EVOLUTION OF THE NUCLEOLI DURING OOGENESIS IN XENOPUS LAEVIS STUDIED BY ELECTRON MICROSCOPY

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SUMMARY

Xenopus laevis tadpoles and toads were killed at several ages. The structure of the nuclei of the germinal cells has been observed by light and electron microscopes. We distinguish 11 successive stages in nucleolar structure: (1) a single, essentially granular nucleolus in the oogonium (10 μm diameter), (2) a reticulated nucleolus in the leptotene oocyte, (3) fragmentation of this nucleolus into a few smaller nucleoli, (4) multiple tiny nucleoli appearing in the cap of the pachytene oocyte, (5) enrichment in the fibrillar constituent of these intra-cap nucleoli, (6) grouped spherical nucleoli, with well segregated granular and fibrillar constituents, as the disintegration of the cap is going on (diplotene A oocyte, 30 μm diameter), (7) dispersion of those nucleoli in the nuclear sap (diplotene B oocyte, 50 μm diameter), (8) formation of long, ribbed nucleoli with multiple DNA-rich spots (diplotene C oocyte, 100 μm diameter), (9) fragmentation of the nucleolar ribbons into multiple spherical nucleoli with eccentric fibrillar core and granular cortex (diplotene D oocyte, 150 μm diameter), (10) multiple purely fibrillar nucleoli (diplotene E oocyte, between 150 and 400 μm diameter), and (11) multiple classical nucleoli with concentric fibrillar core and granular cortex (diplotene F oocyte, between 400 and 1000 μm diameter).

The multiplication of the nucleoli in Xenopus laevis may occur successively (a) by the fragmentation of the single oogonium nucleolus at the leptotene stage, (b) by de novo formation of nucleolar bodies inside the cap at the pachytene stage, and (c) by the growth of those nucleoli lying free in the nuclear sap at the early diplotene stage. They evolve into nucleolar ribbons which later on fragment into spherical bodies.

Four successive phases during the growth of an oocyte can be distinguished with respect to the ribosomal system. (I) The first phase is characterized by the nucleolar DNA amplification. (II) During the second phase, the multiplication of the nucleoli is going on. Ribosomes are present in the cytoplasm and the rate of cellular growth is very high. (III) During the third phase, the synthesis of rRNAs seems to be repressed while the synthesis of heterogenous small RNAs is going on. Ribosomes are no longer visible in the cytoplasm. The nucleoli are purely fibrillar. The rate of cell growth is lower than in the preceding phase. (IV) During the fourth (= Duryee lampbrush stages 3—6), or vitellogenic phase, rRNAs are actively synthesized and numerous ribosomes appear in the cytoplasm. The nucleoli have the classical structure and the rate of growth is about the same as during phase III.

INTRODUCTION

The nucleus of a ripe Xenopus laevis oocyte contains from 1400 to 1600 nucleoli (Perkowska, Macgregor & Birnstiel, 1968). Each nucleolus shows a granular cortex and a fibrillar core (Miller, 1962; Wartenberg, 1963) associated with DNA (Miller, 1966; Ebstein, 1967; Van Gansen & Schram, 1968). Ribosomal RNA, which accounts for more than 90% of the total RNA of the ripe oocyte (Davidson, Allfrey & Mirsky,
1964) is synthesized in the nucleoli (Izawa, Allfrey & Mirsky, 1963; Edström & Gall, 1963; Davidson & Mirsky, 1965; Macgregor, 1967). Nucleolar DNA consists mainly of ribosomal DNA (Evans & Birnstiel, 1968) although its density differs somewhat from the rDNA of the somatic DNA (Dawid, Brown & Reeder, 1970). Thus the isolated nucleolar DNA could provide the first portraits of genes – ribosomal cistrons – in full activity (Miller & Beatty, 1969a, b; Miller, Beatty, Hamkalo & Thomas, 1970).

The nucleolar rDNA of the *Xenopus* oocyte nucleus is some 1400- to 2000-fold more abundant than the rDNA of a somatic nucleus (Evans & Birnstiel, 1968). We know from Gall (1968) that the amplification of the ribosomal cistrons occurs very early during oogenesis, at the pachytene stage of meiosis. The excess DNA, identified in vitro as rDNA by molecular hybridization experiments, is localized in a dense Feulgen-positive 'cap' which appears in the pachytene oocyte (Gall, 1968), incorporates thymidine (Ficq, 1968; Macgregor, 1968; Gall, 1969) and hybridizes, on cytological slides, with labelled rRNA (Gall & Pardue, 1969).

The oogonium has a single large nucleolus (Gall, 1969). In the young diplotene cell the cap, surrounding several voluminous nucleoli grouped together, is progressively dispersed into the nuclear sap, as can be seen with the light microscope (Ficq, 1970).

The aim of the present work is to describe, at an ultrastructural level, the formation and evolution of the nucleoli from the oogonia up to the young diplotene oocytes. The succeeding previtellogenic stages have been studied by our colleague, Dr C. Thomas (1967, 1970, 1971).

**MATERIAL AND METHODS**

**Material**

*Xenopus laevis* tadpoles and young toads were killed at different ages before and after metamorphosis, defined as being the time when the tail regression is complete (= + 0 days). Thus, we fixed the ovaries of metamorphosing animals: -8, -3, +1, +5, +8, +10 and +15 days and +1 and +2 months. The *Xenopus* were previously anaesthetized by a brief immersion in a 0.1% MS 222 (Sandoz) solution and dissected in cold TC 199 Difco medium.

**Electron microscopy**

The small ovaries were fixed in 3% glutaraldehyde in cacodylate buffer at pH 7.4 for 2 h at 4°C, washed overnight in cold cacodylate buffer and postfixed in OsO₄, 1% in Millonig buffer at pH 7.4 for 1 h at 4°C. The specimens were dehydrated in ethanol and embedded in Araldite. The ultrathin sections were successively stained by uranyl acetate and by lead citrate.

For autoradiography after ³H-actinomycin treatment of the fixed material we followed the technique described by Steinert & Van Gansen (1971). The ovaries were fixed for 30 min at 4°C either in 3% glutaraldehyde in Sorensen buffer at pH 7.4, occasionally postfixed in 1% OsO₄ in Millonig buffer, or in a methanol-acetic acid mixture (6:4 by vol) or in freshly distilled acrolein 3% in Sorensen buffer. The fixed specimens were incubated in the dark for 1 h, at room temperature, in a 10 µg/ml (about 100 µCi/ml) solution of ³H-actinomycin (Schwarz; specific activity: 8.4 Ci/mmol). The photographic emulsion used was Ilford L4. The autoradiograms were developed after being exposed for 3, 7, 8 and 9 months. Observations were made with an AEI EM 6B electron microscope.
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Light microscopy

Some ovaries were fixed in a Zenker solution and paraffin embedded. The sections were stained by the Unna mixture and the Feulgen method. Control slides were digested by RNase and DNase. The sensitivity of the Feulgen method was greatly enhanced by the use of a fluorescent microphotometer (Leitz).

Observations

Oogonia

The very small ovaries of the tadpoles killed a few days before complete metamorphosis contain numerous oogonia, leptotene oocytes and even a few pachytene (cap) oocytes. Exceptionally, a young diplotene may be seen. The oogonia are small cells, about 10 μm in diameter, with a very conspicuous single nucleolus, about 2 μm diameter, the nucleus itself being very voluminous (Figs. 3, 4). The nucleolus is almost entirely granular, only small fibrillar patches being present (Fig. 5). After 3H-actinomycin treatment (Fig. 6) it becomes intensely labelled. It is Feulgen-negative at the light-microscope level. The cytoplasm (Fig. 3) contains numerous ribosomes as well as β-glycogen particles (controls were made by the Thiery (1967) technique), centrioles, microtubules and dictyosomes.

Leptotene oocytes

The condensing chromosomes become clearly apparent (Fig. 7). The nucleolus seems to undergo a kind of reticulation as a material of low density segregates from the granular constituent (Fig. 8). The leptotene nucleolus, Feulgen-negative, is labelled after 3H-actinomycin treatment. Some oocytes, belonging to nests of typical leptotenes, contain 2–4 nucleoli (Figs. 9, 10).

Zygotene-pachytene oocytes

Pachytene oocytes are particularly numerous in young toads between 5 and 10 days after complete metamorphosis. After Unna-staining, the green chromosomes can clearly be distinguished from the purple-red cap as described by Ficq (1970). Inside the cap, small red bodies are visible: they lose their stain after RNase treatment (Fig. 12). Ultrastructural examination reveals the paired chromosomes and the cap, surrounding nucleolar bodies (Fig. 13). Tangential sections of the cap show clearly that numerous nucleolar bodies are present (Fig. 11), in which dense fibrillar patches occur between the granular constituent (Fig. 14). Tritiated actinomycin binds both to the chromosomes and to the cap (Fig. 15). Occasionally, caps are seen which seem to be reticulated and contain nucleolar bodies having an important fibrillar core surrounded by granular blebs (Fig. 16).

At the pachytene stage the cytoplasm still resembles that of the oogonia in possessing numerous ribosomes.
Diplotene oocytes

In somewhat larger and rounder oocytes the thick bivalents are no longer seen and the cap seems to disintegrate into the nuclear sap (Fig. 17). The structure of the still-grouped nucleolar bodies is clearly the same as in the reticulated cap (Fig. 18). This stage seems to mark the beginning of cytoplasmic growth: we will call it 'diplotene A'. When no trace of the cap is left, the nucleoli are seen to be dispersed in the nuclear sap. In such oocytes, about 50 μm in diameter, stained by the Unna technique, the nucleoli have a very typical bipartite structure (Fig. 20) which corresponds, as seen with the electron microscope (Fig. 19), to a fairly good segregation of the granular and fibrillar constituents (Fig. 21). At this stage the nucleoli failed to give a positive Feulgen reaction (the chromosomes dispersed in the nuclear sap are well stained). The binding of 3H-actinomycin to the nucleoli is irregular and weak even after methanol-acetic fixation where the morphological preservation is very poor but the labelling on the chromosomes very high: these cells will be referred to as 'diplotene B'.

In oocytes about 100 μm in diameter, which are very common in the ovaries of toads killed 1 month after complete metamorphosis, the nucleoli take on a very remarkable ribboned appearance. After Feulgen staining they display numerous small positive dots (Fig. 23). Their colour and fluorescence disappear after DNase treatment. Seen with the electron microscope (Fig. 22) the ribboned nucleoli appear to be formed of alternate areas of fibrillar and granular constituents (Fig. 24). They characterize what we will call 'diplotene C' oocytes. In the largest oocytes of the same ovaries the ribboned nucleoli are no longer seen but numerous spherical nucleoli are dispersed in the nuclear sap most frequently at the periphery of the nucleus (Figs. 25, 26). Each nucleolus displays the double granular-fibrillar constitution where the granular constituent has a typical excentric position (Fig. 27). In those 'diplotene D' oocytes, the cytoplasm contains ribosomes mixed with a fine fibrillar material, very similar to the one discovered and described by Thomas (1967, 1970) in somewhat older oocytes where the cytoplasmic accumulation of small RNAs is going on. Glycogen particles are no longer detectable. The latter, 'diplotene E' oocytes, which have diameters ranging from 150 to 400 μm, are very numerous in ovaries of Xenopus killed about 3 months after metamorphosis: their nucleoli are purely fibrillar and their cytoplasm contains no ribosomes but is largely filled with a fibrillar ribonucleoprotein material (Thomas, 1967, 1971). In the vitellogenic oocytes (with diameters of 400–1000 μm), or 'diplotene F' oocytes, the numerous large nucleoli (about 10 μm in diameter) show the classical constitution: a central fibrillar core and a concentric granular cortex. Their cytoplasm is progressively enriched with typical ribosomes (Thomas, 1967, 1971).

DISCUSSION

A tentative interpretation of nuclear evolution throughout Xenopus oogenesis is shown in Fig. 1. The single essentially granular nucleolus of the oogonium (Fig. 1, 1) becomes reticulated at the leptotene stage (2) and is fragmented into smaller bodies (3).
The amplified nucleolar DNA appears in the immediate vicinity of those nucleoli at the pachytene stage. As the DNA-cap becomes larger and larger, numerous bodies become apparent (4) inside it. Each of them has a granular and a fibrillar constituent, the latter becoming progressively more and more important (5). After the disintegration of the cap (6, diplotene A), which corresponds to the beginning of the oocyte growth, the nucleoli present well segregated constituents (7, diplotene B). They undergo an extensive transformation to form long ribboned nucleoli (8, diplotene C) containing numerous DNA-rich spots. The ribboned nucleoli fragment into spherical bodies where the fibrillar core has a typical excentric position (9, diplotene D). Later on, when the accumulation of small RNAs in the cytoplasm becomes obvious, the nucleoli lose their granular constituents, becoming purely fibrillar (10, diplotene E). These fibrillar nucleoli were already described by Wartenberg (1963) in *Xenopus* previtellogenic oocytes. Finally, when the yolk appears in the cytoplasm together with the ribosomes, the numerous nucleoli, each of which is as large as the entire...
initial oogonium, present their classical concentric core and cortex constitution (II, diplotene F). If our interpretation is correct, the multiplication of the nucleoli would occur successively: (a) by the fragmentation of the single oogonium nucleolus, (b) by de novo formation of nucleolar bodies inside the cap and (c) by the growth of those nucleoli lying free in the nuclear sap and evolving into nucleolar ribbons which later on fragment into spherical bodies. This interpretation can be tested by high-resolution autoradiography which is now being carried out in our laboratory. Favard-Sereno (1968), observing the evolution of the nucleoli throughout oogenesis in the cricket, comes to a rather similar conclusion: first that the nucleoli multiply from 1 or 2 to 6 or 8, then each of these nucleoli undergoes a considerable growth, and finally fragments into numerous nucleolar bodies dispersed through the nuclear sap.
Moreover, regarding the ribosomal system, we can distinguish 4 successive phases during the growth of an oocyte. Fig. 2 shows an approximative growth curve of a single oocyte obtained by measuring the most common type of oocyte observed at each studied *Xenopus* stage. The first phase is characterized by the nucleolar DNA amplification; ribosomes are present in the cytoplasm; there is no (or very slight) cellular growth. During the second phase, the multiplication of the nucleoli occurs; ribosomes are still present in the cytoplasm; cell growth is very important: the cell increases more than 300-fold in volume in less than 1 month. Surprisingly, during the third phase the synthesis of rRNAs seems to be repressed because the nucleoli are purely fibrillar, true ribosomes are no longer visible in the cytoplasm and the synthesis of heterogenous small RNAs is going on in these previtellogenic oocytes (Thomas, 1967, 1970, 1971; Ford, 1970; Mairy & Denis, 1970, 1971). Cellular growth is slower than in the preceding phase whereas cell volume increases about 20-fold over a period of at least 2 months. The fourth phase corresponds to the Duryee (1950) lampbrush stages 3–6 as defined for *Xenopus* by Davidson et al. (1964). Ribosomal RNAs are actively synthesized (Davidson et al. 1964; Brown & Littna, 1964; Brown, 1966; Gall, 1966) and true ribosomes accumulate in the cytoplasm of the vitellogenic oocytes (Thomas, 1967). The cellular growth rate is about the same as in the preceding phase.

REFERENCES


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Fig. 3. *Xenopus laevis* oogonium. Glutaraldehyde (cacodylate)–osmium fixation. *g0*, glycogen particles; *nu*, single nucleolus; *pf*, prefollicular cell; *r*, ribosomes. × 30 000.

Fig. 4. *Xenopus laevis* oogonium. Zenker fixation, Unna staining. × 1000.
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Fig. 5. Single nucleolus of *Xenopus oogonium*. Glutaraldehyde (cacodylate)–osmium fixation. f, fibrillar constituent; g, granular constituent. ×50,000.

Fig. 6. Single nucleolus of *Xenopus oogonium*. Acrolein (Sørensen) fixation, ³H-actinomycin treated, 3 months exposure. ×30,000.
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Fig. 7. *Xenopus laevis* leptotene oocyte. Glutaraldehyde (cacodylate)-osmium fixation. c, centriole; ch, condensing chromosomes; d, dictyosome; nu, single reticulated nucleolus; pf, prefollicular cell. × 15000.
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Fig. 8. Single nucleolus of *Xenopus* leptotene oocyte. Glutaraldehyde (cacodylate)–osmium fixation. *f*, fibrillar constituent; *g*, granular constituent. × 50,000.

Fig. 9. Constricted nucleolus of *Xenopus* leptotene oocyte, which may be a transitional figure to nucleolar fragmentation. Glutaraldehyde (Sörensen)–osmium fixation, ³H-actinomycin-treated, 8 months exposure. *ch*, chromatin; *nu*, nucleolus. × 15,000.

Fig. 10. Two reticulated nucleoli (*nu*) are seen in this section of a probably late leptotene oocyte. Glutaraldehyde (Sörensen)–osmium fixation, ³H-actinomycin treated, 7 months exposure. × 15,000.
Fig. 11. Pachytene oocyte. Glutaraldehyde (cacodylate)–osmium fixation. Tangential section of the cap (cp) containing numerous nucleolar bodies (nu). × 15,000.

Fig. 12. Pachytene oocyte. Zenker fixation, Unna staining. ch, chromosomes; nu, nucleolus. × 1000.
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Fig. 13. Pachytene oocyte. Acrolein fixation. ch, chromosomes; cp, cap; nu, nucleolus. × 15000.

Fig. 14. Nucleolar body inside the cap of a pachytene oocyte. Glutaraldehyde (caco-
dylate)-osmium fixation. cp, cap; f, fibrillar constituent of nucleolus; g, granular
constituent of nucleolus. × 50000.
Fig. 15. Pachytene oocyte. Glutaraldehyde (Sørensen)-osmium fixation, $^3$H-actinomycin treated, 7 months exposure. $cp$, cap; $nu$, nucleolus. $\times 10000$.

Fig. 16. Late pachytene oocyte. Acrolein fixation. Reticulated cap ($cp$) containing several nucleoli where both the fibrillar ($f$) and the granular ($g$) constituents are clearly seen. $\times 15000$. 

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[Image of cellular structures labeled with 'nu' and 'cp']

[Image of cellular structures labeled with 'cp', 'g', and 'f']
Fig. 17. Diplotene A oocyte. Glutaraldehyde (cacodylate)–osmium fixation. The vanishing cap (cp) seems to disintegrate into the nuclear sap. Only one nucleolus (nu) can be seen in this section. Numerous ribosomes and mitochondria are present in the growing cytoplasm. pf, prefollicular cell. × 6000.

Fig. 18. Nucleolus of diplotene A oocyte. The fibrillar (f) and granular (g) constituents of the nucleolus are seen. cp, cap. × 30 000.
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Fig. 19. Diplotene B oocyte. Acrolein fixation. Several nucleoli (nu) are dispersed into the nuclear sap. The fibrillar (f) and granular (g) constituents of each nucleolus are well segregated. \( \times 9000 \).

Fig. 20. Diplotene B oocyte. Zenker fixation, Unna staining. \( \times 1000 \).

Fig. 21. Nucleolus of diplotene B oocyte. Acrolein fixation. \( f \), fibrillar constituent; \( g \), granular constituent. \( \times 50000 \).
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Fig. 22. Diplotene C oocyte. Glutaraldehyde (Millonig–osmium fixation.) Ribboned nucleoli (mi) with well segregated constituents lying in the nuclear sap. × 15,000.

Fig. 23. Diplotene C oocyte. Zenker fixation, Unna staining. × 1000.

Fig. 24. Part of a ribboned nucleolus of a diplotene C oocyte. Glutaraldehyde (Millonig)–osmium fixation. f, fibrillar constituent; g, granular constituent. × 50,000.
Fig. 25. Diplotene D oocyte. Glutaraldehyde (cacodylate)–osmium fixation. The spherical nucleoli, dispersed in the nuclear sap, are particularly numerous at the nuclear border. nu, nucleolus. \( \times 10000 \).

Fig. 26. Diplotene D oocyte (D). Zenker fixation, Unna staining. B, diplotene B oocyte; C, diplotene C oocyte. \( \times 200 \).

Fig. 27. Nucleolus of diplotene D oocyte. Glutaraldehyde (cacodylate)–osmium fixation. The fibrillar constituent (f) has a typical excentric position inside the granular constituent (g). \( \times 50000 \).
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[Image 25: Electron micrograph showing nucleoli in Xenopus oocytes.

Image 26: inset showing different stages of nucleoli.

Image 27: High magnification of a nucleolus.

Legend:
- "nu": Nucleolus
- "g": Granular component
- "r": Reticular component
- "B": Early stage
- "C": Intermediate stage
- "D": Late stage
- "1 μm" scale bars]