Accumulation of pigment granules around nuclei in early embryos of Anura (Amphibia)

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SUMMARY

A dark spot was found to appear in each blastomere of the vegetal surface of blastulae of *Rana rugosa, Hyla arborea*. The spot divided into two before division of the blastomere, so that one new spot was allotted to each daughter cell. These dark spots were formed at early blastula stage, and persisted until the end of yolk plug stage.

Cytological observations showed that each dark spot corresponds to a mass of accumulated pigment granules around the nucleus of a blastomere. The accumulation increases with development during the cleavage period more rapidly in blastomeres of the vegetal hemisphere than in those of the animal hemisphere.

This accumulation of pigment granules around nuclei during development indicates that the granules are transported toward the nuclei during the cleavage period, suggesting some sort of directional flow of cytoplasm in blastomeres of early amphibian embryos.

INTRODUCTION

Amphibian embryos have been used as suitable materials for investigation of developmental biology. Detailed descriptions of normal development and experimental investigations, however, have been done on relatively few species of amphibians, including *Xenopus laevis, Ambystoma mexicanum, Rana pipiens* and *Cynops pyrrhogaster*.

Working on the development of various Japanese species, the author observed the formation of dark spots in the vegetal half of blastulae of *Rana rugosa* and *Hyla arborea*, that had never been noticed in the 'common' species. As will be shown, these dark spots correspond to the accumulation of pigment granules around the nuclei during development in the cleavage period.

This accumulation of pigment granules is found in embryos of several families (genera) of anura, such as Ranidae (*Rana*), Hylidae (*Hyla*) and Bufonidae (*Bufo*). It is therefore conceivable that the accumulation of pigment granules around nuclei is of general occurrence in early amphibian embryos. The accumulation of pigment granules suggests intracellular movement toward the nuclei in blastomeres of early amphibian embryos. This paper describes the dark spots and

accumulation of pigment granules as observed mainly in *Rana rugosa* and *Hyla arborea*.

MATERIALS AND METHODS

Embryos

Sexually mature females and males of *Rana rugosa* and *Hyla arborea* were collected during their breeding season (June and July) in the suburbs of Kyoto and Fukuoka. They were stored at a low temperature (10–15 °C) until use.

Fertilized eggs were obtained either by artificial insemination or by mating females and males in the laboratory. For artificial insemination, mature eggs were squeezed out from a female and placed into a dry dish. Sperm suspension was prepared by mincing isolated testes with forceps in a small volume of 1/10-strength modified Steinberg's solution '1/10 MSS' (NaCl, 3·4 g; KCl, 0·05 g; $Ca(NO_3)_24H_2O$, 0·08 g; $MgSO_47H_2O$, 0·205 g; in 1000 ml distilled water buffered to pH 7·2 by 3 mM HEPES–NaOH), and immediately mixed with the eggs.

Naturally laid early embryos of *Rana japonica* and *Bufo bufo japonicus* were collected in the suburbs of Kyoto in their breeding season (February and April respectively).

Observation of intact embryos

Fertilized eggs were deprived of jelly coats before the first cleavage by gentle swirling in 1/10 MSS containing 1 % sodium thioglycollate, pH 9–10, for a few minutes. They were rinsed a few times with 1/10 MSS and allowed to develop within intact vitelline membranes at room temperature (23–27 °C).

Embryos were observed from the vegetal side with an inverted microscope and photographed at intervals of some minutes.

Observation of the sectioned materials

At intervals, embryos were fixed with 10 % formalin (1/10 diluted formaldehyde solution) at room temperature. They were dehydrated with ethanol, cleared with xylene, embedded in paraffin and cut into 8–10 μm thick serial sections. The sections were stained with Azan for histological observation. Some sections were mounted without staining.

Sections were observed mainly under dark-field illumination with a condenser (Nikon Dark Field Condenser). The advantage of the use of dark-field illumination for observation of sectioned materials will be shown in the Results.

RESULTS

When early blastulae of *R. rugosa* and *H. arborea* were observed from the vegetal side, numerous dark spots were found, distributed all over the non-pigmented vegetal surface of the embryos. Fig. 1 shows a series of photographs of an external view of the vegetal hemisphere of a *R. rugosa* blastula. The dark spots are clearly recognized. Occasionally some dark spots divided into two (indicated with arrowheads in Fig. 1). This was immediately followed by the division of the blastomere, which partitioned the two spots, one into each daughter blastomere. These dark spots were recognizable from the early blastula to the end of the yolk plug stage (Fig. 2). Fig. 3 shows the dark spots in *H. arborea*, behaving in almost the same way as in the *R. rugosa* blastulae.

The dark spots were observed in *Rana japonica* and *Bufo bufo japonicus* also, but only in a restricted region around the vegetal pole, and were less distinct than those in *R. rugosa* and *H. arborea*.
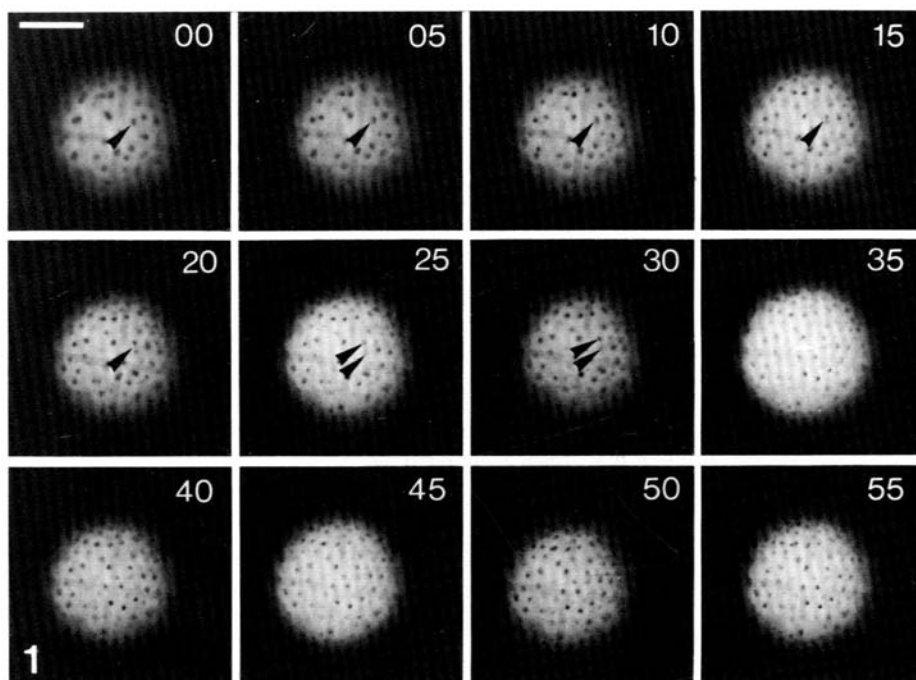
Fig. 1. Continuous observation of the vegetal hemisphere of a *Rana rugosa* blastula. Numerals are the time in minutes after the early blastula stage when the observation started. Arrowheads indicate one of the dividing dark spots. Indistinct images of a glass reticle in the microscope are superimposed in the pictures. Bar equals 0·5 mm.

Sectioned blastulae stained with Azan revealed a mass of pigment granules accumulated around each nucleus (Fig. 4A,B). In unstained sections such accumulated pigment granules could be clearly identified as dark areas. No other structure exhibited such a dense appearance: yolk granules and nuclei were hardly recognizable (Fig. 4C,D). Thus it is certain that the dark spot, observed in intact embryos, represents the mass of pigment granules accumulated around the nucleus. Dividing spots as observed to take place prior to cytokinesis thus indicate nuclear division.

By dark-field illumination, the accumulated pigment granules were seen to shine brilliantly against a dark background (Fig. 4A'–D'). Moreover this illumination showed small bright spots scattered in the cytoplasm, most probably representing less accumulated or single granules, which could not be identified with an ordinary microscope. In the stained sections, other organelles such as yolk granules and nuclei became faintly visible (Fig. 4A',B'), which reduced the sharp contrast of pigment granules seen in non-stained materials (Fig. 4C',D'). The distribution of pigment granules in the blastomeres was, therefore, observed in detail by dark-field illumination of the unstained sections.

This illumination enables us to get a broad view of the distribution of accumulated pigment granules in a whole embryo (Fig. 5). At the blastula stage,

the accumulation is observed not only in vegetal blastomeres but also in blastomeres in the animal hemisphere.

The process of accumulation of pigment granules in early development was somewhat different between *R. rugosa* and *H. arborea*. In the embryos of *R. rugosa* up to the 4-cell stage, pigment granules were scattered in the periphery of each blastomere and absent in the vicinity of the nucleus. The pigment granules were often found to line the cell surface which had been formed by the cleavage furrow (marked by arrowheads in Fig. 6), but few pigment granules were present in the endoplasm. At the 8-cell stage, some pigment granules were observed at the periphery of each blastomere, and at the 16-cell stage the granules occupied the whole cytoplasm (Fig. 6. 8-cell,16-cell). Radial arrays of pigment granules converging on the nucleus were sometimes noticed (Fig. 6. 16-cell,v).
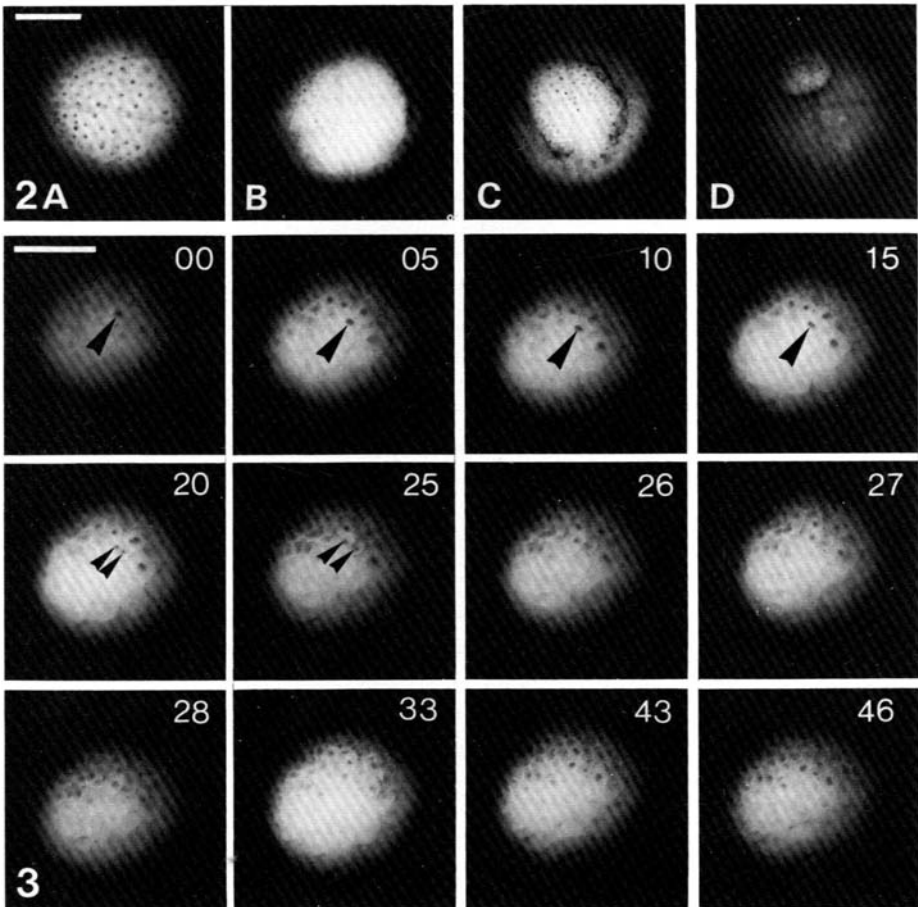

Fig. 2. Vegetal hemisphere of *R. rugosa* embryos in late stage. (A) Blastula. (B) Early gastrula. (C) Mid gastrula. (D) Late gastrula (yolk plug stage). Bar equals 0·5 mm.

Fig. 3. Continuous observation of the vegetal hemisphere of a *Hyla arborea* blastula. Numerals are the time in minutes after the beginning of observation. Arrowheads indicate one of the dividing dark spots. Bar equals 0·5 mm.

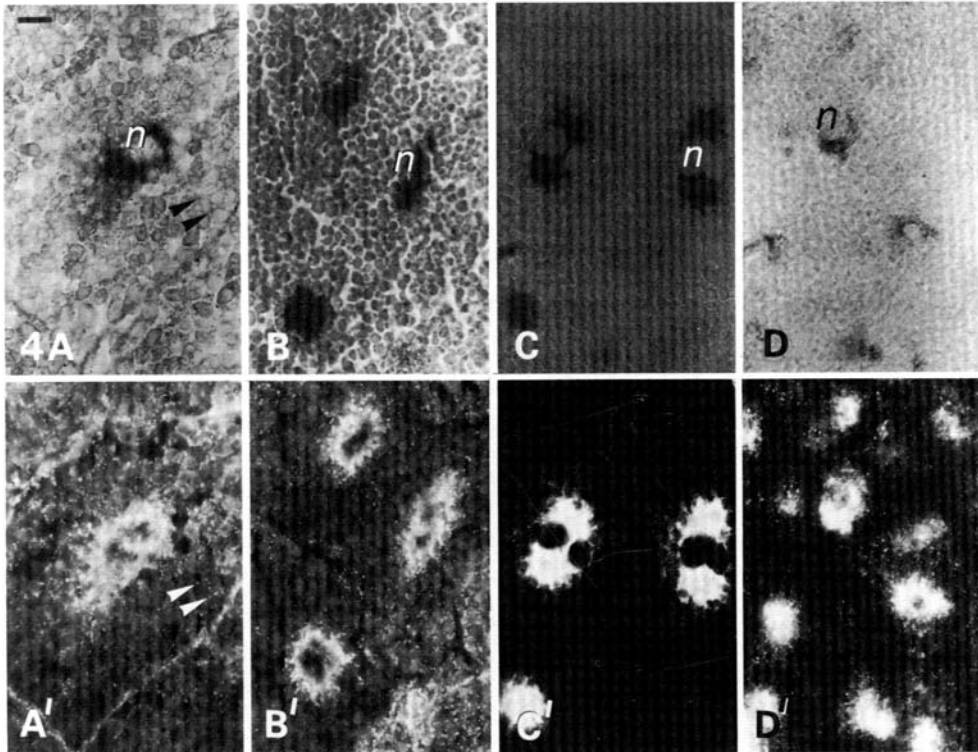B.bufo   R.japonica   R.rugosa   H.arborea

Fig. 4. Accumulation of pigment granules observed in four species of frogs. (A),(A'), (B) and (B') are stained with Azan. (C),(C'),(D) and (D') are non-stained sections. Observation with an ordinary microscope (A–D) is compared with that observed under dark-field illumination (A'–D'). In the latter condition the pigment granules are recognized as bright spots against dark background. Arrowheads in (A) and (A') indicate single pigment granules. *n*, nucleus. Bar equals 10 μm.
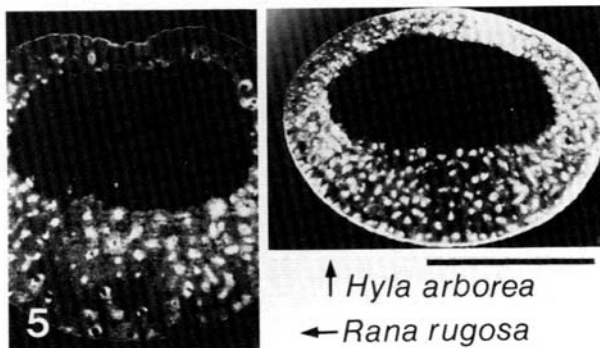

↑ *Hyla arborea*
← *Rana rugosa*

Fig. 5. Views of whole blastulae with dark-field illumination. Bar is 0·5 mm.

Accumulation of the granules around the nucleus began at the 32-cell stage (Fig. 6. 32-cell,v) and progressed through the 256-cell stage. As development continued pigment granules accumulated more densely around nuclei in blastomeres of both
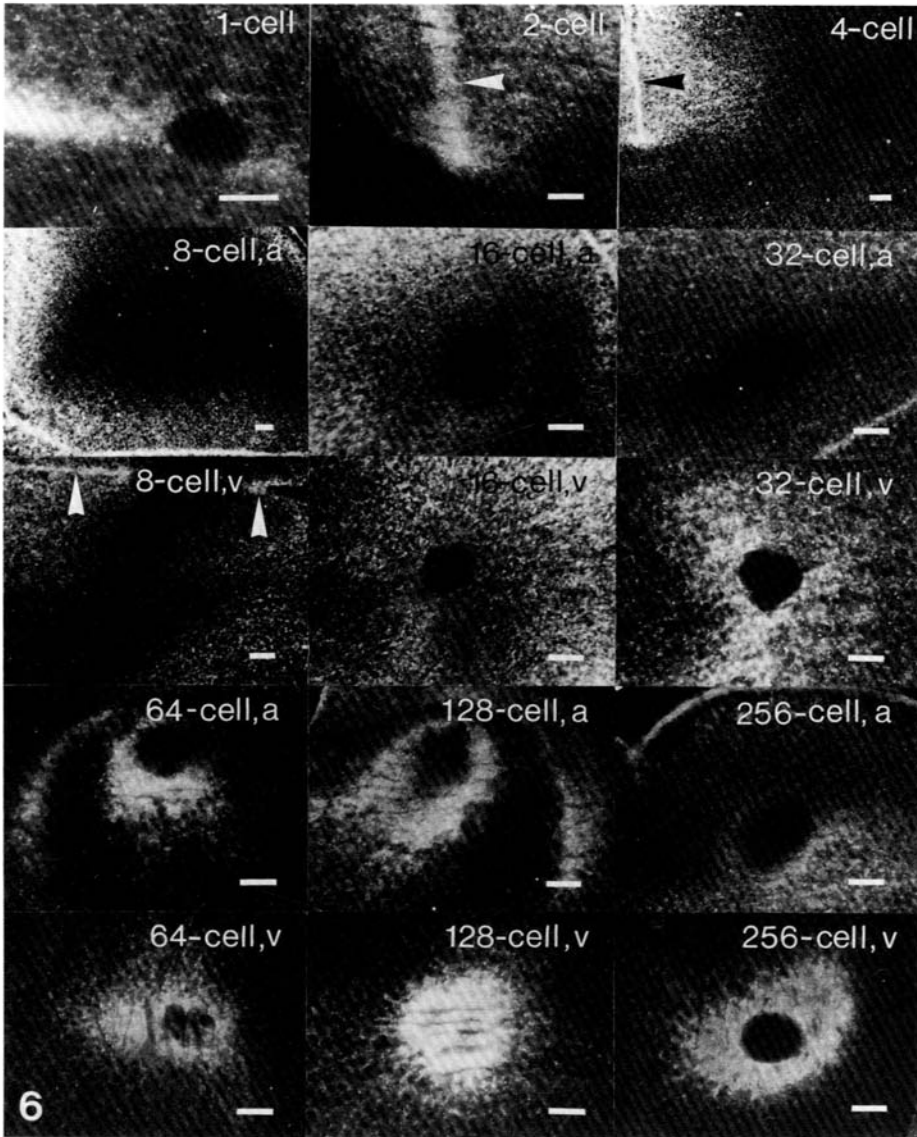
Fig. 6. Series of unstained sections of *R. rugosa* embryos, at indicated stages, observed with dark-field illumination. For each stage from 8 to 256 cells, both blastomere in animal half (a) and vegetal half (v) are shown. (1-cell) Some pigment granules tailed after the sperm nucleus. (2-cell) Diastema of the first cleavage furrow is indicated by an arrowhead and the neighbouring cytoplasm contains abundant pigment granules. (4-cell) Diastema of second cleavage furrow is indicated by an arrowhead. The neighbouring cytoplasm contains abundant pigment granules in the same way as the 2-cell-stage embryo. (8-cell) Most pigment granules are still in the periphery of the blastomere, though in the vegetal half some extend deep into the cytoplasm. Arrowheads indicate the third cleavage furrow which is abundant in pigment granules. (16-cell) Pigment granules are distributed throughout the whole cytoplasm. (32-cell) Pigment granules begin to accumulate around nucleus in the vegetal half blastomere. Each bar indicates 10 μm.

the animal hemisphere and the vegetal hemisphere during the cleavage period (Fig. 6). Corresponding to this accumulation of pigment granules, the dark spots became visible in the intact embryo at the 64-cell stage especially in the blastomeres located near the vegetal marginal zone (Fig. 7). The appearance of the dark spots in the most vegetal cells occurred later, owing probably to the larger cell size which would reduce the visibility of accumulated pigment granules as dark spots. Fig. 7 also shows, before the appearance of the perinuclear spots of granules, a dark area with irregular shapes around the vegetal pole at the 1-cell stage. This area, most probably indicating the distribution of the pigment granules, became gradually fainter as the cleavage furrows passed through the area in later divisions.

In contrast to *R. rugosa*, in the embryos of *H. arborea* some degree of pigment granule accumulation was observed as early as the 1-cell stage (Fig. 8). The accumulation became denser with development during the cleavage period. The density of accumulation in blastomeres of the vegetal hemisphere was higher than in those of the animal hemisphere during early cleavage stages.

DISCUSSION

The appearance of dark spots in cells at the vegetal surface of intact blastulae in *R. rugosa* and *H. arborea* has led to the present finding of an accumulation of pigment granules around the nuclei. The visibility of such spots is rather limited: they are observed only in particular species of frogs, only in cells of the non-pigmented vegetal surface of the blastulae and only in later stages (after the 64-cell stage) when the size of the blastomeres is reduced so the nuclei come close to the surface of the embryos. Future studies will depend of course on direct observation of the pigment granules. The dark spots, however, are natural markers for the
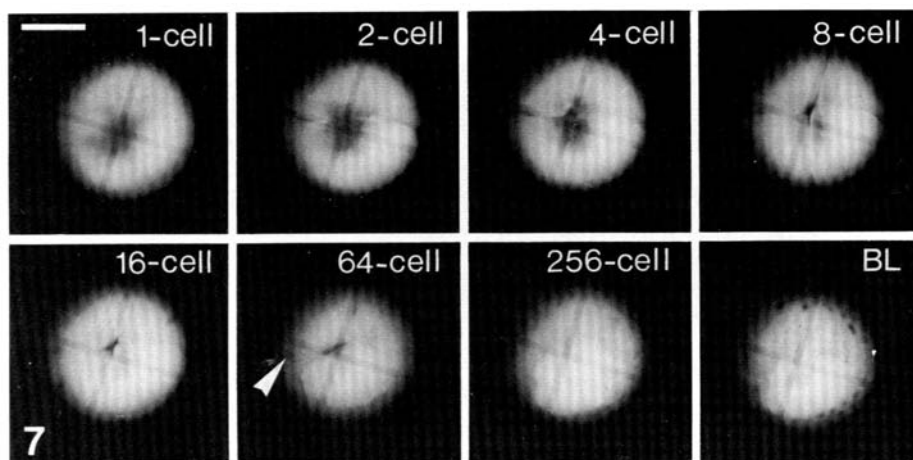

Fig. 7. Continuous observation of the vegetal hemisphere of an early embryo of *R. rugosa* at 1, 2, 4, 8, 16, 64, 256-cell and early blastula (BL) stage. Indistinct images of a glass reticle in the microscope are superimposed in the pictures. Arrowhead indicates the first appearance of the dark spot. Bar is 0·5 mm.
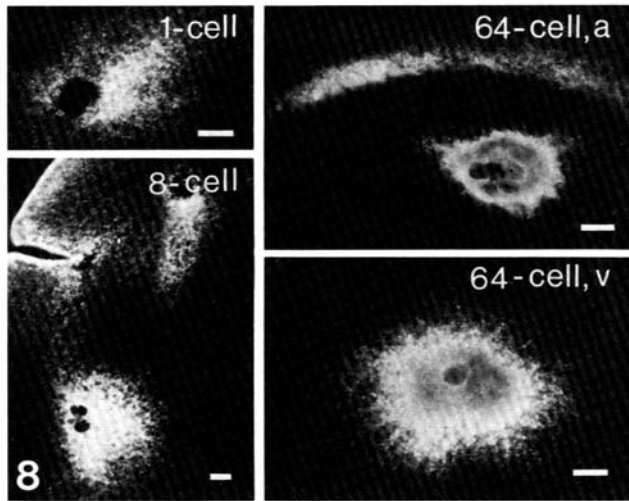
Fig. 8. Unstained sections of *H. arborea* embryos, observed with dark-field illumination. (1-cell) Some pigment granules are already observed around the nucleus. (8-cell) Accumulation of the pigment granules is more advanced in the vegetal blastomere than in the animal one. (64-cell) Full accumulation of the granules as in a 64-cell-stage embryo of *R. rugosa* (Fig. 6. See in text). Each bar indicates 10 μm.
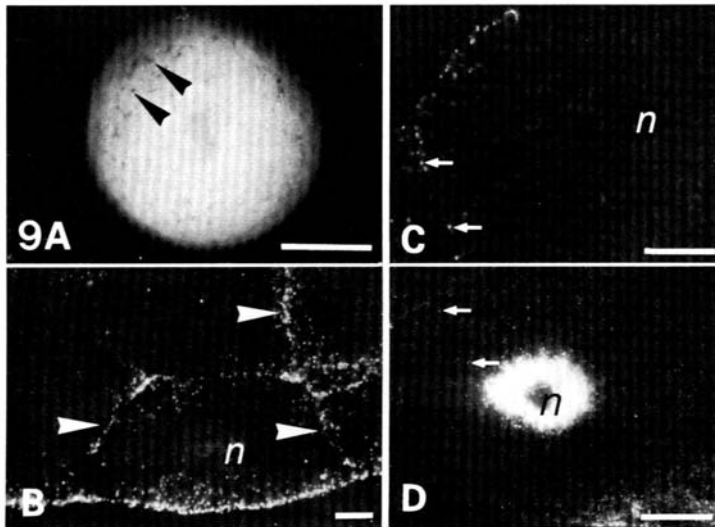

Fig. 9. (A) Vegetal hemisphere of *Xenopus laevis* at the blastula stage. Arrowheads indicate accumulated pigment granules at the boundaries of the blastomeres. Bar is 0.5 mm. (B) Unstained section of *X. laevis* at the stage of early blastula observed with dark-field illumination. Arrowheads indicate pigment granules which are lined up along the boundary lines between the blastomeres. Bar is 50 μm. (C),(D) One of the vegetal blastomeres in an unstained section of *X. laevis* (C) and *H. arborea* (D) at the 256-cell stage. Each arrow indicates a single pigment granule. Pigment granules in *X. laevis* are clearly larger than those in *H. arborea*. Bar is 50 μm. *n*, nucleus.

localization of the nuclei in vegetal blastomeres. The two species, *R. rugosa* and *H. arborea*, will provide good materials for studying cell-cycle timing in amphibian embryos.

During the process of pigment granule accumulation, the appearance of the granules first in the periphery of the blastomere followed by the gradual concentration of the granules around the nuclei indicates some sort of directional transport system in cytoplasm, rather than *de novo* formation of the pigment granules around the nuclei. The accumulation clearly demonstrated in three families of amphibians (cf. Fig. 4) suggests that such a directional transport system is widely distributed in early amphibian embryos. The appearance of a perinuclear zone of yolk-poor RNA-rich cytoplasm in the 4-cell stage in *Xenopus laevis* embryos (Imoh, 1984) may be a result of such a cytoplasmic flow.

In a preliminary observation, however, the accumulation of pigment granules around the nuclei was not seen in *X. laevis*. During the cleavage period most of the pigment granules are found along the boundary lines between the blastomeres (Fig. 9B,C). On the vegetal surface of *Xenopus* embryos the pigment granules are also distributed mainly along the boundaries of the blastomeres, so sometimes a mass of pigment granules on the surface of the vegetal half looks like the 'dark spot' of *R. rugosa* and *H. arborea* (Fig. 9A). The pigment granules in *X. laevis* are very large when they are compared with the pigment granules in *R. rugosa* or *H. arborea* (Fig. 9C,D). This may be a reason why the pigment granules do not accumulate around the nuclei though the yolk-poor RNA-rich cytoplasm accumulates around the nuclei during the cleavage period in *Xenopus laevis*.

Since endoplasmic granules first appear at the periphery of the blastomere, it seems obvious that the pigment granules have migrated from the cortex. Abundant pigment granules in the cortical layer of the animal hemisphere will be sources of perinuclear granules in blastomeres in the animal hemisphere. There is still the question: where do the pigment granules come from in the vegetal half at early stages? The small number of pigment granules incorporated on insemination, visualized as the penetration path of the sperm nucleus (Fig. 6. 1-cell) does not seem to be enough to explain the origin of accumulated pigment granules around the nuclei in later stage (blastula) embryos (Figs 4 & 5).

Nicholas (1945), Ballard (1955) and Harris (1964) reported on the so-called 'cortical ingression' in embryos during the cleavage period: the vitally stained cortex of the lateral and vegetal parts of eggs migrated inward (toward the blastocoel), along newly formed cleavage furrows. The disappearance of pigment granules from the surface of the vegetal pole region by the 5th cleavage (cf. Fig. 7) suggests such a 'cortical ingression' in *R. rugosa* embryos. In fact, the abundance of pigment granules at the first and second cleavage furrows or diastemas (arrowheads in Fig. 6) clearly shows the transport of pigment granules from the surface of the embryo to a deeper region. Thus a pair of animal and vegetal blastomeres formed by the third cleavage will be equally endowed with the pigment granules. It is highly probable that the pigment granules thus transported are utilized in a future stage of accumulation around the nuclei.

It is obvious that the accumulation of pigment granules reported in this paper reflects the movement of cytoplasmic components in the blastomeres. Radial arrays of pigment granules sometimes observed to converge toward nucleus may indicate that the movement is due to astral rays in mitosis. The significance of such centripetal movement is not known, but it may be relevant to segregation of cytoplasmic components which could play a role in differentiation among the blastomeres. Alternatively, it may be related to some interactions between nucleus and cytoplasm in early amphibian development.

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