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Synthesis of Amplified DNA That Codes for Ribosomal RNA

(density gradient centrifugation/2′,5′-dimethyl-*N*(4′)benzyl-*N*(4′)[desmethyl]rifampicin/extrachromosomal DNA/*Xenopus laevis*/ovaries)

MARCO CRIPPA* AND GLAUCO P. TOCCHINI-VALENTINI†

* CNR Laboratory of Molecular Embryology, Arco Felice, and † CNR International Institute of Genetics and Biophysics, Naples, Italy

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ABSTRACT **During the amplification stage in ovaries, the complete repetitive unit of the DNA that codes for ribosomal RNA in *Xenopus* appears to be transcribed. This large RNA transcript is found in a complex with DNA. Substitution experiments with 5-bromodeoxyuridine do not show any evidence that a complete amplified cistron is used as a template for further amplification. A derivative of rifampicin, 2′,5′-dimethyl-*N*(4′)benzyl-*N*(4′)[desmethyl]rifampicin, preferentially inhibits the DNA synthesis responsible for ribosomal gene amplification. These results are consistent with the hypothesis that RNA-dependent DNA synthesis is involved in gene amplification.**

Amplification of the DNA genes that code for ribosomal RNA has been shown to occur in amphibian oocytes and in the ovaries of various insects (1–4). In oocytes of *Xenopus laevis*, it occurs during the pachytene stage of the meiotic prophase (1), when the number of ribosomal cistrons present in the oocyte nucleus increases more than a thousand times over the expected tetraploid amount. Two possible mechanisms for this amplification have been envisaged (5). According to the first mechanism, the oocyte DNA that codes for ribosomal RNA (rDNA) is synthesized exclusively on the chromosomal nucleolar organizer and the copies are subsequently detached. However, since a correlation between the rate of DNA synthesis and the amount of DNA present in the "cap" region of *Xenopus* oocytes nuclei has been reported (6), it is likely that the nucleolar organizer is not the only site of synthesis; new copies of rDNA, according to the second proposed mechanism, are synthesized extra-chromosomally (5).

In this paper we present three types of experiments consistent with the hypothesis (7), that rDNA amplification occurs through RNA-dependent DNA synthesis (8, 9).

MATERIALS AND METHODS

Immature ovaries from young tadpoles were dissected and incubated according to Gall (1) to label DNA. At the end of the incubation, the ovaries were homogenized in 2 ml of 0.30 M NaCl–0.030 M Na citrate containing 1% sodium dodecyl sulfate; previously digested pronase (200 μg/ml) was added and the mixture was incubated 4 hr at 45°C. The solution was then brought to 1.0 M $NaClO_4$ and repeatedly extracted with phenol. The aqueous phase was precipitated with alcohol and the precipitate was dissolved in 0.30 M NaCl–0.030 M Na citrate. The RNA was removed by incubation for 1 hr at 37°C with 100 μg/ml of boiled pancreatic ribonuclease. After phenol extraction, the DNA was again precipitated with alcohol and dissolved in 10 mM Tris, pH 8.1. When DNA–RNA hybrids were studied, the ribonuclease treatment was either omitted or performed as indicated in the text.

For equilibrium density analysis, solid CsCl was added to the DNA solution to a refractive index of 1.4010; centrifugation was in a Spinco 40 rotor at 33,000 rpm for 48 hr. Fractions were collected manually and the radioactivity was determined after precipitation with Cl_3CCOOH.

To label RNA, the ovaries were incubated in a medium containing 400 μCi/ml of [^{3}H]uridine (1). The RNA was extracted in 0.1 M acetate, pH 5.0 (10). In some experiments, sucrose density analysis was performed with 15–30% sucrose gradients prepared in 0.01 M acetate (pH 5.0) with 0.5% sodium dodecyl sulfate. When the RNA from these gradients was used for hybridization, each fraction was first dialyzed extensively against 0.30 M NaCl–0.030 M Na citrate.

For BrdU-substitution analysis, the ovaries were incubated in a medium containing 50 μM FdU; after 1 hr [^{3}H]BrdU was added to a final concentration of 7.5 μM (2 μCi/ml) and the incubation was allowed to proceed for the times indicated in the text. In the "chase" experiments, at the end of the desired incubation period the ovaries were carefully washed and placed in a fresh medium containing 0.1 mM thymidine and 50 μCi/ml of $^{32}PO_4$. The DNA was then extracted and analyzed in CsCl as described above.

DNA–RNA hybridization was done according to Gillespie and Spiegelman (11). Schleicher & Schuell B-6 filters were loaded with 1 or 2 μg of DNA per filter and annealed in 1 ml of 0.30 M NaCl–0.030 M Na citrate for 20 hr at 66°C. After incubation, the samples were treated for 30 min at 37°C with a mixture of pancreatic ribonuclease (20 μg/ml) and T1 RNase (0.5 μg/ml).

The DNA coding for ribosomal RNA was prepared according to Dawid *et al.* (12).

"Spacer"-rich DNA was prepared by differential melting on hydroxylapatite columns of purified rDNA previously disrupted sonically to a molecular weight of 2.5×10^5. The DNA that eluted from hydroxylapatite with 0.5 M phosphate buffer, after the temperature was brought to 94°C and the column was extensively washed with 0.12 M phosphate buffer, was concentrated and recycled. This procedure selects for GC-rich DNA sequences. The fraction eluted with 0.5 M phosphate buffer at 94°C, after three cycles on hydroxylapa-

Abbreviations: rDNA, ribosomal DNA, including gene sequences homologous to ribosomal RNA, together with spacer DNA; chromosomal rDNA, the rDNA that can be purified from somatic cells; amplified rDNA, extrachromosomal rDNA that has been amplified in oocyte nuclei.

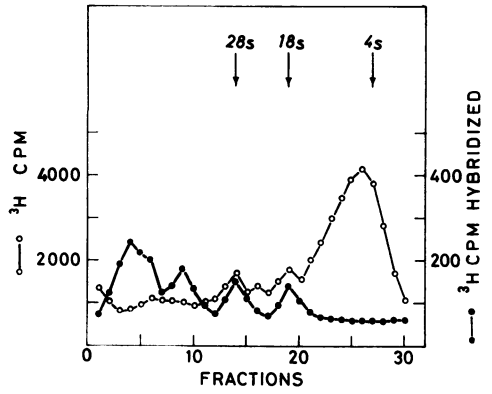

FIG. 1. Sucrose density analysis of RNA extracted from ovaries during amplification stage. The ovaries were incubated for 48 hr with 400 μCi/ml of [^{3}H]uridine (Amersham, TRK), and the RNA was extracted, acid-precipitable counts O—O—O; counts hybridized to purified rDNA ●—●—●.

tite, represented about 10–12% of the DNA put on the first column.

RESULTS

Transcript of ribosomal DNA

Xenopus rDNA is made of repetitive units that comprise the sequences coding for the 40S ribosomal precursor, and a portion of higher GC content called "spacer." The molecular weight of the repetitive unit is around 9×10^6; 5×10^6 daltons are accounted for by the 40S sequences, and the remaining 4×10^6 daltons by the spacer (5, 12). There is strong evidence that in the cases so far analyzed, which are limited to somatic tissues (kidney cells in culture and nerula cells), the spacer is not transcribed (13–15).

We have studied the transcription of rDNA in amplification-stage ovaries. Fig. 1 shows the sedimentation profile of the total ovarian RNA in a sucrose gradient. It is clear that most of the RNA synthesized is small, with a sedimentation coefficient of about 4–5S, as in somewhat larger oocytes (16–18).

If the fractions of the gradient are analyzed by hybridization with purified rDNA, four peaks of hybridization are detected (Fig. 1). Besides the peaks corresponding to the 18S and 28S RNA, there are two additional peaks sedimenting at 37S and 47S, with molecular weights of 2.6×10^6 and 4.2×10^6, respectively. It is clear that in this system, with long labeling periods, the small (4S and 5S) RNA species accumulate much more than the larger RNA species; therefore the larger species do not appear as peaks of Cl_3CCOOH-precipitable counts, but can only be detected by hybridization. We interpret the 37S peak as the normal ribosomal precursor (15), and the 47S peak as the transcript of the total ribosomal DNA unit. We suggest that the 47S RNA is synthesized by the oocytes during the amplification stage.

When the 47S and the 37S peaks are allowed to compete (under saturation conditions) for hybridization to rDNA with an equimolar mixture of unlabeled 28S and 18S ribosomal RNA, a competition plateau of 50% and 95%, respectively, is obtained (Table 1). The rDNA used in the hybridization experiments was essentially pure by the criteria described in the literature (19); consequently, we do not expect that any other RNA species sedimenting in the 47S and 37S regions of the gradient affect our hybridization results.

If the 47S peak is the transcript of the total ribosomal unit, then the sequences that do not compete with unlabeled 28S and 18S RNA should hybridize to the spacer. "Spacer"-rich DNA was prepared by differential melting on hydroxylapatite (*Methods*). Contaminant sequences coding for 28S and 18S RNA are, however, still present, as indicated by the 5% saturation level obtained when the "spacer"-rich DNA is hybridized with radioactive 28S and 18S RNA. Furthermore, some spacer sequences of lower GC content are most probably lost during the purification procedure and are, therefore, not represented in our "spacer"-rich DNA.

Table 1 shows that the "spacer"-rich DNA hybridizes with the 47S RNA extracted from amplification-stage ovaries and that the counts hybridized only partially compete with cold 28S and 18S RNA.

When ovaries during the amplification stage are incubated with [^{14}C]thymidine, and the extracted DNA is analyzed in CsCl, two distinct bands are detected; the heavier is rDNA and the lighter is bulk chromosomal DNA (1). When ovaries are simultaneously incubated with[^{3}H]uridine and [^{14}C]-thymidine, a peak of [^{3}H]uridine incorporation banding with the heavy region of the ribosomal DNA is detected (Fig. 2). This material is resistant to RNase and becomes sensitive after heat denaturation. These findings are consistent with the idea that this RNA is part of a DNA–RNA hybrid structure.

We were able to recover the RNA from the DNA–RNA hybrid structure present *in vivo*. When sedimented in a sucrose gradient, this RNA has the profile shown in Fig. 3. Most of the material has a sedimentation coefficient of 45S.

Analysis of the BrdU-substituted DNA

When ovaries during the amplification stage were incubated with [^{3}H]BrdU in the presence of FdU in order to inhibit endogenous thymidylate synthetase, and consequently to maximize substitution of dT by BrdU, the pattern in Fig. 4 is observed. After 6 hr of incubation (Fig. 4*b*) two peaks of radioactive incorporation (peaks *A* and *C*) are observed, banding at densities of 1.780 and 1.723, respectively. After 12 hr (Fig. 4*c*), a shoulder of intermediate density appears (peak *B*) that bands at 1.745. After 24 hr of incubation, the radioactive profile is unaltered except that the amount of radioactivity banding at 1.745 (peak *B*) is markedly increased in respect to peak *C* (Fig. 4*d*).

Peak *A* contains sequences that hybridize with radioactive 28S and 18S RNA. Peak *C* is most probably bulk chromo-

TABLE 1. *Analysis by DNA–RNA hybridization of the RNA synthesized by ovaries during the amplification stage*

DNA	Radioactive RNA	Unlabeled competitor	cpm
rDNA	47S	none	695
rDNA	47S	28S + 18S	352
rDNA	37S	none	421
rDNA	37S	28S + 18S	38
"spacer"-rich	47S	none	514
"spacer"-rich	47S	28S + 18S	455

The 47S region (tubes 3, 4, 5) and the 37S region (tubes 8, 9, 10) of the gradient shown in Fig. 1 were separately pooled, dialyzed against 0.30 M NaCl–0.030 M Na citrate and hybridized with purified ribosomal DNA or "spacer"-rich DNA.

somal DNA, partially substituted in one strand with BrdU. It is lighter than native amplified rDNA and hydridizes with complementary RNA synthesized *in vitro* by *Escherichia coli* RNA polymerase on bulk *Xenopus* DNA that is free of ribosomal cistrons. Peak *B* contains bulk chromosomal DNA substituted with BrdU in both strands. In fact, its average displacement in density from the unsubstituted DNA is double the shift of peak *C*.

In another experiment, the ovaries were chased after 24 hr of incubation with FdU and [^{3}H]BrdU with cold thymidine for 24 more hr, and were simultaneously incubated with ^{32}P. The tritium counts show the profile with the three peaks (*A*, *B*, *C*). The ^{32}P counts show a peak of incorporation coincidental with peak *C*, a second, lighter peak coinciding with the unsubstituted DNA marker, and a shoulder of incorporation in the density region where the unsubstituted amplified ribosomal cistrons are expected to band (Fig. 4*e*).

No ^{32}P incorporation is detectable in the regions of [^{3}H]-BrdU-containing peaks *A* and *B*. These results are consistent with the idea that peak *A* and peak *B* represent, respectively, amplified ribosomal cistrons and bulk chromosomal DNA, both substituted with BrdU in both strands. No ^{32}P incorporation is seen at a density at which amplified ribosomal cistrons substituted in only one strand would be expected to band.

Drug sensitivity

We have tested the effect of 2′,5′-dimethyl-N(4′)-benzyl-N(4′)-[desmethyl]rifampicin, a derivative of rifampicin, that has been shown to inhibit RNA-dependent DNA polymerase (21), on DNA synthesis in *Xenopus* ovaries during the amplification stage.

Fig. 5 shows that the heavier peak is sensitive to the drug, suggesting that the target is a factor involved in the amplification of the ribosomal genes, but not in the replication of chromosomal DNA. Alternatively, the drug could interact directly with the ribosomal cistrons that are known to have a base composition very different from the bulk DNA (5).

If exogenous DNA and radioactive precursors are injected into unfertilized eggs, thymidine counts are incorporated

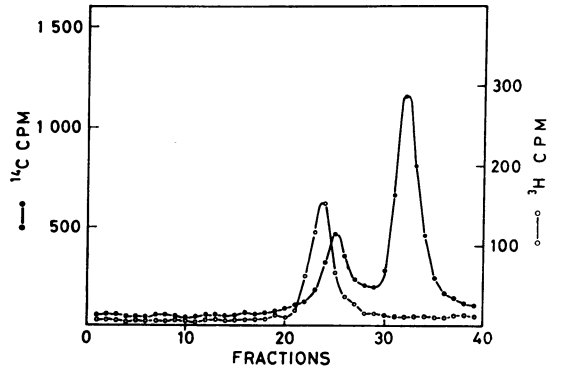

FIG. 2. Equilibrium density centrifugation of labeled nucleic acids extracted from ovaries during the amplication stage. The ovaries were incubated for 48 hr with 50 μCi/ml of [^{14}C]thymidine (Amersham, 400 Ci/mol) and with 400 μCi/ml of [^{3}H]uridine (Amersham, TRK). The extracted nucleic acids were incubated with 20 μg/ml of boiled pancreatic RNase for 1 hr at 37°C, subsequently reextracted with phenol, alcohol precipitated, and resuspended in 10 mM Tris·HCl (pH 8.1) for the CsCl centrifugation.

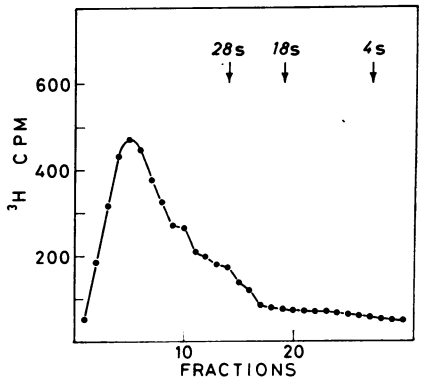

FIG. 3. Sucrose density analysis of the RNA recovered from a DNA–RNA hybrid. The ovaries were incubated as described in Fig. 2. The RNase treatment was omitted and the extracted nucleic acids were centrifuged to equilibrium in CsCl. The tubes corresponding to the amplified ribosomal cistrons (tubes 20–30 of Fig. 2) were pooled,d ialyzed against 15 mM NaCl–5 mM $MgCl_2$, heated for 10 min at 100°C, and digested for 1 hr with electrophoretically pure DNase (100 μg/ml). The solution was extracted with phenol and precipitated with alcohol after the addition of unlabeled *E. coli* tRNA as a carrier. The pellet was then suspended in 0.01 M acetate buffer pH 5.0–0.5% sodium dodecyl sulfate, and analyzed in sucrose.

into a DNA that bands in CsCl at the density of the injected DNA (20). Table 2 shows that ribosomal DNA injected into unfertilized eggs stimulates thymidine incorporation into DNA, and that the rifampicin derivative has no effect on this incorporation. These results indicate that the target of this drug is not the ribosomal DNA itself, but rather some factor present in young oocytes that operates in the amplification.

In the experiment reported in Fig. 5, 100 μg/ml of drug was used in the incubation mixture; under these conditions, the morphology of the oocytes remains perfectly normal (Prof. J. Brachet, personal communication).*

We have also studied the effect of 4′-N-desmethylrifampicin, another derivative of rifampicin (21), on this system. We found no detectable effect up to 400 μg/ml; higher concentrations are toxic.

DISCUSSION

If the mechanism of amplification of ribosomal genes involves RNA-dependent DNA synthesis, the overall picture would be as follows: (*a*) transcription of the ribosomal DNA sequences localized in the nucleolar organizer region of the chromosome; (*b*) utilization of the transcript for RNA-dependent DNA synthesis, with the formation of an intermediate RNA–DNA complex.

Since amplified rDNA, like chromosomal rDNA, is made of repetitive units, each containing the spacer, the transcript isolated from ovaries during the amplification stage, in order to be used as a template for gene amplification, should also contain the sequences corresponding to the spacer. We have verified this prediction. By hybridization with ribosomal DNA, we have identified a 47S RNA species carrying se-

* In an accompanying paper [*Proc. Nat. Acad. Sci. USA, 68*, 2774 (1971)], A. Ficq and J. Brachet present cytological evidence that this rifampicin derivative acts at the "caps" to inhibit rDNA synthesis in *Xenopus*.

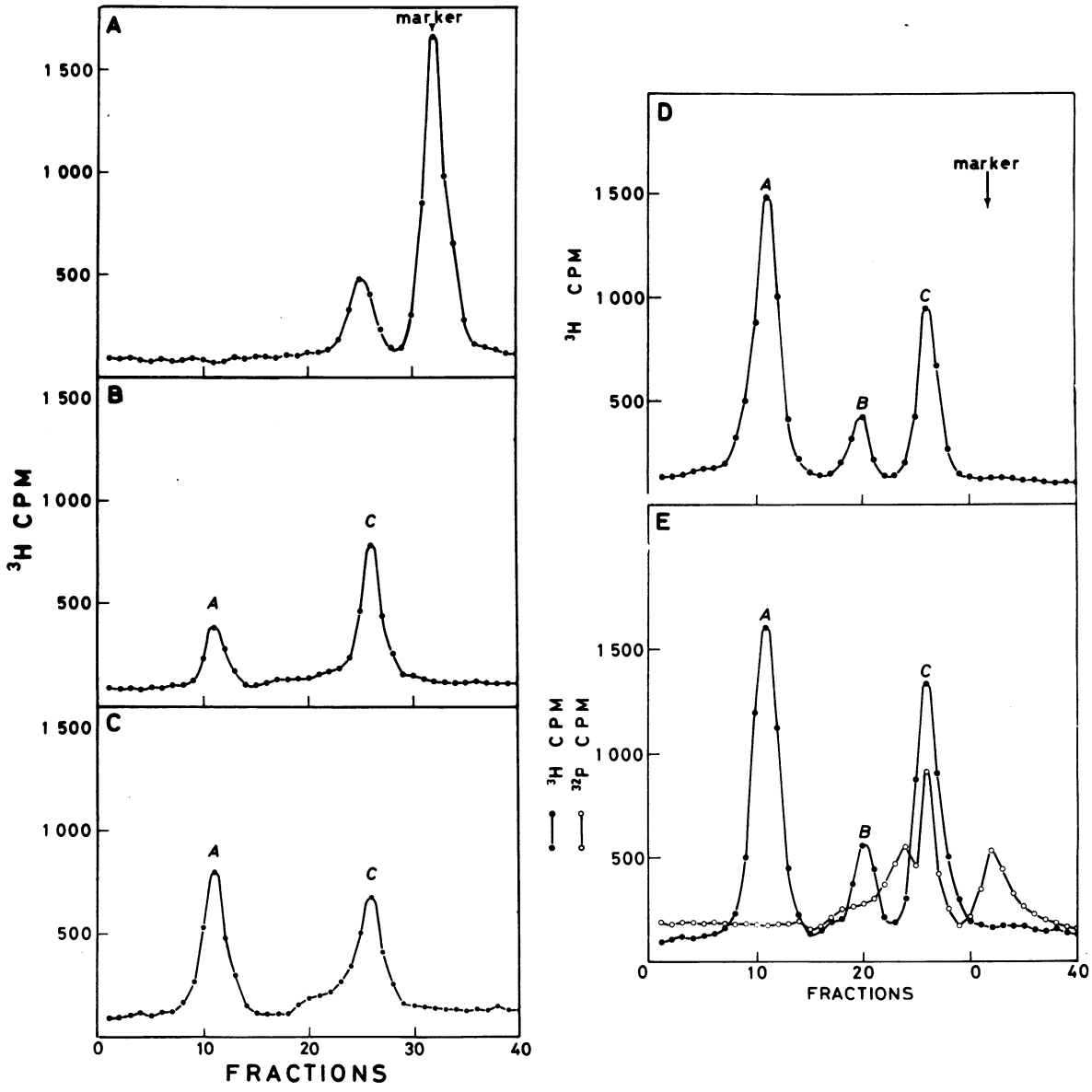

FIG. 4. Equilibrium density centrifugation of BrdU-substituted DNA extracted from ovaries during the amplification stage. The ovaries were incubated with FdU and BrdU and, when indicated, chased with unlabeled dT and labeled with ^{32}P. *a*) control: 48 hr of incubation with [^{3}H]thymidine; *b*) 6 hr incubation with FdU and [^{3}H]BrdU; *c*) 12 hr incubation with FdU and [^{3}H]BrdU; *d*) 24 hr incubation with FdU and [^{3}H]BrdU; *e*) 24 hr incubation with FdU and [^{3}H]BrdU and then chased with unlabeled dT and incubated with ^{32}P for 24 more hr. *Marker* marks the position of normal *Xenopus* DNA.

TABLE 2. *Effect of 2',5'-dimethyl-4'-benzyl-(4')[desmethyl]-rifampicin on DNA synthesis*

	cpm/20 eggs
Unfertilized eggs	2,000
Unfertilized eggs + drug	1,970
Unfertilized eggs + *Xenopus* rDNA	24,600
Unfertilized eggs + *Xenopus* rDNA + drug	29,100

Unfertilized eggs were injected as described by Gurdon (20). 40 ngrams of *Xenopus* rDNA were injected per egg, together [^{3}H]-thymidine (Amersham, TRK) and, when indicated, drug to with give a final concentration inside of the egg of 0.01 μCi/ml and 100 μg/ml, respectively. After injection, the eggs were incubated for 8 hr at 20°C. After incubation, the eggs were homogenized and the acid-precipitable counts were determined.

quences complementary to the 28S and 18S genes and to the spacer. The molecular weight of this RNA species (4.2×10^6) is in fairly good agreement with the value expected for the transcript of the complete unit. Moreover, we have isolated from young ovaries a DNA–RNA complex. From this complex, we have been able to recover an RNA molecule sedimenting at 45S, with a corresponding molecular weight of 4.4×10^6.

The interpretation of the BrdU-substitution experiments is difficult. We should not forget that ovaries during the amplification stage contain different types of cells that are synthesizing different DNAs. The oocytes are in the pachytene stage of meiosis, and have already gone through the *S* phase of their cell cycle; the only DNA synthesis occurring in the nucleus of these cells is that due to the amplification of the ribosomal cistrons. However, the other cells present in the

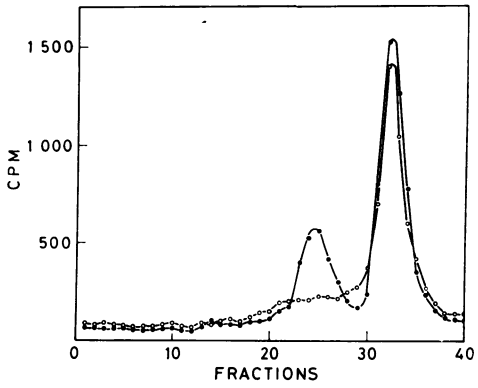

FIG. 5. Equilibrium density centrifugation of labeled DNA extracted from ovaries during the amplification stage. The ovaries were incubated for 48 hr with 100 μCi/ml of [^{3}H]thymidine (Amersham, TRK). The DNA was extracted and banded in CsCl ●—●—●, control; O—O—O, ovaries treated with100 ug/ml of dimethylbenzylrifampicin during the *in vitro* incubation period.

ovary are normally dividing and are, therefore, synthesizing DNA to replicate their chromosomal complement.

In different cells, the degree of BrdU-substitution varies, possibly because of differences in pool size and because the enzymes involved in DNA synthesis could have different affinities for the BrdU-TP. Our results indicate that after 6 hr of incubation in BrdU there is a peak (*A*), banding at a density of 1.780, that we assume is rDNA substituted in both strands by BrdU. Alternatively, peak *A* could represent a complex structure containing RNA and BrdU-substituted DNA.

We were unable to detect any rDNA of intermediate density between unsubstituted rDNA and peak *A*. Moreover, after a chase and ^{32}P labeling, we failed to observe a shift of the heavy rDNA towards a lighter region. This observation seems to argue against the possibility that a complete amplified cistron could be used as a template for further amplification. There are, however, trivial reasons that could explain our failure to observe the shift, i.e. the BrdU-substituted DNA might be unable to undergo a second round of replication.

The dimethyl derivative of rifampicin is an inhibitor of RNA-dependent DNA polymerase and, in our system, acts preferentially on the amplification process. This drug, however, has been shown to also inhibit DNA-dependent RNA polymerase and, at high concentrations, DNA-dependent DNA polymerase (21). We shall report elsewhere the isolation from *Xenopus* ovaries of a synthetic polymer-dependent DNA polymerase (manuscript in preparation) that is inhibited by this drug.

Our results are consistent with the possibility that RNA-dependent DNA synthesis is involved in ribosomal gene amplification.

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