Temporal pattern of cleavage and the onset of gastrulation in amphibian embryos developed from eggs with the reduced cytoplasm

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SUMMARY

Fertilized eggs of the Japanese newt, Cynops pyrrhogaster, were divided into two or four equal-sized parts with fine glass rods before the first cleavage. In such cases one of the egg fragments, at least, proceeds to cleavage and gastrulates.

The temporal pattern of cell division and the onset of gastrulation in such half or quarter embryos were investigated and compared with normal development. The following results were obtained: (1) desynchronization starts two divisions earlier in quarter embryos and one division earlier in half embryos compared with whole embryos, (2) the time from the first cleavage to the onset of gastrulation does not widely vary among quarter, half and whole embryos and (3) the numbers of blastomeres which constitute embryos at the pigment stage decrease in proportion to the diminution of egg volume.

INTRODUCTION

Gastrulation is the first dramatic event of morphogenetic movement in animal development. Several investigations of gastrulation in amphibian embryos have been concerned with either the cell movement or specific activation of mRNA or rRNA synthesis (Holtfreter, 1943, 1944; Denis, 1968; Woodland & Gurdon, 1968; Nakatsuji, 1975a, b, 1976; Keller, 1975, 1976, 1978; Keller & Schoenwolf, 1977). We have as yet, however, limited information as to how the timing of the onset of gastrulation is determined. In order to analyse this problem, the relationship between cell divisions and the onset of gastrulation has been investigated. Cell divisions before gastrulation can be divided into two stages. The first is the period of synchronous cleavage in which cells divide synchronously or metachronously at a high rate. After the mid-blastula stage, embryos enter the period of asynchronous cleavage and a certain length of interphase appears in this period. The number of synchronous divisions is 13 in Xenopus (Satoh, 1977), 10 in axolotl (Hara, 1977) and 12 in Cynops (Suzuki, Kuwabara &

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Kuwana, 1976). In common in the above three species, gastrulation starts after the 15th division of the most rapidly dividing blastomeres in the animal hemisphere. Dettlaff (1964) stated 'nuclear activity during asynchronous division is... directly related to the onset of gastrulation'.

Experimental analysis of this problem has also been done in relation to the nucleo-cytoplasmic interactions. Chulitskaia (1970) has reported that in sturgeon embryos injected with cytoplasm or the supernatant of its homogenate from mature unfertilized eggs, desynchronization started later as compared with normal untreated embryos. However, these injections had no effect on the onset of gastrulation. Landström, Løvtrup-Rein & Løvtrup (1975) on the other hand, reported that injection of four kinds of deoxyriboside triphosphate into fertilized eggs prolonged the period of synchronous divisions and suppressed the onset of gastrulation in *Xenopus*.

To study this problem quantitatively in relation to the nucleo-cytoplasmic ratio, we developed a method to divide an uncleaved egg into several fragments, by which the cytoplasmic volume of the embryo was decreased to one half or one quarter, and the relationships between cytoplasmic volume and the proportion of cell divisions or the time required for the onset of gastrulation were studied in the Japanese newt, *Cynops pyrrhogaster*.

**Materials and Methods**

Adult newts, *Cynops pyrrhogaster*, were collected in the suburbs of Kyoto, and both females and males were kept in the same pond in the courtyard of the laboratory. Spawning of fertilized eggs was induced by injecting about 50 i.u. of human chorionic gonadotropin (Gonatropin, Teikoku Zoki Co. Ltd) under the skin of females every other day.

**Egg fragments**

After removal of the jelly capsule with forceps and oculists' scissors, uncleaved fertilized egg was put on an agar plate (1% agar) on the bottom of a dish filled with 1/10 strength modified Steinberg's solution (NaCl: 3.4 g, KCl: 0.05 g, Ca(NO₃)₂ 4H₂O: 0.08 g, MgSO₄ 7H₂O: 0.205 g in 1000 ml D.W., buffered to pH 7.2 by 3 mM HEPES-NaOH). The vitelline membrane was removed with a pair of forceps, and the egg was placed with its animal hemisphere upward (Fig. 1a). In order to divide the egg into two equal-sized parts, a fine glass rod (0.1-0.2 mm in diameter, 1–2 cm in length) was put on it (Fig. 1b). The egg was squeezed gently by the weight of the rod, and eventually separated into halves, without loss of eggplasm (Fig 1d). Repeating the same process on each egg fragment, it was possible to divide an egg into four equal-sized parts (Fig. 1e–h). The differences in the diameters of the four fragments from an egg were less than ±9.7% of the mean fragment diameter.
Fig. 1. (a) Configuration of the uncleaved fertilized egg just after the removal of vitelline membrane. (b) Just after putting a glass rod on the egg. (c) 20 min after (b). The part of the egg pressed down gently under the weight of the glass rod became translucent. (d) The egg has been divided into two parts without detectable leakage of eggplasm. 45 min after (b). (e) The two egg fragments from the egg shown in (a). (f) Just after putting a glass rod on each egg fragment perpendicularly to the first dividing plane shown in (b). (g) 10 min after (f). The part of each egg fragment under the glass rod resembles that in fig. (c). (h) 60 min after (f). The egg has been divided into four equal sized parts. Bar equals 1 mm.
Onset of gastrulation

As it was impossible to know the exact time of fertilization or karyogamy, the time between the onset of first cleavage and the beginning of pigmentation was defined as the time required for the initiation of gastrulation. The quarter, half and whole embryos without vitelline membranes were reared in holes in agar plates on the bottoms of dishes filled with 1/10 strength modified Steinberg's solution at 20 ± 0.5 °C. The time of the onset of first cleavage and the beginning of pigmentation were observed with stereoscopic microscopes and inverted microscopes.

Time-lapse cinematography and analysis of films

Whole eggs, with or without the vitelline membrane, half eggs and quarter eggs were filmed from the top with the aid of time-lapse cinematographic apparatus (camera: Bolex H 16 Reflex, timer: Nikon CMFA) from the onset of first cleavage to the gastrula stage at 20 ± 0.5 °C with frame intervals of one or four min. The films were studied with the aid of film analyzer (Nac Dynamic Frame Analyzer).

Counting of cell number

At the beginning of the pigment stage, in which the accumulation of pigment granules at the future blastopore is observable, the embryo was transferred to an agar-bottomed (1 % agar in D. W.) dish filled with Ca²⁺-, Mg²⁺-free modified Steinberg's solution with 1 mM ethylenediamine-tetraacetic acid 4 Na salt (EDTA 4 Na salt), and a few rents were made with a tungsten needle into the blastocoel. After two hours, most of the blastomeres were dissociated. They were then fixed by addition of 0.5 % glutaraldehyde into the dish. Blastomeres still clumped together were dissociated manually by a tungsten needle or a hair loop. The number of blastomeres at pigment stage was thus counted either directly under a stereoscopic microscope, or on photographic prints of the dissociated blastomeres spread as a monolayer. In this counting, each cell showing a cleavage furrow was scored as two.

RESULTS

Feasibility of the separation of eggs and development of egg fragments

The egg is not suitable for the operation immediately after spawning, when its swelling by absorption of water is not complete and its surface stiffness is low, since the resultant fragments are of irregular shape. Conversely, the gradual rise of surface stiffness which starts about one hour prior to the first cleavage makes the egg very resistant to successful division into fragments. Therefore we had to operate on the egg during the period from one hour after spawning until two hours before the first cleavage. If operated during this period, the eggs were
Timing of the onset of gastrulation

Fig. 2. Vegetal side views of whole (a) half (b) and quarter embryo (c) at pigment stage. The pigmentation point in each embryo is shown by an arrow. Bar equals 1 mm.

divided completely by 30–60 min, depending on batches and temperature. Since the first cleavage begins usually as late as 6–7 h after fertilization in Cynops at 20 °C, it was not difficult to repeat the operation to divide the uncleaved egg into four fragments. In some cases it was even possible to obtain eight fragments from a single uncleaved egg.

Usually one of such half or quarter egg fragments, probably containing the zygote nucleus, proceeded to the cycle of cleavage and gastrulated (Fig. 2), but in some cases two or more fragments divided from a single egg cleaved and developed to the gastrula stage. Since it is known that polyspermy occurs naturally in newt’s egg, all such fragments but one will be androgenic merogons. Among fragments, the first one to undergo cleavage proceeded to the latest stage of development (Fig. 3), while others ceased to develop at earlier stages or became abnormal. In the experiments to follow, only the fragment that cleaved first was used for observation, on the premise that the first one contained the zygote nucleus.

Time required for the onset of gastrulation

The time required for the onset of gastrulation in the quarter, half and whole embryo was respectively 35.9 ± 1.3 h, 34.8 ± 1.8 h and 33.6 ± 1.0 h at 20 ± 0.5 °C (mean ± s.D.). These results show that the time from the first cleavage to the onset of gastrulation does not widely vary in spite of the decrease in cytoplasmic volume (Table 1).

Period of synchronous cleavage

Cinematographic records on the whole embryos confirmed the twelve cycles of synchronous cleavage as reported by Suzuki et al. (1976) on newt’s eggs. By projection of the time-lapse film at a speed of 24 frames per sec each of the synchronous divisions, especially from the fifth cleavage onward, was very
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Fig. 3. (a) Whole embryo (top) and half embryo (bottom) at neural tube stage. (b) Larva from a quarter egg. (c) Normal larva at the same stage as that in fig. (b). Each bar equals 1 mm.

Table 1. Time required from onset of first cleavage to pigmentation (hr: min)

<table>
<thead>
<tr>
<th>Type</th>
<th>Time (hr: min)</th>
<th>Mean (hr: min)</th>
<th>S.D. (hr: min)</th>
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<td>33:35</td>
<td>1:00</td>
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<tr>
<td>Half embryos</td>
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<td>34:47</td>
<td>1:48</td>
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<tr>
<td>Quarter embryos</td>
<td>36:45, 36:25, 36:20, 35:50, 35:40</td>
<td>35:55</td>
<td>1:16</td>
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clearly observed by the propagation of the ‘cleavage wave’ from the animal hemisphere expanding toward the vegetal hemisphere as described in *Bufo* (Shirakami, 1958) and in axolotl (Hara, 1977). In the case of quarter embryos, such a synchronous cleavage with the obvious cleavage wave was also found to occur up to the 10th cleavage, with the same rhythm as in whole embryos although in a few quarter embryos the synchrony extended to the 11th cleavage. In other words, in quarter embryos the stage of asynchronous division starts mostly two cycles earlier than the whole embryos. In half embryos, 11 or 12 cycles of synchronous cleavage were observed.

For more detailed analysis of temporal pattern of cleavage, 14 blastomeres from the animal hemisphere of each embryo were selected randomly on the films at blastula stage and their ancestor cells were traced back to the 2-cell stage.
Timing of the onset of gastrulation

Whole embryo

Round of cleavage

Quarter embryo

Fig. 4. Diagram of continuous tracing of the 14 blastomeres which were selected randomly in the animal hemisphere of a whole (top) and quarter embryo (bottom) at blastula stage. Each solid circle shows the time of appearance of a visible cleavage furrow in the ancestor cell. The number with thick bar shows the round of cleavage, and the length of the bars show the duration from the first appearance of visible cleavage furrow in the selected blastomeres to the last appearance of that. Bottom scales indicate min after second cleavage.

and descendant cells were traced to the 11th or 12th cleavage. Figure 4 clearly demonstrates the marked contrast between the whole and the quarter embryos as to the number of synchronous divisions (12 in the whole embryos and 10 in the quarter embryos). This figure also indicates the coincidence of the synchronous division between the two embryos up to the 10th cleavage. The generation time of blastomeres in the animal hemisphere from G-3 to G-12 (here, G-n represents the generation of blastomeres which are between the (n-1)th cleavage and the (n)th cleavage) was measured on the films (Table 2). Rapid increase of the calculated standard deviation of the generation time at G-11 in quarter embryos clearly distinguishes the transition from synchronous to asynchronous division.

Number of blastomeres at pigment stage

The numbers of cells constituting an embryo at pigment stage decreased apparently in proportion to the diminution of egg volume. They were 5466 ± 1574 (mean ± s.d.) in quarter embryos, 9304 ± 1618 in half embryos and 14818 ± 1184 in whole embryos (Table 3).
Table 2

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<td>14</td>
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<td>Number of blastomeres examined</td>
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<tr>
<td>Average generation time (min)</td>
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<td>102</td>
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<td>5.5</td>
<td>4.4</td>
<td>8.2</td>
<td>29.4</td>
<td>40.4</td>
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</table>
Timing of the onset of gastrulation

Table 3. Numbers of cells at pigment stage

<table>
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<th>Whole embryos</th>
<th>Half embryos</th>
<th>Quarter embryos</th>
</tr>
</thead>
<tbody>
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<td>15775, 15187, 14990, 13268, 15210, 14818</td>
<td>9215, 7333, 9076, 8494, 9042, 9304</td>
<td>4350, 6895, 4003, 9227, 3956, 5466</td>
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<tr>
<td>16040, 17299, 13638, 13169, 15168, 1184</td>
<td>7846, 9545, 13941, 7786, 7895, 1618</td>
<td>7064, 7166, 3628, 6229, 4609, 1574</td>
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<td>13856, 14212,</td>
<td>8899, 10998, 8637, 10986, 9869,</td>
<td>5350, 4375, 6429, 3832, 4871,</td>
</tr>
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</table>

Discussion

Relationship between cytoplasmic volume and the temporal pattern of cleavage

Landström et al. (1975) reported that injection of deoxyribonucleotides (dXTP) into fertilized eggs brought about prolongation of the duration of synchronous division in Xenopus. It was suggested that the pool of dXTPs, which is possibly utilized for DNA synthesis, maintains the synchrony of early cleavages. Rott & Shevelera (1968) reported that in haploid loach embryos, the decrease in the rate of cell division occurred one cell generation later than in diploids.

To analyse this problem quantitatively, we have reduced the cytoplasmic volume of a fertilized egg to one half or one quarter. The results clearly showed that the desynchronization started two divisions earlier in a quarter embryo and one division earlier in a half embryo compared with a whole embryo. This result supports the idea that the synchrony of early cleavages is maintained by the pool of cytoplasmic contents.

Relationship between cytoplasmic volume and the onset of gastrulation

According to Dettlaff’s suggestion, the onset of gastrulation is determined by an accumulation of a certain threshold amount of some substances, produced in interphases, bringing about a prerequisite change in the state of the cytoplasm. In the experiments by Landström et al. (1975) injection of dXTPs suppressed the morphological development and inhibited the synthesis of all kinds of RNA, including mRNA required for differentiation to occur.

However, the present results indicate the contrary: the start of gastrulation was not accelerated regardless of the advance in the start of asynchronous cleavage induced by diminution of cytoplasm (Fig. 5). Similar results were also shown in Chulitskaia’s experiments: artificial prolongation of the synchronous cleavage period by injection of cytoplasm or the supernatant of its homogenate from mature unfertilized eggs did not lead to a delay of gastrulation. Thus the length of the asynchronous period before gastrulation could be variable, without
changing the timing of the onset of gastrulation. This indicates that the transition from synchronous to asynchronous division is not an event which directly triggers the preparation for the start of gastrulation, and does not support any theory which advocates the necessity of a fixed period of asynchronous division for accumulation of some substances responsible for the onset of gastrulation.

**Cell number at the onset of gastrulation**

Suzuki *et al.* (1976) estimated the cell number of *Cynops* embryo at the pigment stage as 18000, by counting the number of nuclei in an aliquot taken from the suspension of dissociated blastomers, whereas in the present study we have directly counted the total cell number of the whole embryo, which amounted to 15000. The difference in the cell number between Suzuki *et al.* and us will be due to the method employed.

During the period of synchronous division the cell number is doubled by each round of division. The rate of cell proliferation then becomes lower as embryos enter the asynchronous period in normal development (Suzuki *et al.* 1976; Hara, 1977). This situation can be applied to quarter and half embryos as the generation time of blastomers begins to lengthen from the loss of synchrony (Table 2 and Fig. 4). The reduction in cell number in quarter and half embryos as compared with whole embryos may be explained by the diminution of the cycles of synchronous division (twice or once). On the assumption that the rates of cell division in the asynchronous period are nearly equal in quarter and whole embryos, a surplus of the cell number in quarter embryos (5500) over one-quarter of the whole embryo (3750 = 15000/4), despite the loss of two cycles of synchronous division, would be due to the fact that the period of asynchronous division in quarter embryos was longer than that in whole embryos (Fig. 5). The same will apply to the half embryos, in which the cell number of 9500 is slightly more than one-half of the cell number of the whole embryo (7500 = 15000/2).

These figures on cell numbers might lead one to expect embryos to begin gastrulation when a certain nucleo-cytoplasmic ratio is established in the blastomers. However, Cooke (1973) reported that when an embryo was treated with mitomycin C at mid-blastula stage, the embryo began gastrulation with a large volume of blastomers in *Xenopus*. Thus the nucleo-cytoplasmic ratio itself may not determine the timing of the onset of gastrulation.
Timing of the onset of gastrulation

As to the clock mechanism for the onset of gastrulation the work on pseudo-gastrulation is suggestive (Holtfreter, 1943; Smith & Ecker, 1970; Malacinski, Ryan & Chung, 1978). Oocytes of some amphibian species treated with progesterone underwent morphogenetic movements which resembled normal gastrulation both morphologically and temporally, without any cleavage, in Ringer's solution. Further, enucleated oocytes when they were treated with hormone showed this phenomenon though they stopped the movement at pseudo-mid-gastrula stage. This may imply that a biological clock for determining the timing of the onset of gastrulation is set up in the cytoplasm.

Recently it was reported by Sawai (1979), Hara, Tydeman & Kirschner (1980) and Sakai and Kubota (unpublished) that cyclic changes of surface stiffness or the periodic appearance of surface contraction waves occurs in non-nucleate fragment of uncleaved amphibian eggs. Investigation on the correlation between these periodic activities of the cytoplasm and the clock mechanism for determining the timing of morphogenesis is in progress.

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REFERENCES


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