DIFFERENTIAL SYNTHESIS OF THE GENES FOR RIBOSOMAL RNA DURING AMPHIBIAN OÖGENESIS

By JOSEPH G. GALL

DEPARTMENT OF BIOLOGY, KLINE BIOLOGY TOWER, YALE UNIVERSITY

Communicated by Norman H. Giles, November 7, 1967

Molecular hybridization experiments have shown that the genes for ribosomal RNA are located in or near the nucleolus organizer in *Drosophila melanogaster*^{1, 2} and in the toad *Xenopus laevis*.^{3, 4} Under normal circumstances the number of organizers per genome will be characteristic of the organism, and DNA from various tissues should contain the same proportion of ribosomal genes. This conclusion has been confirmed for several tissues of the chicken.² However, cytological evidence has long suggested that the nucleolus organizer undergoes a differential replication in oöcytes of certain animals. Data supporting this view will be summarized later in this article. If such a differential replication occurs, oöcyte DNA should be enriched with respect to the ribosomal genes. The cytological picture is particularly striking in ovaries of the toads *Bufo* and *Xenopus*, in which the differential synthesis occurs during pachytene. In recently metamorphosed toads the ovary contains a sufficiently high proportion of pachytene oöcytes to permit detection of the differential synthesis by biochemical means.⁵ This communication describes the ovarian DNA of *Xenopus* and demonstrates that it contains an excess of sequences coding for rRNA. Recently Brown and co-workers^{6, 7} have studied the DNA from older oöcytes of *Xenopus* and have reached similar conclusions.

Materials and Methods.—(1) *Animals*: Tadpoles of the African clawed toad *Xenopus laevis* were grown in the laboratory. During the second and through the fourth weeks after metamorphosis, a large number of oöcytes are in the pachytene stage. At this time the testes and ovaries can be distinguished by gross inspection (cf. Figs. A and B in Blackler⁸). When the oöcytes pass into diplotene and begin their growth phase, they have completed the differential DNA synthesis. If individual oöcytes are detectable with the dissecting microscope (10×–20×), the ovary will show little incorporation into the nucleolar DNA.

(2) *DNA extraction*: Ovaries from 8–10 toads were dissected under sterile conditions and cultured 20–60 hr in a medium containing 9 μ c thymidine-C¹⁴ (47 mc/mM) or 100 μ c thymidine-H³ (14 c/mM). The ovaries were rinsed in SSC and homogenized in 2 ml tris-EDTA-sucrose solution. Predigested pronase (100 μ g) and SDS (0.2%) were added and the mixture incubated several hours at 37°C. The solution was mixed with phenol and centrifuged, after which the aqueous layer was extracted three times with ether and finally bubbled with air. Previously boiled RNase (100 μ g) was added and allowed to act 1–2 hr. Pronase (100 μ g) was added and digestion continued for 1–2 hr. The solution was next mixed with 2 vol of ethanol and held in the freezer for 3 hr or longer. No precipitate was visible, but the DNA could be deposited on the walls of the test tube by centrifuging at 10,000 *g* for 30 min. The DNA was redissolved in 1 ml 0.1 × SSC. Denaturing was accomplished by heating 10 min in a boiling water bath or by adding NaOH and neutralizing with HCl. Samples labeled with C¹⁴ had a specific activity of 500–1500 cpm/ μ g; those with H³ had about 10,000 cpm/ μ g. A total of 1.5–3.0 μ g of DNA was recovered per pair of ovaries.

(3) *RNA*: Radioactive RNA was prepared from immature ovaries of *Xenopus* cultured 3–4 days in a medium containing 300–600 μ c uridine-H³ (25 c/mM). Labeled mouse RNA was obtained from cultured L cells. In both cases the RNA was prepared essentially

as recommended by Brown and Littna.⁹ Purification was carried out on sucrose gradients, and the 28S, 18S, and 4S fractions were collected separately. The specific activity of *Xenopus* RNA samples varied between 10,000 and 30,000 cpm/ μ g, the mouse RNA between 50,000 and 140,000 cpm/ μ g.

(4) *Centrifugation*: Density equilibrium centrifugation was performed in the Spinco No. 50 angle-head rotor.¹⁰ Samples of 4.5 ml were placed in polyallomer tubes, covered with mineral oil, and spun for 20–24 hr at 42,000 rpm and 20°C. Fractions were collected by puncturing the bottom of the tube. All samples were filtered onto nitrocellulose membranes. Denatured DNA was collected in $6 \times$ SSC and filtered immediately. Native DNA was either collected in 5% TCA and filtered after chilling 1–3 hr, or was denatured with alkali and filtered in $6 \times$ SSC.

(5) *Annealing*: The filter technique described by Gillespie and Spiegelman¹¹ was used for forming molecular hybrids between RNA and DNA. Annealing was routinely carried out at 66°C for 16–20 hr.

(6) *Counting procedure*: All radioactive samples were deposited on nitrocellulose membrane filters and counted in toluene-PPO-POPOP. For double-labeling experiments representative H³ and C¹⁴ spectra were measured *on filters* and appropriate channels chosen for minimizing spillage of C¹⁴ counts into the H³ channel. In annealing experiments the filters which contained C¹⁴-labeled DNA were counted both before and after hybridization. In this way the fraction of C¹⁴ counts in the H³ channel was directly determined, and the retention of DNA during the hybridization step was monitored.

(7) *Abbreviations*: rRNA, ribosomal RNA; rDNA, the DNA sequences coding for rRNA; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl, 0.015 M Na citrate, pH 7.0; toluene-PPO-POPOP, 4 gm 2,5 diphenyloxazole and 50 mg 1,4 bis [2-(4-methyl-5-phenyloxazolyl)]-benzene in 1 liter toluene; tris-EDTA-sucrose, 0.05 M tris-(hydroxymethyl)-amino methane, 0.1 M ethylenediaminetetraacetate, 27% w/v sucrose, pH 8.4; TCA, trichloroacetic acid.

Results.—As used here the term *ovarian DNA* refers to unfractionated DNA from the whole ovary. It is derived primarily from follicle cells and oöcytes, but contains contributions from primordial germ cells, oögonia, erythrocytes, and connective tissue cells.

Buoyant density of ovarian DNA: Labeled DNA from young ovaries of *Xenopus* separates into two bands of radioactivity when centrifuged to equilib-

FIG. 1.—Separation of labeled ovarian DNA into two bands after equilibrium centrifugation in CsCl. (—) Native DNA; (---) sample treated with DNase before centrifugation.

FIG. 2.—Ovarian DNA of *Xenopus* denatured by boiling and centrifuged to equilibrium in CsCl. Major and minor peaks retained their relative heights and positions in the gradient, although both are denser than in native sample. The minor peak contained 30% of the radioactivity.

FIG. 3.—Labeled ovarian DNA of *Xenopus* (---) and unlabeled *E. coli* DNA (—) centrifuged together in CsCl. The density scale was determined from refractive index measurements on a second CsCl gradient run simultaneously; the absolute values were adjusted to give $\rho = 1.710$ at the *coli* peak. Main *Xenopus* peak, $\rho = 1.700$; minor *Xenopus* peak, $\rho = 1.729$.

FIG. 4.—Saturation curves for annealing of *Xenopus* ribosomal RNA with *Xenopus* ovarian DNA. The plateau levels were much higher than with *Xenopus* somatic DNA. See Table 1 for further details.

rium in CsCl. Both bands are eliminated by prior treatment with DNase (Fig. 1). The majority of the radioactivity is in the lighter band, which has the same buoyant density as DNA derived from liver and other somatic tissues of *Xenopus*. The minor, more dense band is seen only when ovaries of the appropriate age are labeled; detectable radioactivity is not found in this part of the gradient when labeled DNA is isolated from older ovaries or from liver. During the second and through the fourth weeks after metamorphosis, up to 30 per cent of the total radioactivity may be found in the minor peak (Fig. 2); after this time, labeling of the minor peak drops off rapidly as many oöcytes pass into the diplotene stage of meiosis. The minor peak can be detected in older ovaries by optical density measurements (Fig. 7).

The buoyant density of the peaks was estimated by centrifuging radioactive ovarian DNA with unlabeled *E. coli* DNA as a density standard (Fig. 3). The densities of the major and minor peaks are 1.700 and 1.729, corresponding to guanine + cytosine contents of 41 per cent and 70 per cent, respectively.¹² When ovarian DNA is boiled or treated with strong alkali before centrifugation both peaks increase in density, while retaining the same relative heights and positions in the gradient (Fig. 2). In one experiment the new densities were determined as 1.714 and 1.746. These observations suggest that the minor peak, like the major, contains double-stranded DNA that is denatured in the usual way by alkali.¹²

Annealing of ovarian DNA: If the minor peak represents nucleolar DNA in whole or in part, then we should expect ovarian DNA to be enriched in the cistrons for rRNA. This has been tested by means of annealing experiments utilizing ovarian DNA and several RNA samples.

Wallace and Birnstiel^{3, 4} showed that somatic DNA of *Xenopus* hybridizes with about 0.1 per cent of its weight of rRNA; the saturation levels for 28S and 18S rRNA being, respectively, 0.04–0.09 per cent and 0.025–0.04 per cent.

TABLE 1. *Details of an annealing experiment between Xenopus ovarian DNA and Xenopus rRNA (mixed 28S and 18S).*

DNA on Filter Before Annealing (cpm)		DNA on Filter after Annealing (cpm)		RNA used for annealing (μg)	RNA in RNase- Resistant Hybrid (cpm)		RNA/ DNA (%)
574	0.845	430	0.633	0.5	112	0.0100	1.58
555	0.817	416	0.613	1	133	0.0119	1.94
558	0.822	403	0.594	2	161	0.0144	2.42
559	0.823	407	0.599	4	202	0.0180	3.01
562	0.828	392	0.577	8	217	0.0193	3.34
35 μg <i>E. coli</i> unlabeled		Not determined		2	2	—	—

C^{14} -labeled ovarian DNA (679 cpm/μg) was boiled and placed on nitrocellulose filters. The filters were annealed with the indicated amounts of H^3 -labeled rRNA (11,200 cpm/μg) for 16 hr at 66°C. After treatment with RNase (20 μg/ml in $2 \times$ SSC) for 1 hr at room temperature the filters were washed in $6 \times$ SSC and the radioactivity determined in a liquid scintillation counter. The counts of hybrid were made at restricted channel settings to minimize spillage of C^{14} into the H^3 channel. All values are corrected for spillage (10%) and are adjusted to full channel.

Their values were obtained by annealing in solution. In the present experiments the filter technique of Gillespie and Spiegelman¹¹ was used. DNA from *Xenopus* liver gave a saturation value of 0.10–0.15 per cent when annealed with a mixture of 28S and 18S rRNA. The saturation levels for 28S and 18S separately were about 0.09 per cent and 0.05 per cent. Thus there is reasonable agreement between the two techniques.

When unfractionated DNA from early ovaries was annealed with rRNA much higher saturation levels were found. Although only very small amounts of ovarian DNA were available, the annealing could be studied with no more than 0.5–1.0 μg DNA on each filter. The results with one sample of ovarian DNA are shown in Table 1 and Figure 4. In this sample the saturation levels for 18S and 28S rRNA are about 1.5 per cent and 2.0 per cent, respectively, and about 3.2 per cent for the mixture. A second DNA sample saturated at 0.8 per cent and 2.0 per cent with 18S and 28S rRNA, and a third at 2.8 per cent with 28S rRNA. These values correspond to a 20- to 30-fold enrichment of the ribosomal genes in ovarian DNA as compared to somatic DNA. Since the oöcytes constitute a minority of the cells in the ovary, their specific enrichment must be considerably higher than this figure.

Annealing of gradient fractions: Wallace and Birnstiel^{3, 4} demonstrated that the sequences in somatic DNA which hybridize with 28S rRNA are denser than the bulk of the DNA. This they did by separately hybridizing the fractions from a CsCl gradient. A repetition of their experiment is shown in Figure 5, where *Xenopus* liver DNA was fractionated and hybridized with a mixture of 28S and 18S rRNA. Annealing experiments were next carried out with fractions from CsCl gradients of ovarian DNA. In Figures 6 and 7 one can see that nearly all of the hybridization takes place in the heavy peak. In Figure 7 the DNA was derived from ovaries with diplotene oöcytes, and the DNA of the minor peak did not label; however, it was detectable by optical density measurements. A saturation value has not been determined for the heavy peak alone. Since it represents only a small fraction of the total DNA (Fig. 7), its saturation level must be well above the 3 per cent found for unfractionated ovarian DNA.

Discussion.—The experiments reported here demonstrate a differential synthesis of rDNA in ovaries of recently metamorphosed *Xenopus*. The rDNA has a buoyant density of 1.729, corresponding to a G + C content of 70 per cent. This value is somewhat higher than that reported for the rDNA of *Xenopus* somatic tissues, namely 1.723.⁴ A direct comparison of ovary and somatic DNA must be made before this difference can be considered significant. However, it seems possible that the G + C content of the ovary rDNA exceeds the G + C content of *Xenopus* rRNA (about 60%^{7, 9}). Perhaps certain high G + C regions in the rDNA are not transcribed, or, if transcribed, are eliminated in the transformation from precursor 45S rRNA to definitive 28S and 18S rRNA. Alternatively, the buoyant density of the rDNA may be unusual for its base composition.

There is compelling cytological evidence that the excess rDNA is made in the oöcytes and that it is involved in the formation of the multiple nucleoli characteristic of these cells. The cytological features will be briefly summarized. In many Amphibia the oöcyte nucleus reaches a diameter of over 0.5 mm and contains 1000 or more nucleoli.^{13, 14} Oöcyte nucleoli are similar to somatic nucleoli in most respects,¹⁵ including fine structure,^{16–18} but they are not attached to the chromosomes; during most of oögenesis they are situated just inside the nuclear envelope. They incorporate uridine and other RNA precursors rapidly,^{17, 18} and the incorporation is actinomycin-sensitive.¹⁹ In the newt *Triturus*, the RNA of the oöcyte nucleoli has a base composition similar to that of rRNA.²⁰

Each of the multiple nucleoli contains a small amount of DNA, which is frequently, though not invariably, demonstrable as a Feulgen-positive granule.^{21–23} During part of oögenesis the nucleoli take the form of beaded circles or necklaces, and these are fragmented by DNase.^{24, 25} Finally the nucleolar DNA has been demonstrated by the specific binding of actinomycin.²⁶ Electron micrographs suggest that the DNA may exist as circles of several sizes.²⁴

The origin of the nucleolar DNA was first studied by King in 1908.²⁷ She showed that extra “chromatin” arises in the oöcytes of the toad *Bufo* during pachytene, and that this “chromatin” is associated with the forming nucleoli. Later Painter and Taylor²² used the Feulgen reaction to demonstrate that the material observed by King was in fact DNA. Photometric measurements show that in *Bufo* the synthesis of nucleolar DNA results in a two to threefold increase

FIG. 5.—An experiment to demonstrate the ribosomal complements in somatic DNA. *Xenopus* liver DNA (200 μ g) was boiled and then centrifuged to equilibrium in CsCl (—). Each gradient fraction was placed on a nitrocellulose filter and annealed with 1 μ g of mouse ribosomal RNA. The RNase-resistant hybrid (---) was formed with DNA sequences of high density. Mouse rRNA anneals well with amphibian DNA,⁷ and was available at the high specific activity needed for this experiment (60,000 cpm/ μ g).

FIG. 6.—An experiment to demonstrate the ribosomal complements in ovarian DNA. *Xenopus* ovarian DNA (12 μ g) was centrifuged to equilibrium in CsCl (·---·). Each gradient fraction was denatured, placed on a nitrocellulose filter, and annealed with 1 μ g of mouse ribosomal RNA (120,000 cpm/ μ g). As in somatic DNA (Fig. 5), the DNA sequences complementary to rRNA occurred at a high density, coincident with the minor DNA peak.

The biochemical and cytological data taken together suggest the following sequence of events. During pachytene, specific replication of the nucleolus organizer region takes place; this involves the genes for both 18S and 28S rRNA. The extra organizers, possibly in the form of circular DNA molecules, migrate to the nuclear envelope, where they serve as the templates for the synthesis of rRNA precursors. The formation of the multiple nucleoli is the cytological expression

FIG. 7.—*Xenopus* ovarian DNA (40 μ g) was boiled and centrifuged to equilibrium (·---·). Each fraction was then annealed with 1 μ g of mouse ribosomal RNA. This DNA sample was derived from older ovaries in which the minor peak did not label with thymidine; the minor peak was nevertheless demonstrable by optical density measurements.

in nuclear DNA content.²⁸ Early meiotic nuclei contain the expected 4C amount of DNA, but by late pachytene this has increased to more than 10C. Macgregor²⁹ has found that in *Xenopus* the DNA content during pachytene rises from 4C to about 14C. Thus in these two species most of the DNA in the late pachytene nucleus is newly synthesized nucleolar DNA. The extra DNA is in the form of minute granules or fibers separate from the chromosomes (Fig. 8). During the period of DNA increase the oöcyte nuclei exhibit a marked uptake of thymidine- H^3 into the nucleolar granules, while the chromosomes remain unlabeled.^{28, 29}

of these events. The synthesis of the extra DNA is limited to the pachytene stage, but the DNA itself is metabolically stable and persists throughout oögenesis in association with the nucleoli.

The phenomenon described here is of widespread occurrence in animal oögenesis. DNA granules associated with the multiple nucleoli can be demonstrated in many amphibians, both urodele and anuran.³⁰ The nucleoli of grasshopper oöcytes occur as long strands which are disrupted by DNase,³¹ while in the cricket they are small circles which arise in association with Feulgen-positive material.³² In the fly *Tipula*, excess DNA occurs in the oöcyte along with

FIG. 8.—*Xenopus* oocyte nuclei during the period of nucleolar DNA synthesis. Feulgen stain, $\times 1700$.

(A) Very early stage containing normal pachytene chromosomes with the 4C amount of DNA. Arrow indicates nucleolus.

(B) Excess DNA begins to accumulate as granules around the nucleolus. Such nuclei show intense thymidine incorporation.

(C) After synthesis the nuclear DNA content has more than tripled and the excess DNA appears as a compact mass. Subsequently the mass will break up into hundreds of granules which become associated with the multiple nucleoli.

nucleolar material,³³⁻³⁵ and the same has been shown in several other insects,³³ including a number of beetles.³⁶ Among the latter, "Giardina's ring" in oocytes of *Dytiscus* is particularly striking.

The synthesis of extra rDNA is related to the extreme demand for rRNA which arises during oögenesis. The oöcyte faces a unique problem: its single nucleus must supply RNA to a mass of cytoplasm which in other tissues would contain several thousand nuclei. Polyploidy or polyteny as mechanisms for the increase of DNA templates are ruled out by the need for an orderly meiosis. Differential replication provides a genetically "acceptable" mechanism whereby those templates in demand, namely the rDNA, can be produced in abundance. Painter and Taylor²² recognized the essential features when they concluded, "If these nucleolar organizers are genetically the same as those which form nucleoli in ordinary somatic cells, then we may say that the germinal vesicle of the toad is highly polyploid in nucleolar organizers, but otherwise lampbrush chromosomes are normal meiotic structures."

Summary.—The DNA synthesized in ovaries of recently metamorphosed toads (*Xenopus laevis*) consists of two fractions, one of which has the same buoyant density as somatic DNA ($\rho = 1.700$). The other is a double-stranded DNA of unusually high buoyant density ($\rho = 1.729$) which contains up to 30 per cent of the thymidine incorporated by the ovary. Molecular hybridization experiments demonstrate that unfractionated ovarian DNA is enriched 20-30 times over somatic DNA in the genes for rRNA. The extra genes are located in the heavy DNA fraction. The differential synthesis of the genes for ribosomal RNA is correlated with the appearance of hundreds of nucleolus organizers during the pachytene stage of meiosis. The multiple nucleoli of the oöcyte later arise in association with these organizers, which provide extra templates for the massive synthesis of rRNA that occurs during oögenesis.

This study was aided by a U.S. Public Health Service grant from the National Institute of General Medical Sciences (GM 12427). The technical assistance of Miss Carolyn Staehly is gratefully acknowledged.

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- ³⁰ The author and H. C. Macgregor have seen granules in the following species: *Bufo bufo*, *B. terrestris*, *B. americanus*, *B. marinus*, *Xenopus laevis*, *Rana clamitans*, *Eleutherodactylus johnstonii*, *Triturus viridescens*, *T. cristatus*. The situation in *Triturus* and *Rana* needs further study. Biochemical analysis of isolated germinal vesicles has yielded values of 36C for *Triturus viridescens* (Izawa, M., V. G. Allfrey, and A. E. Mirsky, these PROCEEDINGS, **50**, 811 (1963) and over 500C for *Rana pipiens* (Finamore, F., D. Thomas, G. Crouse, and B. Lloyd, *Arch. Biochem. Biophys.*, **88**, 10 (1960); Haggis, A., *Science*, **154**, 670 (1966)). Feulgen preparations of early oöcytes give little indication that such large amounts of DNA are present, at least in pachytene and early diplotene, although some Feulgen-positive material is associated with the nucleoli.
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