PRENUCLEOLAR BODIES OF URECHIS OOCYTES

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SUMMARY

The immature oocyte and unfertilized eggs of Urechis caupo, a marine echiurid worm, contain many nuclear bodies and a main nucleolus. These bodies are similar to prenucleolar bodies of other materials with respect to their origin, structure, cytochemical and biochemical characteristics. They are formed in large numbers during the diffuse diploctene stage, perhaps by an aggregation of nucleolar materials which had been associated with chromosomes. They are fibrillar in structure, made up mainly of some nucleolar proteins, and are inactive in RNA synthesis. Biochemical analyses suggest the possibility that these structures contain a small amount of ribosomal RNA precursors which may have originated from the main nucleolus. An accumulation of prenucleolar bodies in Urechis oocytes may result from the lack of formation of multiple functional nucleoli, such as those found in amphibian oocytes.

INTRODUCTION

The formation of nucleoli at the end of telophase in adult cells and also during early embryonic development is the morphological manifestation of activation of ribosomal genes. Some proteins (perhaps also a small amount of RNA) accumulate in the form of prenucleolar bodies prior to the formation of new nucleoli. Various light- and electron-microscopic studies suggest that these bodies give rise to new nucleoli: (a) Prenucleolar bodies are similar to nucleolar cores with respect to their submicroscopic structures (Karasaki, 1965, 1968; see review by Hay, 1968). (b) They show similar staining characteristics with silver as the nucleoli (Das, 1962; Das & Alfert, 1966). (c) They decrease in number as new nucleoli are formed (Das & Alfert, 1963) and persistent accumulation of such bodies is seen in organisms in which the nucleolar organizer is absent or inactivated (Hay, 1968; Swift & Stevens, 1966). (d) Their presence in close association with chromosomes and their aggregation into new nucleoli at the end of mitosis is also suggested by some electron-microscopic studies (Stevens, 1965; see review by Lafontaine, 1968). A detailed study on the formation, accumulation, and a biochemical and histochemical characterization of prenucleolar materials seems necessary in order to learn whether they are implicated in the regulation of ribosomal RNA (rRNA) synthesis. The immature oocytes and unfertilized eggs (mature oocytes) of Urechis are excellent materials for such studies. These cells contain numerous nuclear bodies (previously called ‘pseudonucleoli’, see Das & Alfert, 1970) and a prominent main nucleolus (Das, Luykx & Alfert, 1965).

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In the present report we describe the origin, structure, and some cytochemical and biochemical characteristics of these bodies. The results presented show that these structures are identical with prenucleolar bodies in other kinds of cells. They originate during oogenesis, perhaps by aggregation of some nucleolar ribonucleoproteins that might have remained associated with chromosomes. An accumulation of such pre-nucleolar bodies seems to be correlated with absence of functional multiple nucleoli.

MATERIALS AND METHODS

The echiuroid marine worms *Urechis caupo* were collected from a mudflat at Bodega Bay, near San Francisco, California. They were kept at 12-14 °C in aerated seawater. Only worms collected within a month were used in the present experiments.

A large number of oocytes at various stages of maturation is present, free floating, in the coelomic cavity of female worms (Newby, 1940). Samples of coelomic fluid, containing such oocytes, were collected (see Das, 1968). Samples of unfertilized eggs were also collected from the storage sacs (see Das *et al.* 1965). In some experiments unfertilized eggs were kept at about 4-5 °C for various times prior to fixation. All samples were fixed in acetic alcohol or in 10 % neutral formalin and paraffin sections (5 μm in thickness) were then prepared. Nuclei from unfertilized eggs were also isolated (see below), spread on slides, and dried. Slides were stained with haematoxylin, acid fast green, azure B, or with our silver staining procedures (Das, 1962). The alkaline fast green (Alfert & Geschwind, 1953) and bromphenol blue (Bloch, 1966) staining techniques for basic proteins have also been applied. The submicroscopic structures of nuclear bodies have been studied as described earlier (Das, Micou-Eastwood, Ramamurthy & Alfert, 1970).

The rate of RNA and protein synthesis and the time of accumulation of nuclear bodies in immature oocytes and fertilized eggs have been determined. Tritiated cytidine (500 μCi/worm; spec. act. 2.34 Ci/mM) or uridine (450 μCi/worm; spec. act. 200 Ci/mM) was injected into the coelomic cavity of female worms. Samples of coelomic fluid containing immature oocytes were collected at various times after labelling. Unfertilized eggs were exposed (see Das *et al.* 1965) to tritiated uridine (100 μCi/ml; spec. act. 26-5 Ci/mM) or phenylalanine (100 μCi/ml; spec. act. 1.6 Ci/mM) for various times. Some unfertilized eggs exposed to [3H]uridine were fertilized 25 h later in order to determine the fate of rRNA precursors after fertilization. Cells exposed to labelled RNA precursors were fixed in acetic alcohol, while those exposed to [3H]phenylalanine were fixed in 10 % neutral formalin. Autoradiographs of paraffin sections (5 μm in thickness) were prepared by using liquid emulsion (NTB2). Autoradiographs were stained with haematoxylin through the processed emulsion and silver grains were counted over labelled oocytes. Some autoradiographic preparations were used for staining with silver (Das, 1962). Nuclear bodies from unlabelled unfertilized eggs were also isolated (see below) and autoradiographs prepared.

A relatively simple technique was developed to isolate intact nuclei from unfertilized and fertilized eggs (Figs. 5-7). The technique involves partial fixation of the nuclear membrane, but not the cell membrane, in cold dilute formalin solution. Samples were fixed for 15 min in 10-15 vol. of cold 5 % neutral formalin, centrifuged, and then suspended in 10-15 vol. of cold deionized water. The suspension was thoroughly mixed with a Pasteur pipette. As soon as eggs began to lyse, they were spun down, mixed again with 5-6 vol. of cold deionized water, and gently mixed with the pipette. The intact nuclei from lysed eggs were centrifuged and further cleaned (2-3 times) with cold 0-1 % Triton X-100 containing 0-25 % formalin which prevents excessive swelling of nuclei. The centrifugation was done at about 1000-2000 rev/min for 2-3 min. All operations during nuclear isolation (also isolation of other cell fractions, see below) were done at ice-bath temperature.

Attempts have also been made to isolate nuclear bodies from unfertilized eggs. Eggs were rapidly broken, by passing them through a hypodermic needle, in 5-6 vol. of cold deionized water with added 0-1 % Triton X-100 and 0-25 % neutral formalin. They were centrifuged (2000 rev/min for 2 min) to remove main nucleoli and membranes. The supernatant was then centrifuged at 8000 rev/min for 10 min. The precipitate was suspended in fresh solution and
fractions of nuclear bodies of various sizes, with some contaminating pigments, chromosomes, and nucleoplasmic fragments, were obtained by differential centrifugation. Clean nucleolar samples were recovered from the precipitate obtained after the first centrifugation. This precipitate was mixed in 5–6 vol. of cold deionized water and centrifuged for 4 min at 2000 rev/min. The precipitate at the bottom of the tube contained mainly nucleoli; the gelatinous precipitate over the nucleolar pellet contained membranes with some trapped nucleoli. Most of these nucleoli were recovered from the gelatinous precipitate after several washings and centrifugations (3–4 times). A slightly different procedure for nucleolar isolation has been described earlier (Das et al. 1970).

RNA from whole eggs (unfertilized and fertilized), nuclei (with nucleoli), nucleoli alone, and from various fractions of nuclear bodies was extracted by the phenol-SDS technique (see Das et al. 1970) and then characterized by acrylamide (2-0 %)-agarose (0-5 %) gel electrophoresis (Dingman & Peacock, 1968). The gel was stained with 0-2 % methylene blue (Peacock & Dingman, 1967), scanned with the Joyce-Loebl densitometer, and sectioned at 1-2 mm thickness. Each section was left overnight at room temperature in 10 ml of toluene-based scintillation fluid with added 0-5 % NCS (Nuclear-Chicago Corp.) to extract RNA, then counts were made. The mild formalin fixation used in isolation of various cell fractions did not produce any effect on the profiles of different species of RNA of *Urechis* eggs (Das et al. 1970).

**RESULTS**

Many nuclear bodies are present in immature oocytes, unfertilized eggs, and in embryos at various stages of development (Figs. 4–7). In unfertilized eggs, which are at the diakinesis stage of meiosis, some of these bodies accumulate in the vicinity of the main nucleolus (Figs. 7, 8). The size of these bodies is variable; in eggs from freshly collected worms the largest bodies had diameters of 1–2 μm. The average diameter of the main nucleolus is about 9 μm.

Some nuclear bodies remain associated with chromosome pairs. Such an association is very striking in old eggs and also in eggs incubated at 4–5 °C (Figs. 8–12). At low temperature the size of these bodies also increases (Figs. 8–12). Such an increase seems to be caused by swelling, since the enlarged bodies often appear vacuolated (Figs. 10, 12). The increase in size is also brought about by fusion of nuclear bodies. The number of these bodies decreases at low temperature. The average number per nucleus was 50 ± 2-0 (n = 112) in eggs kept for 2–10 h at 17–18 °C, but in other batches of eggs up to about 150 of such bodies have been seen. However, at low temperature their number decreases markedly: the numbers were 35 ± 1-0 (n = 28), 20 ± 2-0 (n = 28), 19 ± 1-0 (n = 28), and 15 ± 1-0 (n = 28) in eggs incubated at 4 °C for 2, 4, 6 and 10 h, respectively. Many large nuclear bodies disintegrate after a prolonged incubation at low temperature, while numerous new small bodies appear around diffuse chromosomes (Figs. 10–12). Such small newly formed bodies swell up with continued incubation of eggs at low temperature (Fig. 12).

Nuclear bodies begin to accumulate during early oogenesis. A few such bodies are already present in small oocytes (Fig. 13); their number increases as oocytes become larger (Fig. 14). The large oocytes are at the diffuse diplotene stage which may correspond to the lampbrush stage of amphibian oocytes (Callan, 1966; Hay, 1968). Thus, there is a correlation between the increase in the number of nuclear bodies and the diffuse state of chromosomes. These oocytes are very active in RNA synthesis, especially at the site of the main nucleolus (Figs. 15, 16; see also Das & Alfert, 1966). A comparison of per cent cytoplasmic and nucleolar grains from autoradiographs of
oocytes of different sizes shows, as expected, that the cytoplasmic RNA originates from the nucleolus (Fig. 1); as the cytoplasmic label increases, there is a corresponding decrease in the nucleolar label. In fact, the curves for the cytoplasmic grains are mirror images of those for nucleolar grains. Recent studies by Gould (1969a) and by Davis & Wilt (1972) show that rRNA is primarily made in such oocytes of *Urechis*.

Electron-microscopic examination of both isolated nuclear bodies (Fig. 17) and those present in intact eggs shows that they are composed mainly of fibrillar components, similar to those seen at the core of the main nucleolus (Das et al. 1970). The isolated nuclear bodies, shown in Fig. 17, are still surrounded by nucleoplasmic materials. One of these bodies contains some materials similar in density to chromosomal materials. This was apparently still attached to a pair of chromosomes at the time of isolation.

Figs. 18–20 present the cytochemical staining characteristics of nuclear bodies.
Fig. 2. Densitometer tracing (thin line in Fig. 2A) of carrier RNA and radioactivity profiles of RNA from different fractions of unfertilized eggs which have been exposed to [3H]uridine for 24 h at 18 °C. Fractions 2, 3 and 4 (Fig. 2B) contain mainly large, medium, and small nuclear bodies, respectively. Various species of RNA were separated by acrylamide-agarose gel (3 mm in thickness) electrophoresis run at 200 V for 2 h in Tris-EDTA-borate buffer, pH 8.3. 2-mm gel slices were used for radioactivity determinations. In 2A: O---O, nuclei; ---, nucleoli. In 2B: C---C, fraction no. 2; ---, fraction no. 3; ---, fraction no. 4. Insets: autoradiographs of a heavily labelled nucleus with the main nucleolus (Fig. 2A) and some lightly labelled nuclear bodies (Fig. 2B).
Nuclear bodies give a positive reaction with acid fast green for proteins (Fig. 18A). These same nuclear bodies show very little staining with alkaline fast green, while the main nucleolus is well stained (Fig. 18B); thus, relative to the main nucleolus, the nuclear bodies contain very little histone(s) stainable with alkaline fast green. Some basic proteins other than histones may be present in these bodies, since they stain with bromphenol blue (Fig. 20). The nuclear bodies also give a positive reaction for silver-stainable nucleolar proteins (Figs. 13, 14; see also Das & Alfert, 1966). Compared to the main nucleolus, nuclear bodies contain very little RNA. Fig. 19A shows haematoxylin-stained nuclear bodies and nucleoli in a sectioned egg. When the same section is stained with azure B after removing haematoxylin in 45% acetic acid, practically no RNA staining can be seen in nuclear bodies (Fig. 19B).

A small amount of RNA is present in some of the nuclear bodies. This is evident from the autoradiographic localization of labelled RNA in them. However, unlike the main nucleoli, nuclear bodies never become rapidly labelled with labelled RNA pre-
Oocyte prenucleolar bodies

Only very few nuclear bodies from unfertilized eggs show some activity after an exposure of eggs to 100 µCi/ml of [3H]uridine for 4 h or longer (Figs. 21, 22) or to 100 µCi/ml of [3H]phenylalanine for 24 h (Figs. 23, 24). Such labelled bodies might have been formed from ribonucleoproteins synthesized elsewhere during the experimental period.

Figs. 2 A, B present densitometer tracing and radioactivity profiles of RNA extracted from whole nuclei, nucleoli alone, and from various fractions of nuclear bodies. Fractions 2, 3 and 4 contain large, medium and small nuclear bodies, respectively. In this experiment, eggs were labelled for 24 h with [3H]uridine. The autoradiographs of the isolated nucleus (Fig. 2A) and nuclear bodies (Fig. 2B) show that the nucleus and the main nucleolus are heavily labelled, while a few nuclear bodies are lightly labelled. As expected, the nucleolar and the whole nuclear fractions (Fig. 2A) contain predominantly the 38S rRNA precursor and its immediate derivatives, 30-28S and 23S RNAs (Das et al. 1970). In the 3 fractions of nuclear bodies (Fig. 2B) some labelled RNA around the 38S region is seen. The 30-28S, 23S, and other low molecular weight RNAs are also present in these fractions.

Labelled nucleolar RNA precursors, made in unfertilized eggs, are also retained in embryos in which new nucleoli are not yet formed. Figs. 3A, B show that unfertilized eggs, collected 25 and 29-5 h after labelling with [3H]uridine, contain rRNA precursors (38 and 23S) and mature rRNA (28 and 18S); the 30S rRNA precursors in these samples cannot be distinguished from the 28S rRNA. The labelled rRNA precursors persist in 4-5 h-old embryos (about 64-cell stage) produced from eggs which have been labelled with [3H]uridine for 25 h before fertilization. These embryos do not contain nucleoli and do not synthesize rRNA (our unpublished results; also see Gould, 1969b and Schwartz, 1970).

DISCUSSION

The growing oocytes of various organisms are highly active in rRNA synthesis (see Brown & Dawid, 1968; Gall, 1969; Maden, 1970). The size and, in some cases, the number of nucleoli increase during oogenesis; this is preceded by an amplification of ribosomal DNA (Brown & Dawid, 1968; Gall, 1969). While some amplification of ribosomal DNA occurs during oogenesis in Urechis, the extra copies are apparently maintained in the single nucleolus (Dawid & Brown, 1970). The numerous nuclear bodies present in immature oocytes and in unfertilized eggs are not similar to functional multiple secondary nucleoli of amphibian oocytes (Hay, 1968; Gall, 1969); nor are they similar to active micronucleoli seen in some organisms (Kalnins, Stich & Bencosme, 1964; Gabrusiewycz-Garcia & Kleinfeld, 1966). Nuclear bodies are mainly fibrillar in structure and they are inactive in RNA synthesis even in the highly active immature oocytes. These bodies contain very little RNA and, therefore, are not similar to nuclear structures reported to be involved in the transport of rRNA into cytoplasm (Allen & Cave, 1968) or to perichromatin granules described by Monneron & Bernhard (1969).

With respect to their origin, structure, some cytochemical staining behaviour, and their inability to synthesize RNA, these bodies resemble the prenucleolar bodies seen
at the end of mitosis (Das, 1962; Stevens, 1965; Lafontaine, 1968; Stevens & Prescott, 1971; Phillips, 1972), during early embryogenesis (Karasaki, 1965, 1968; Miller, 1966), and also in anucleolate cells of *Xenopus* (Hay, 1968; Hay & Gurdon, 1967) and corn (Swift & Stevens, 1966). The nuclear bodies accumulate during oogenesis in *Urechis*. The presence of such bodies in maturing oocytes of other organisms has also been reported by several workers (Sareen, 1967; Bier, Kunz & Ribbert, 1967; Hay, 1968; Bal, Jubiville & Cousineau, 1969; Mahowald & Tiefert, 1970). An aggregation of mainly proteinaceous materials around chromosomes may be involved in producing such bodies during oogenesis, especially in the diffuse diplotene stage. It is apparent from the present results that when chromosomes of unfertilized eggs become diffuse at low temperature, such bodies appear in contact with chromosomes and increase in number and size. It has been reported in earlier studies that prenucleolar materials are present around the decondensing telophase chromosomes (Tandler, 1959; Das, 1962; Das & Alfert, 1963; Stevens, 1965; Lafontaine, 1968). Nucleolar materials dispersed into the cytoplasm during mitosis may appear around chromosomes at the end of telophase (Das & Alfert, 1963). It is also possible that some nucleolar materials are used to form chromosomal 'matrix' during mitosis and that these materials are later used to form new nucleoli (McClintock, 1934; Heitz, 1931; see also Stevens, 1965; Phillips, 1972).

Similar to prenucleolar bodies, the nuclear bodies of *Urechis* show some cytochemical similarities with normal nucleoli. They give positive staining reactions with silver for nucleolar proteins (Das, 1962); both structures stain with bromphenol blue for some basic proteins (Bloch, 1966). Their response to low temperatures is also similar. At low temperature stickiness and nucleolar aggregation is known to occur (Ehrenberg, 1946). In *Urechis*, separation, swelling, and final disintegration of nucleolar components occur during prolonged incubation of eggs at low temperature (Das, Ramamurthy & Alfert, 1969). Nuclear bodies also fuse, swell, and then disintegrate at low temperature.

A small amount of labelled RNA, as well as proteins, has been detected in some of these bodies. The gel electrophoretic analyses indicate that such bodies may contain some rRNA precursors. The possibility exists that most of these rRNA precursors and other RNA species present in various fractions of nuclear bodies, might have come from contaminating chromosomes and nucleoplasm. Main nucleoli were absent in these fractions. In HeLa cells (Fan & Penman, 1971) and in salivary gland cells of *Chironomus* (Ringborg & Rynlander, 1971), an association of rRNA precursors with chromosomes has been observed. Such RNAs, with conjugated proteins, are incorporated and processed in new nucleoli (Fan & Penman, 1971). It may be that in *Urechis* oocytes and eggs some rRNA precursors leave the nucleolus, become associated with chromosomes, and later appear in prenucleolar bodies. The rRNA precursors, made in unfertilized eggs of *Urechis*, are maintained at least for several hours after fertilization when new nucleoli are not yet formed (see also Gould, 1969b). It is, however, not known whether or not this RNA remains associated with chromosomes and/or cytoplasm, or whether such rRNA precursors are finally processed in the new nucleoli formed during the gastrula stage (Gould, 1969b).
Oocyte prenucleolar bodies

The present results show that a large number of prenucleolar bodies, containing nucleolar proteins and perhaps also a small amount of rRNA precursors, accumulate during oogenesis in *Urechis*. Thus, prenucleolar materials are available for organization into multiple nucleoli during oogenesis. However, multiple functional nucleoli are not formed because the amplified rDNA seems to remain contained within the main nucleolus (Dawid & Brown, 1970). This situation is analogous to those in which prenucleolar bodies accumulate in somatic cells which lack, or contain inactivated, nucleolar organizers (Hay & Gurdon, 1967; Swift & Stevens, 1966; Stevens & Prescott, 1971; Phillips, 1972). The nucleus at the end of telophase, during oogenesis, and in early development appears to maintain a pool of mainly proteinaceous materials for the formation of new nucleoli. While their exact role is unknown, one might speculate that their interaction with the nucleolar organizer results in the initiation of rRNA synthesis. Puffing in certain fly-salivary chromosomes also involves an accumulation of protein prior to RNA synthesis (see Berendes, 1968).

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REFERENCES


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Oocyte prenucleolar bodies


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ABBREVIATIONS ON PLATES

chr chromosome  nb nuclear body

cyt cytoplasm  nu nucleolus

n nucleus

Figs. 4–7. Isolated nuclei, stained with Harris's haematoxylin, from immature oocytes (Fig. 4), embryos at the 8-cell stage (Fig. 5), and unfertilized eggs (Figs. 6, 7). All these contain nuclear bodies, some of which are associated with chromosome pairs. Figs. 4, 7, x 1000; Fig. 5, x 900; Fig. 6, x 200.

Figs. 8–12. Isolated nuclei, stained with Harris's haematoxylin, from unfertilized eggs which had been exposed to 4 °C for 2, 6, 12, 24 and 48 h, respectively. Note the association of many nuclear bodies with chromosomes and also an increase in the size of these bodies. Some nuclear bodies (pointed by arrows) become vacuolated with time and small new bodies are formed around diffuse chromosomes (Figs. 10–12). x 1000.
Oocyte prenucleolar bodies

4. Image 4
5. Image 5
6. Image 6
7. Image 7
8. Image 8
9. Image 9
10. Image 10
Figs. 11, 12. For caption see p. 792.
Figs. 13, 14. Paraffin sections (5 μm) of immature oocytes stained with silver to show an accumulation of nuclear bodies. × 1000.
Figs. 15, 16. Autoradiographs of sections (5 μm) of immature oocytes collected one day after injection of [3H]cytidine. The inset in Fig. 16 shows silver grains over the main nucleolus of this oocyte. Autoradiographs were exposed for 4 days. × 900.
Fig. 17. Electron micrograph of isolated nuclear bodies which are mainly fibrillar in structure. The arrow points to the presence of some materials in one of these bodies, similar in density to chromosomal materials. Nucleoplasmic materials are also present around these bodies. × 31,000.
Oocyte prenucleolar bodies
Figs. 18–20. Cytochemical staining characteristics of nuclear bodies.

Fig. 18a. A section of an unfertilized egg stained with acid fast green.

Fig. 18b. The same section stained with alkaline fast green after removal of acid fast green in water and 70% ethanol.

Fig. 19a. A section of an unfertilized egg stained with Harris's haematoxylin.

Fig. 19b. The same section stained with azure B after removing haematoxylin in 45% acetic acid.

Fig. 20. A section of an unfertilized egg stained with bromphenol blue. Note that the nuclear bodies are stained only with acid fast green, haematoxylin, and bromphenol blue. × 1000.

Figs. 21–24. Autoradiographs of isolated nuclei (Figs. 21, 23) and nuclear bodies (Figs. 22, 24) from unfertilized eggs exposed to $[^3H]uridine$ for 4 h (Figs. 21, 22) and to $[^3H]phenylalanine$ for 24 h (Figs. 23, 24). Insets in Figs. 21 and 23 show silver grains over the nucleolus of these nuclei. Nuclei and nucleoli are heavily labelled, while some nuclear bodies (Figs. 22, 24) are lightly labelled. Autoradiographs were exposed for 3 weeks (Figs. 21, 22) and 1 week (Figs. 23, 24). × 1200.