ABSENCE OF rDNA AMPLIFICATION IN THE UNINUCLEOLATE OOCYTE OF THE COCKROACH BLATTELLA GERMANICA (ORTHOPTERA: BLATTIDAE)

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ABSTRACT

Amplification of the genes coding for ribosomal RNA occurs in the oocytes of a wide variety of organisms. In oocytes of various species of crickets (Orthoptera: Gryllidae) the amplified DNA is contained in a large extrachromosomal DNA body. Multiple nucleoli form about the periphery of the DNA body during the diplotene stage of meiosis I. In contrast to the general pattern of orthopteran oocytes, oocytes of the cockroach Blattella germanica demonstrate a single large nucleolus instead of many nucleoli. In order to determine whether the genes coding for rRNA are amplified in the oocyte of B. germanica, the relative amount of rDNA in oocytes was compared with the rDNA content of spermatocytes and somatic cells. An extrachromosomal DNA body similar to that present in crickets is not present in B. germanica. A satellite DNA band which contains nucleotide sequences complementary to rRNA accounts for approximately 3–5% of the total DNA in somatic and in male and female gametogenic tissues. Female cells contain approximately twice as much rDNA as do male cells. An XX-XO sex-determining mechanism is operative in B. germanica. In situ hybridization with rRNA indicates that the nucleolar organizer is located on one end of the X chromosome and that oocytes do not contain more than twice the amount of rDNA found in spermatocytes. The data indicate that rDNA is not amplified in the uninucleolate oocyte of B. germanica.

Amplification of the DNA coding for ribosomal RNA (rDNA) has been described in the oocytes of various species of mollusks (4), echiuroid worms (9), arthropods (15, 22), and chordates (4, 14, 28). In the oocytes of many orthopteran insects, rDNA amplification appears to be correlated with the presence of a large, extrachromosomal DNA-containing body about which hundreds of nucleoli form during the diplotene stage of oogenesis (6, 7). In crickets (family: Gryllidae) such a DNA body has been identified in the pachytene-stage oocytes of representatives of the subfamilies Gryllinae, Nemobiinae, Brachytrupi-}

The Journal of Cell Biology • Volume 71, 1976 • pages 49-58

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tidae) differ from those of other orthopterans in that they are uninucleolate (30) rather than multinucleolate. The number of genes coding for rRNA was determined in somatic and gametic-genic tissues of the German cockroach Blatella germanica, in order to determine whether the genes coding for rRNA are amplified in this uninucleolate oocyte.

MATERIALS AND METHODS

Collection of Tissues

A colony of B. germanica was raised from five animals which were collected in Pulaski County, Arkansas. The colonies were reared in large glass tanks, the upper portions of which were coated with petroleum jelly to prevent escape. The animals were fed water, mouse pellets, apple, and potato. They received 14 h of light daily. The animals were dissected with the aid of a stereomicroscope. Gonads were collected from second to third instar nymphs which weighed less than 30 mg. Gonadal sex was determined at the time of dissection. The gonads were stored in 70% ethanol at -20°C. The femurs of several hundred adult animals, the sex of which had been identified prior to dissection, were frozen and stored at dry-ice temperature.

Preparation of DNA

Testes and ovaries (approximately 0.5 ml packed volume) were washed with several changes of 2× SSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). They were extracted with 100 μg/ml beta-amylase (Sigma Chemical Co., St. Louis, Mo.) for 1 h at 37°C, and subsequently with 100 μg/ml ribonuclease A ( Worthington Biochemical Corp., Freehold, N. J.) for 2 h at 37°C. They were then washed with 2× SSC and suspended in 1 ml of lysing solution (0.05 M Tris; 0.05 M EDTA; 0.5% Sarkosyl NL-97; Geigy Chemical Corp., Ardsley, N. Y.). They remained in the lysing solution for 1 h at 37°C, and then were homogenized in a motor-driven glass Teflon homogenizer. The homogenate was centrifuged for 20 min at 1,500 g. The supernate was mixed with CsCl and brought to a density of 1.7 and a total vol of 5 ml. DNA was further purified by banding in gradients of CsCl. The DNA was centrifuged at 32,000 rpm, 20°C, for 60 h in the Spinco SW-50.1 rotor (Beckman Instruments, Palo Alto, Calif.).

DNA was extracted from frozen femurs by a modification of the Pronase procedure. Tissues were homogenized in 5 ml of 0.1 M Tris, 0.05 M EDTA, and 0.5% sodium dodecyl sulfate. The homogenate was made 1 mg/ml Pronase (Calbiochem, San Diego, Calif.) and digested for 3 h at 37°C. The digest was phenol-extracted twice at 37°C for 30 min. Nucleate was precipitated from the aqueous phase by making it 0.2 M sodium acetate and 70% ethanol. After several ethanol washes, the precipitate was suspended in 2× SSC and extracted for 1 h each with 200 μg/ml beta-amylase, 100 μg/ml RNase-A, and 50 μg/ml Pronase. After two subsequent phenol extractions, DNA was precipitated with ethanol and wound onto a glass rod. The DNA was mixed with CsCl which was brought to a density of 1.70 and a vol of 5 ml. It was banded in CsCl as described above.

Preparation of Labeled RNA

Previous studies have demonstrated extensive homologies between the rRNA's of a wide variety of organisms (26). The rRNA of the South African clawed toad Xenopus laevis has been utilized extensively in such hybridization experiments. Labeled 18S and 28S rRNA were prepared by growing a cell line of X. laevis kidney cells (line CCL 102 A, obtained from the American Type Culture Collection, Rockville, Md.) in modified Lebowitz L-15 medium with 15% fetal calf serum. Cells were grown for 3-5 days in medium containing 25 μCi/ml [5-3H]uridine (sp act 29.2 Ci/mmol, New England Nuclear Corp., Boston, Mass.) and then for an additional 24 h in two changes of fresh medium containing unlabeled uridine. Subsequently, RNA was extracted from the cells by a modification of the phenol procedure and 18S and 28S RNA were isolated as described elsewhere (6). Ribosomal RNA was reconstituted by combining one part 18S RNA with two parts 28S RNA (wt/wt).

Cytological Procedures

Ovaries were fixed for light microscopy in three parts ethanol:one part acetic acid. They were embedded in paraffin for sectioning or immersed in 45% acetic acid for squashing. Sections and squashes were stained with Azure B according to the procedure of Gabruszewycz-Garcia and Kleinfeld (13), according to the Feulgen procedure (12), and with Giemsa stain (16).

For electron microscopy, ovaries were fixed for 1 h at 4°C in freshly prepared 5.0% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Subsequently, the ovaries were washed overnight in several changes of 0.1 M buffer and then postfixed for 1 h at 4°C in 1% osmium tetroxide in 0.1 M phosphate buffer at pH 7.4. After dehydration, the ovaries were embedded in Epon-Araldite and sectioned with a diamond knife on a Porter-Blum MT-2 ultramicrotome. The sections were mounted on naked grids, stained with uranyl acetate and lead citrate, and viewed in a Siemens 101A electron microscope.

Nucleic Acid Hybridization

RNA-DNA hybridization on Millipore HAWP filters (Millipore Corp., Bedford, Mass.) was carried out as described previously (6).

RNA-DNA hybridization in situ was carried out according to a modification of the technique of Gall and Pardue (16). Treatment of the slides with 0.2 N HCl was omitted. Autoradiographs were prepared with full-
strength Kodak NTB2 emulsion (Eastman Kodak Co., Rochester, N. Y.). The autoradiographs of the hybrid structures were stained with Giemsa stain. In some cases, the hybridization was carried out on sectioned ovaries. The DNA in sections was denatured by placing the sections in 95% formamide in 0.1 x SSC for 2 h at 70°C.

RESULTS
In early diplotene-stage oocytes (Fig. 1), the nucleolus is seen as a single mass of material which stains blue-violet with Azure B (RNA). The nucleolus remains singular long after vitellogenesis has begun (Fig. 2). No Feulgen-positive material can be detected within it. Phase-contrast microscopy of whole, living oocytes confirms that there is but a single nucleolus in each germinal vesicle (Fig. 3). The nucleolus is visualized by transmission electron microscopy as an anastomosing network of ribbons or plates connecting larger masses of nucleolar material (Fig. 4). These larger masses of nucleolar material (Fig. 4) appear to have a fibrillar core and granular cortex. Areas that are primarily fibrillar and others that are granular are also associated with the ribbons (Fig. 5).

In neutral CsCl, the bulk DNA of B. germanica (main band DNA) has a density of 1.698 g/cm³ (39% G+C), and DNA hybridizing with rRNA (satellite DNA) has a density of 1.714 g/cm³ (55% G+C) (Fig. 6).

A satellite DNA of the same buoyant density as the DNA that hybridizes with rRNA ($\rho = 1.714$) is visualized by isopycnic banding in neutral CsCl in the analytical ultracentrifuge (Fig. 7). In gradients that were overloaded with DNA (0.5 OD$_{260}$ U/gradient), the satellite is seen to account for 3–5% of the total DNA. No consistent differences were noted between male and female tissues or between somatic and gametogenic tissues in the relative proportions of satellite and main band DNA.

Approximately 0.02% of the DNA extracted from male tissues (testis and leg muscle) and 0.035% of the total DNA from female tissues (ovary and leg muscle) hybridize with labeled Xenopus laevis 18S and 28S RNA (Fig. 8). The results suggest that female cells have approximately twice as much rDNA as do male cells. An XX-XO sex-determining mechanism is operative in this species. If the genes coding for rRNA are localized on the X chromosomes, female cells with two X chromosomes would be expected to have approximately twice as many genes coding for rRNA as male cells. Chromosome counts made on the nuclei of spermatocytes confirm that $n = 11$ (2$n = 22$ female, 2$n = 21$ male [29]) (Fig. 9).

A Feulgen-positive extrachromosomal DNA body similar to that found in oocytes of gryllid crickets is not observed in either oocytes or spermatocytes of B. germanica. A small heteropycnotic knob is localized on the end of the presumptive X chromosome of spermatocytes (Fig. 11) and on its bivalent counterpart in oocytes (Fig. 10). The nucleolus persists well into the pachytene stage of meiosis in spermatocytes (Fig. 13). In autoradiographs of pachytene-stage spermatocytes hybridized with rRNA, most of the silver grains are localized over the nucleolus which is associated with the heteropycnotic knob on the presumptive X chromosome (Fig. 12).

Ovary DNA contains DNA derived from somatic cells as well as from oocytes. Perhaps the somatic cell DNA dilutes the oocyte DNA to such an extent that amplification cannot be detected in DNA extracted from whole ovaries. In order to determine whether rRNA is amplified in oocytes, autoradiographs of oocytes hybridized with rRNA were compared with autoradiographs of spermatocytes hybridized with rRNA. Since amplification of rDNA has been detected in premeiotic germ line cells in males of other species (23), it is necessary to determine whether the amount of rDNA increases during spermatogenesis. Grain counts made over nuclei of interphase somatic cells in the testis were compared with grain counts made over the chromosomes of pachytene-stage spermatocytes (Figs. 14 and 15; Table I). The data indicate that somatic cells and spermatocytes contain the same amount of rDNA (i.e., there is no amplification of rDNA during spermatogenesis). Next, comparisons were made between oocytes and spermatocytes which had been hybridized with labeled rRNA in situ (Figs. 16 and 17; Table I). Oocytes demonstrate less than two times the number of silver grains found over the nucleolar organizer regions of spermatocytes (i.e., there is no amplification of rDNA during oogenesis).

Finally, in sections of ovaries hybridized with X. laevis rRNA in situ, virtually no label was visualized over the nucleoli of oocytes. Labeling of oocyte nucleoli was the same as or less than that of the remainder of the nucleus, and less than that over the nucleolar area of interphase follicle cells.

DISCUSSION
The uninucleolate condition is common in the oocytes of invertebrates and in some vertebrates.
including the mammals. Multiple nucleoli are common in oocytes of fish, amphibians, birds, reptiles, and some arthropods (30). Exceptions to these general rules are common. The cockroaches...
FiguRe 8. Filters containing DNA extracted from ovaries (△) and testis (▲) of animals weighing less than 30 mg, and femur muscle of male (●) and female (○) adult animals were hybridized with 3H-labeled X. laevis rRNA (sp act 285,000 cpm/μg). Filters were hybridized for 18 h at 68°C in solutions containing 4X SSC and 0.4% SDS. The amount of DNA on each filter was determined after hybridization by hydrolyzing the filters in 5% perchloric acid for 30 min at 70°C and measuring the A_{260} of the hydrolysate.

TABLE I
Grain Counts Made From Autoradiographs of B. germanica Testes and Ovaries Hybridized with 3H-Labeled rRNA*

<table>
<thead>
<tr>
<th></th>
<th>Mean no. silver grains (±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>Meiotic prophase cells</td>
<td></td>
</tr>
<tr>
<td>Grains over chromosomal nucleolar organizer</td>
<td>8.80 (±0.36)</td>
</tr>
<tr>
<td>Grains over chromosomes (exclusive of nucleolar organizer)</td>
<td>6.57 (±0.54)</td>
</tr>
<tr>
<td>Interphase somatic cells</td>
<td></td>
</tr>
<tr>
<td>Grains over nucleolus and perinucleolar chromatin</td>
<td>9.38 (±0.31)</td>
</tr>
</tbody>
</table>

* Grain count comparisons were made on slides which were exposed for 120 days and developed under identical conditions.

represent such an exception. They belong to an order of insects, the orthopterans, most representatives of which demonstrate multinucleate oocytes (30). Bier, Kunz, and Ribbert (2) have interpreted the nucleolar apparatus of B. germanica oocytes as being multinucleolate on the basis of its ultrastructural organization. In the present investigation, small masses of nucleolar material were occasionally visualized separate from the nucleolus. These probably represent material which is being transported to the cytoplasm rather than metabolically active nucleoli.

In Drosophila melanogaster the nucleolus organizer is localized on the X and on the Y chromosomes (25). Male D. melanogaster which are lacking a Y chromosome (X/O males) and females in which one of the X chromosomes has a deletion of the nucleolus organizer (X/X_{NO-} females) would be expected to have about 50% of the rDNA of their X/X mothers. Instead, they contain about 80% the amount of rDNA found in X/X females (27). This increase in rDNA which is somatic in nature (i.e., it is not inherited) is referred to as rDNA compensation. The percent DNA hybridizing with rRNA in male somatic and gametogenic tissues of B. germanica is 60% that of female somatic and gametogenic tissues. rDNA compensation does not appear to occur in B. germanica where the X/O condition is normal for males.

A large extrachromosomal DNA body is present in oocytes representing five subfamilies of crickets (Orthoptera: Gryllidae). RNA-DNA hybridization analysis indicates that the oocytes of
the house cricket *Acheta domestica* contain about 100 times the amount of rDNA found in somatic cells. *In situ* hybridization localizes the amplified copies of rDNA in the oocytes (6). As the cells proceed through diplotene, DNA within the body becomes incorporated into the multiple nucleoli which form at the periphery of the DNA body. In one exceptional species of Gryllidae, the mole cricket *Scapteriscus alectis*, the diplotene-stage oocyte demonstrates a single nucleolus. A large extrachromosomal DNA body is visualized during pachytene. *In situ* hybridization...
FIGURES 12–17  Light micrograph of an Azure B-stained spermatocyte (Fig. 13), and autoradiographs of an interphase male somatic cell (Fig. 14), pachytene-stage spermatocytes (Figs. 12, 15, and 17), and a pachytene-stage oocyte (Fig. 16) which were hybridized with $^3$H-labeled rRNA in situ. Unstained squash preparations of *B. germanica* testis and ovary were hybridized in situ with 1 $\mu$g/ml $^3$H-labeled *X. laevis* 18S and 28S RNA (sp act 950,000 cpm/$\mu$g) in 2x SSC for 18 h at 68°C. The autoradiographs were exposed for 60 days (Fig. 12), 100 days (Figs. 16 and 17), and 120 days (Figs. 14 and 15). The nucleolus persists in many pachytene-stage spermatocytes, staining green with Azure B, and being attached to the presumptive sex chromosome (Fig. 13). In pachytene-stage autoradiographs hybridized with rRNA, most of the silver grains are localized over the heteropycnotic mass which is embedded in the nucleolus (i.e., the nucleolus organizer) (Fig. 12). Approximately the same number of silver grains are found over the nucleolus organizer of pachytene-stage spermatocytes (Fig. 15) as are concentrated over the limited nucleolar area of the interphase somatic cell nucleus (Fig. 14). Approximately the same number of silver grains are localized over the nucleolar organizer regions of pachytene-stage oocytes (Fig. 16) and spermatocytes (Fig. 17). Fig. 12, × 2,170; Fig. 13, × 2,380; Figs. 14-17, × 2,850.
with rRNA demonstrates the presence of amplified rDNA in the DNA body as well as within the oocyte nucleolus (8).

Multinucleolate oocytes amplify rDNA hundreds of times. Amplification occurs at a very low level in uninucleolate oocytes (4, 9) or not at all (28, 21). Thus amplification of rDNA was readily detected in the multinucleolate oocyte of the striped bass, *Roccus saxatilis*, but not in the uninucleolate oocyte of the starfish *Asterias forbesii* (28).

Brown and Dawid (4) and Dawid and Brown (9) have observed approximately a five- to sixfold amplification of rDNA in the uninucleolate oocytes of the echiuroid worm, *Urechis caupo*, and in the surf clam, *Spisula solidissima*. The singular nucleolus in oocytes of the sea urchin *Strongylocentrotus purpuratus* binds [*3H*]actinomycin D to a greater extent than the nucleoli of oogonia, indicating that a limited amount of rDNA amplification may occur during sea urchin oogenesis (11).

More recently, studies on oocytes of the coot clam, *Mulinia lateralis*, indicate that there is no greater than twice the somatic cell amount of rDNA in an oocyte (21). Studies by Vincent on the uninucleolate oocytes of several other species of marine organisms indicate that amplification does not occur (W. Vincent, personal communication).

In some organisms, ribosomes synthesized during oogenesis provide the protein-synthetic machinery of the embryo during the early stages of embryogenesis. When meiosis is completed in *X. laevis*, RNA synthesis ceases and is not initiated again until the onset of gastrulation (5). Synthesis of rRNA does not occur until the late blastemal stage (27 h after fertilization) during early embryogenesis in *A. domesticus* (17). A single *X. laevis* oocyte accumulates an amount of rRNA which is equivalent to the amount of rRNA in liver tissue containing about 200,000 cells (3). Assuming that a *X. laevis* oocyte contains the normal somatic rDNA complement and synthesizes rRNA at a rate equivalent to a rapidly growing cell in culture, it would take some 400 years to synthesize the amount of rRNA found in an oocyte (24). By increasing the number of genes coding for rRNA 1,000 times, this interval becomes 4–5 mo. Thus, amplification of rDNA is extremely advantageous to organisms in which the onset of rDNA synthesis is delayed until late in development.

In some organisms with uninucleolate oocytes, rRNA synthesis begins as early as the four-cell stage (10, 19, 20, 31). The early onset of rRNA synthesis does not appear to be correlated with the size of the egg since early rRNA synthesis has been detected in the relatively large eggs of the sea urchin *Strongylocentrotus purpuratus* (10) and the parasitic nematode *Ascaris lumbricoides* (19), as well as in the small alecithal egg of the mouse (31).

Amplification of rDNA enables the oocyte to synthesize large numbers of ribosomes which provide the protein synthetic machinery of early embryogenesis. Multiple nucleoli and extrachromosomal DNA bodies appear to be expressions of this synthetic activity. In the absence of rDNA amplification the nucleolus remains singular, the demands for ribosomes during early embryogenesis being met by the developing embryonic cells. Thus multiple nucleoli in the oocyte nucleus would appear to be representative of transcription of amplified rDNA.

The author gratefully acknowledges the kindness and assistance of the following people: Ms. Andrea Lippe who provided some of the animals from which our colony arose; Doctors G. Dalrymple and J. Moss, Department of Radiology, Little Rock Veterans Administration Hospital, who allowed me to use their analytical ultracentrifuge; Mr. David Sharp who, with the cooperation of Dr. J. K. Sherman, provided the electron microscope sections; and Mr. Larry Hulse who provided excellent technical assistance throughout the course of this investigation.

The investigation was supported by the U. S. Public Health Service grant no. 5R01-GM-21446.

Received for publication 12 April 1976, and in revised form 1 June 1976.

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