NaCl, 0.67 mM-KCl, 0.34 mM-Ca(NO₃)₂, 0.83 mM-MgSO₄, 0.30 mM-HEPES) at pH 6-8 for 1 min, and the fertilization envelope was removed with forceps.

**Bisection of fertilized eggs**

Bisection of fertilized *Xenopus* eggs was carried out by two procedures:

1. **Early Bisection.** Denuded eggs were placed in a Petri dish covered with 2% agar and rotated 90° off the vertical axis; consequently the animal/vegetal axis of the eggs was oriented horizontally. A fine glass rod was gently put on the egg along the boundary line between the animal and the vegetal hemispheres immediately after the egg was rotated 90° off axis. The region of the egg under the glass rod was gradually squeezed until the egg was separated into halves (Fig. 1). This bisection was always initiated within 30 min after fertilization because at later times the eggs become too rigid to be bisected. The zygote nucleus was usually included in the resulting animal half fragment, which went through the cycles of cleavage (Fig. 1F). After the separation was completed, each half fragment was injected with 25 nl of colchicine dissolved in modified Steinberg's saline (1 mg/ml) so as to arrest cleavages. For precise comparison of the periodicity in an animal half with that in a vegetal half, both should be inhibited from cleavage by injection with colchicine. This avoids the alteration of periodicity caused by formation of a mitotic apparatus in one of the fragments (Sluder, 1979; Shinagawa, 1983). This bisection is called 'Early Bisection' for convenience in the present study.

2. **Late Bisection.** Denuded eggs were placed in a Petri dish and rotated 90° off axis in the same manner as above. They were injected with colchicine soon after the rotation. At 30 min after rotation, a fine glass rod was placed on the boundary line of the hemispheres, to accomplish bisection. This delayed bisection was usually initiated about 50 min after fertilization, when eggs would have normally become too rigid for the operation. However, it was possible to succeed in the bisection because the cytoplasm of the egg had been liquified by colchicine. Thus, in this procedure colchicine was used for liquifying the egg cytoplasm as well as for inhibiting the fragments from cell division. This bisection is called 'Late Bisection'. The timing of the bisection is the main difference between the two procedures.

**Observation of cyclic activity in half fragments**

Cyclic activity in animal and vegetal halves was recorded as a cyclic change in cell diameter, which reflects the cyclic and sequential rounding-up and flattening of the cell. The periodicity of the cyclic rounding-up is known to represent also that of other cyclic activities including SCWs and cyclic change in stiffness (Sakai & Kubota, 1981; Yoneda et al. 1982). Each half fragment was observed from above, and its diameter was measured every 3 min at room temperature (21°C to 23°C). A minimum diameter measured from above corresponds to a maximum rounding-up as viewed from the side. For minimizing the effects of unexpected temperature variation, both halves were placed side by side in a Petri dish. Comparison of the periodicity was always carried out with a pair of fragments derived from a single egg.

**Histology**

After the periodic activity in the half fragments was observed, each was fixed for about half a day in Herry's fixative followed by alcoholic dehydration, and embedding in paraffin wax (m.p.
Periodic activity in the animal and vegetal half fragments formed by Early Bisection

Fig. 2 illustrates the cyclic change in diameter of the animal and vegetal halves formed by bisection of the eggs immediately after 90°-off axis rotation (Early Bisection). Since Fig. 2 shows the diameter of fragments observed from above, the minima of the plot correspond to the times of maximum rounding-up of the fragment, viewed from the side. Though animal halves were prevented from cell

Fig. 1. Bisection of the fertilized Xenopus egg immediately after the egg has been rotated through 90° off the vertical axis. (A) Three minutes after placing a glass rod on the egg (about 15 min after fertilization). Times (min) after placing the glass rod are indicated on the right. The egg is completely separated into two halves at about 30 min after placing the glass rod (G). The animal half fragment alone goes through the cycles of cleavage because of including the zygote nucleus (H). Scale bar equals 1mm.
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division by injection with colchicine, a significant cyclic change in diameter nonetheless occurs. The period of the cyclic change in diameter of the animal halves is definitely longer than that of cleavage cycle (Fig. 2A). The maximum change of diameter for the animal half is 8–15%. Thus, the cyclic change in the animal halves can be equated with that previously observed in anucleate eggs (Sakai & Kubota, 1981; Shinagawa, 1983). On the other hand, vegetal halves do not show as large cyclic changes in diameter (2–5%) as do animal halves, although in many cases, cyclic changes can be detected also in vegetal halves (Fig. 2C). Moreover, some vegetal halves failed to display detectable rounding-up (Fig. 2A,B).

In order to ascertain the difference in periodicity between the two halves, the data of Fig. 2 and of similar experiments are plotted in Fig. 3. The time of the 2nd (circles), 3rd (closed triangles), 4th (squares) and 5th (open triangles) rounding-up in the vegetal halves is plotted against that of the corresponding rounding-up in their animal-half partners. The points of the plot stay above a line with a slope of

![Graph](image)

Fig. 2. Changes in diameter of the animal (●), and vegetal (○) halves formed by bisecting the eggs rotated through 90° off the vertical axis shortly before the bisection (Early Bisection). To facilitate the comparison, the time scale is shifted so as to align the first rounding-up of each half at time 0 in the graph. In reality, the first rounding-up in the vegetal half lags behind that in the animal half by 10 to 40 min (C). Arrows in (A) indicate the cleavage time of the control egg derived from the same batch.
45°, clearly revealing that the period of cyclic rounding-up in the vegetal halves is longer than that in their animal-half partners. The ratio of the former to the latter is actually 1.26 (±0.14 s.d.) on average. These results conclusively indicate that the typical periodic activity is not distributed uniformly throughout an egg but is localized mainly in the animal hemisphere.

**Periodic activity in animal and vegetal halves formed by Late Bisection**

Fig. 4 illustrates the cyclic change in diameter of the animal and vegetal halves formed by bisecting the eggs at 30 min after the 90°-off axis rotation (Late Bisection). The diameter of the animal halves changes as extensively and as quickly as that of the animal halves formed by Early Bisection. However, vegetal halves formed by Late Bisection change their diameter much more than those formed by Early Bisection. As shown in Fig. 4A,B the period and extent of cyclic rounding-up in the Late-Bisected vegetal halves is very similar to that in their animal-half partners, although in some experiments the former remain somewhat

![Fig. 3](image-url)
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slower than the latter (Fig. 2C). However, this difference in periodicity between the two halves is much smaller than in those pairs obtained by Early Bisection.

Fig. 5 further documents the similarity in period of the cyclic rounding-up in both halves of Late-Bisected eggs. In contrast to Fig. 3, each point is located close to a line with a slope of 45°, revealing that the period of cyclic rounding-up in the vegetal halves formed by this procedure has become equalized to that in their animal-half partners. The ratio of the former to the latter is 1.07 (±0.07 s.d.) on average.

Distribution of cytoplasm in the animal and vegetal halves formed by Early or Late Bisection

As seen in Fig. 6A, there is a polarity not only of the cortex but also of the endoplasm of the egg. Externally, the cortex of the animal hemisphere can easily be distinguished from that of the vegetal one because the former is thick and well pigmented, while the latter is thin and poorly pigmented. The animal portion of

Fig. 4. Changes in diameter in the animal halves (●), prevented from mitosis, and the vegetal halves (○), formed by bisecting the eggs rotated through 90° off the vertical axis at 30 min prior to the bisection (Late Bisection). To facilitate the comparison, the time scale is shifted so as to align the first rounding-up in each half at time 0. In reality, 1st rounding-up in the vegetal half sometimes lags behind that in the animal half by up to 15 min.
the surface appears as a clear-cut margin, while the vegetal portion of the surface seems to lack such a marginal structure. Internally, as described by Nieuwkoop (1977), the animal hemisphere of the egg contains a relatively large amount of small to medium-sized yolk platelets embedded in rather abundant cytoplasm, while the vegetal hemisphere is composed of a large quantity of mainly large yolk platelets, the amount of interstitial cytoplasm being scanty.

The distribution of cytoplasmic components in animal and vegetal halves formed by Early Bisection is shown in Fig. 6B and C respectively. The animal half fragment is packed mainly with small to medium-sized yolk platelets embedded in a large amount of yolk-free cytoplasm, while the vegetal counterpart is occupied predominantly by large-sized yolk platelets, apparently lacking yolk-free cytoplasm. The surface of the animal half (Fig. 6B) appears as a clear-cut margin while that of the vegetal half (Fig. 6C) lacks such a marginal structure. Thus, the two halves resemble the animal and the vegetal hemisphere of the intact egg respectively.

![Fig. 5. The time of 2nd (●), 3rd (▲), 4th (■), 5th (△) rounding-up in 12 vegetal halves obtained by Late Bisection plotted against the timing of the corresponding rounding-up in their cleavage-arrested animal half partners.](image-url)
Fig. 7B,C shows the distribution of endoplasmic components in the animal and vegetal half fragments formed by Late Bisection respectively. As pointed out by Elinson (1983), the endoplasm of fertilized *Xenopus* eggs becomes fluid about 15 min after fertilization so that the endoplasmic components can easily be relocated during the incubation of the eggs for 30 min following the 90°-off axis.

Fig. 6. Distribution of the endoplasmic components in an intact egg (A), and in the animal and vegetal halves (B and C respectively) formed by bisecting the egg rotated through 90° off the vertical axis shortly before the bisection. The broken line in (A) indicates the boundary between the animal (a), and vegetal (v) hemispheres, situated upper and lower on the plate respectively. Scale bar equals 500 μm.
rotation. Consequently, heavy yolk granules are translocated toward the lower portion of the rotated egg, and simultaneously, yolk-free cytoplasm is translocated toward the upper portion of the rotated egg, across the pigmentation boundary line between the hemispheres (Fig. 7A).

Fig. 7. Distribution of the endoplasmic components in the intact egg rotated through 90° off the vertical axis at 30 min before the bisection (A), and in the animal and vegetal halves (B and C respectively) formed by bisecting the egg rotated through 90° off the vertical axis at 30 min prior to the bisection (Late Bisection). The broken line in (A) indicates the boundary between the animal (a), and the vegetal (v) hemispheres, situated left and right on the plate respectively. Scale bar equals 500 μm.
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The resulting animal half still contains a large amount of yolk-free cytoplasm and fine yolk platelets, and has gained a small amount of large yolk platelets, probably derived from the endoplasm of the vegetal hemisphere (Fig. 7B). It should be noted particularly that the vegetal counterpart now contains a rather large amount of yolk-free cytoplasm in its upper portion, in an amount similar to the animal half, in contrast to the vegetal half formed by Early Bisection (compare Fig. 7C with Fig. 6C). Figs 7B,C thus clearly show that both halves obtained by Late Bisection contain the same kinds of endoplasmic components including yolk-free cytoplasm, even though the cortex in the two halves remains different in respect to thickness and pigmentation.

On the basis of the above observation of the periodicity and cytoplasmic constitution of the fragments, it could be concluded that the displaceable endoplasmic components lying in the animal hemisphere, probably in the yolk-free cytoplasm, contain the factors inducing the cyclic rounding-up and perhaps other cyclic activities.

DISCUSSION

The amphibian egg clearly has various kinds of polarity along the animal/vegetal axis, such as the polarity of distribution of cytoplasmic components (Nieuwkoop, 1977; Herkovits & Ubbels, 1979), that of the distribution of RNA (Capco, 1982), that of developmental competence (Grunz, 1977), and the structural and functional polarity in the cortex (Goldenberg & Elinson, 1980). It seems plausible that there may also be a polarity in the distribution of the factors producing the periodic activities of the *Xenopus* egg.

The first experiment involving Early Bisection revealed that the typical cyclic rounding-up can be seen only in the animal halves, indicating that the factors inducing it are localized mainly in the animal hemisphere. The second experiment involving Late Bisection further suggested that the factors are present not in the cortex but in the displaceable endoplasmic components, since a vegetal half gains the capacity to show typical rounding-up once it acquires the endoplasmic components displaced from the animal hemisphere. This is in good agreement with the study on the cytoplasmic regulation of the duration of cleavage in amphibian eggs (Aimar, Delarue & Vilain, 1981). The acquisition of the typical cyclic rounding-up in these vegetal halves should exclude the possibility that the absence of shape changes in the vegetal halves formed by Early Bisection is caused merely by the inability of the vegetal half to express the periodic activities, for example, because of shortage of the contractile system. It seems more likely that the vegetal half of a normal egg fails to originate the cyclic changes as vigorously as does the animal half. This lesser activity of the vegetal half is perhaps due to lesser amount of the endoplasmic components containing the factors. The rather typical
cyclic rounding-up in some vegetal halves might be due to a greater amount of the factors, unintentionally introduced during bisection.

Though there are other endoplasmic components in the animal hemisphere such as fine yolk granules, yolk-free cytoplasm would be the most probable candidate for including the factors since it contains abundant organelles and enzyme systems (Herkovits & Ubbels, 1979) lacking in the yolk platelets. The factors present in the endoplasmic components may comprise the 'Master Oscillator', proposed by Kirschner et al. (1980) as controlling the progress of the cell cycle. Although in the present study the factors producing the periodicity were assayed only by recording the cyclic rounding-up of the egg, it seems very likely that other cyclic activities, including SCWs and cyclic change in stiffness, are regulated likewise by the same factors because they seem to be closely linked with the rounding-up movement either temporally or mechanically (Sakai & Kubota, 1981; Yoneda et al. 1982).

If appearance of SCWs is triggered by the factors present in the mass of yolk-free cytoplasm underlying the animal pole, this would explain why SCWs always appear initially at the animal pole of eggs, and propagate toward the vegetal hemisphere. This assumption is consistent with studies demonstrating that the endoplasm plays a much more important role than the cortex in determining the furrow-appearance site and axes of developing embryos (Neff, Malacinski, Wakahara & Jurand, 1983; Ubbels, Hara, Koster & Kirschner, 1983).

For characterization of those factors it may be important to note the relation between them and the maturation promoting factor, thought to be a proteinous substance (Wasserman & Smith, 1978; Lye et al. 1983). The previous, and the present studies reveal that there are many similarities between both factors: (1) each is present in endoplasm, and is translocatable or transplantable (Lye et al. 1983); (2) each causes changes with a periodicity corresponding to the cleavage cycle (Wasserman & Smith, 1978); (3) each is capable of affecting the morphology of a nucleus (Wasserman & Smith, 1978; Sakai & Shinagawa, 1983); (4) each might be derived at least partly from germinal vesicle materials (Herkovits & Ubbels, 1979).

Though the nature of the factors is unrevealed, it might be related to a complex of calcium and the calcium-sequestering system (Harris, 1978; Kiehart, 1981). The free calcium, reportedly affecting the contractile systems, cytoskeletons, and mitotic apparatus, might participate in inducing the autonomous oscillation. Further study is required to characterize, at subcellular and molecular levels, the cytoplasmic factor producing the autonomous oscillation.

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REFERENCES

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