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Time of Synthesis of Genes for Ribosomal Ribonucleic Acid in *Physarum*†

Carol Shaw Newlon,‡ Gail E. Sonenshein,§ and Charles E. Holt*

ABSTRACT: The time in the cell cycle when the genes for ribosomal RNA are synthesized was determined in the plasmodial stage of *Physarum polycephalum*. Three approaches were used. (1) Plasmodia were exposed to [^{3}H]thymidine during the G2 phase of the cell cycle; nuclear satellite DNA, which is preferentially labeled under these conditions, was isolated and hybridized in solution with ribosomal RNA (rRNA). Analysis of the hybridization mixture revealed that 10–25% of the labeled DNA was in DNA–RNA hybrids, which demonstrates synthesis of rDNA during the G2 phase. (2) The per cent of nuclear DNA hybridizable with saturating amounts of rRNA was measured for DNA isolated from plasmodia at different times in the cell cycle. The per cent hybridization increased about 50% during the G2 phase, which also demonstrates synthesis of rDNA during this period. (3) Plasmodia were incubated with the DNA density label iododeoxyuridine, nuclear DNA was isolated, and the buoyant density profile of rDNA was determined. A fraction of the rDNA was shown to be of increased density when the density label was applied during either the S phase, the first half of the G2 phase, or the second half of the G2 phase (there is no G1 phase in *Physarum* plasmodia). Thus, rDNA synthesis occurs during all phases of the mitotic cycle in this organism.

Using density labeling techniques, Mueller and Kajiwara (1966) and Braun and Wili (1969) have shown that the DNA replicated during a given interval of one cell cycle is replicated during the same interval of subsequent cell cycles. In addition, Plaut *et al.* (1966) have shown that DNA in polytene chromosomes from *Drosophila* is synthesized in a defined temporal sequence. These lines of evidence suggest that most genes are synthesized at a particular time in the cell cycle. In order to determine whether this suggestion holds for particular genes, we have begun to study the timing of gene synthesis in *Physarum polycephalum*, an acellular slime mold which displays highly synchronous nuclear division (Rusch, 1970). Our approach is to identify genes by their hybridization to purified RNA species.

In this paper, we report on the time of synthesis of the genes coding for ribosomal RNA. Our results, which corroborate our preliminary report (Sonenshein *et al.*, 1970), and a recent report by others (Zellweger *et al.*, 1972), demonstrate that the genes for rRNA[1] are synthesized in both the S phase and the G2 phase of the mitotic cycle. Studies with other organisms show that rDNA is synthesized throughout the S phase (Amaldi *et al.*, 1969; Balazs and Schildkraut, 1971; Gimmler and Schweizer, 1972); however, these studies do not show whether rDNA is synthesized in the other parts of the cell cycle. In amphibian oocytes, it is clear that rDNA is synthesized in the absence of bulk DNA synthesis (Gall, 1968).

Nuclear division in the multinucleate *Physarum* plasmodium occurs about every 12 hr under the conditions of our experiments. Nuclear DNA synthesis follows metaphase without any measurable gap in time (Nygaard *et al.*, 1960; Sachsenmaier, 1964; Braun *et al.*, 1965); thus, the observation of rDNA synthesis in both the S phase (3–4 hr) and the G-2 phase (8–9 hr) implies an essentially continuous synthesis. However, a brief cessation of rDNA synthesis early in S phase has been reported (Zellweger *et al.*, 1972).

We have approached the problem of determining the time of synthesis of rDNA in three ways. First, we have studied the hybridization of rRNA with a nuclear satellite DNA, that can be preferentially labeled during G2 phase (Holt and Gurney, 1969). Second, we have measured the fraction of the total DNA coding for rRNA as a function of the phase of the mitotic cycle. Finally, we have incubated plasmodia with a density label for DNA during different periods of the mitotic

† From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received October 16, 1972.* This investigation was supported by National Science Foundation Grant No. GB24022. C. S. N. was supported by a Public Health Service Predoctoral Fellowship and G. E. S. was supported by a Public Health Service Predoctoral Traineeship. Parts of this study have been presented previously in abstract form (Sonenshein *et al.*, 1970) and in two theses (Sonenshein, 1970; Newlon, 1971).

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[1] Abbreviations used are: rRNA, 19S and 25S ribosomal ribonucleic acid; rDNA, ribosomal deoxyribonucleic acid, the DNA that contains the sequences homologous to rRNA; nDNA, nuclear deoxyribonucleic acid; SSC, standard saline-citrate (0.15 M NaCl–0.015 M sodium citrate, pH 7.1–7.5); MAK, methylated albumin kieselguhr; MI, MII, etc., the first and second mitoses following preparation (fusion) of a surface culture.

cycle and in each case determined whether a portion of the rDNA increased in density.

Materials and Methods

Strain and Culture Methods. Two strains of *P. polycephalum* were used in this work: M_3A (Holt and Gurney, 1969) and $a + i$ (Dee, 1966). Culture methods were described by Horwitz and Holt (1971). Time of mitosis was determined by the method of Guttes *et al.* (1961).

Preparation of RNA. Radioactive RNA was prepared by incubating cultures at a density of 0.005–0.02 ml of packed plasmodia/ml of culture with [5-^{3}H]uridine (25 Ci/mmol) or [6-^{3}H]uridine (10 Ci/mmol) (New England Nuclear Corp., Boston, Mass.), at a final concentration of 100–250 μCi/ml for 12–16 hr. Nonradioactive uridine was then added to a concentration of 0.1 mg/ml and incubation was continued for an additional 6–10 hr. Crude RNA was prepared by extraction with phenol–cresol–8-hydroxyquinoline (Kirby, 1965) according to Jacobson (1971). The extraction buffer contained 5% sodium triisopropylnaphthalenesulfonate–0.17 M NaCl–0.01 M EDTA–0.01 M Tris-HCl (pH 7.4). After extraction, the aqueous phase was made 0.5 M in NaCl, an additional two or three phenol extractions were performed, and RNA was precipitated from the last aqueous extract with ethanol. Nonradioactive RNA was prepared by the same procedure from cultures harvested at a density of 0.03–0.05 ml of packed plasmodia/ml.

For the preparation of rRNA, crude RNA was dissolved in a small volume of autoclaved buffer (0.01 M MgCl–0.01 M potassium phosphate (pH 6.8)), and treated with 20 μg/ml of deoxyribonuclease I (Worthington, Freehold, N. J., electrophoretically pure) for 20 min at room temperature. Sodium dodecyl sulfate (0.2%) and Pronase (0.1 mg/ml) were added and the solution was incubated at 37° for 5–8 hr and stored frozen. Aliquots were sedimented at 25° through 16-ml linear gradients of 15–30% sucrose in sodium dodecyl sulfate buffer (0.5% sodium dodecyl sulfate–1 mM EDTA–0.1 M NaCl–5 mM Tris-HCl (pH 7.4)) in a Spinco SW-25.3 or SW-27 rotor at 24,000 rpm for 18–19 hr. A typical sucrose sedimentation profile is shown in Figure 1. To prepare total rRNA, fractions containing the 19S and 25S RNA were combined and the RNA was precipitated and washed with ethanol, dried, dissolved in 4 × SSC, dialyzed at 4° against 4 × SSC, and stored frozen. About 85% of the radioactivity in the crude preparation was recovered as rRNA. To prepare 19S or 25S RNA separately, fractions from the middle of the appropriate peaks were combined and the RNA was precipitated with ethanol, redissolved, and repurified on sucrose gradients as above.

For the purification of 4S RNA, crude RNA from 100 ml of culture was dissolved in 15 ml of Tris buffer (0.1 M Tris-HCl (pH 7.4)) and mixed in a centrifuge tube with 15 ml of packed DEAE-cellulose that had been prewashed with 1 M NaCl and then with Tris buffer. All steps were performed at 0–4°. The DEAE-cellulose-bound RNA was washed twice with Tris buffer, mixed with an equal volume of Tris buffer, and pipetted into a 25 cm × 1 cm column. (When crude RNA extracts were layered on prepacked columns of DEAE-cellulose the columns became clogged.) RNA was eluted from the column with a linear 0–0.5 M NaCl gradient in Tris buffer. Ribosomal RNA is not eluted from DEAE-cellulose under these conditions (Monier *et al.*, 1960). About 10% of the radioactivity applied to the column was recovered in a peak at 0.4 M NaCl. The RNA from this peak was precipitated with ethanol, dried, redissolved in a small amount of sodium dodecyl sulfate buffer, and separated from 5S RNA on a 0.9 cm × 110 cm column of Sephadex G-100 (Pharmacia, Piscataway, N. J.) in the same buffer. By this procedure, 7% of the radioactivity in the crude preparation was recovered as 4 S RNA and 1.7% as 5S RNA.

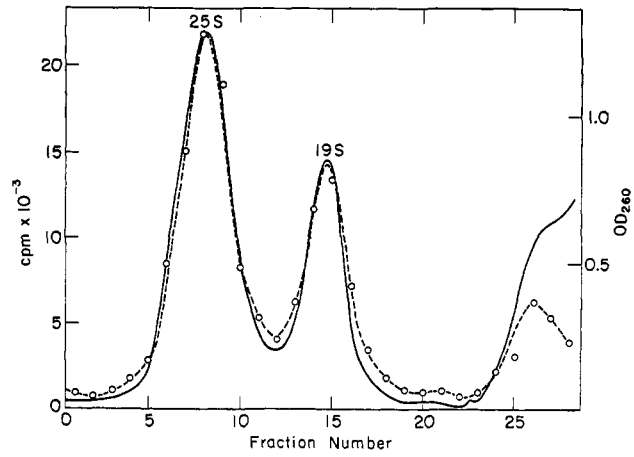

FIGURE 1: Sedimentation of rRNA. Crude RNA prepared from 10 ml of [^{3}H]uridine-labeled culture was dissolved in 3 ml of sodium dodecyl sulfate buffer. An aliquot (0.5 ml) was sedimented through a 15–30% sucrose gradient for 19 hr in an SW-27 rotor. The gradient was collected through a Gilford recording spectrophotometer and radioactivity in 5-μl samples was measured: (O- - - -O) radioactivity; (——) OD.

Preparation of DNA. For the preparation of ^{14}C-labeled nuclear DNA, cultures were grown to a density of 0.025–0.05 ml of packed plasmodia/ml in a medium containing 1 μg/ml of [2-^{14}C]thymidine (1–5 μCi/mg). This concentration of thymidine is enough to ensure continuous labeling of DNA in cultures of densities up to 0.05 ml of packed plasmodia/ml (Newlon, unpublished data). Nuclei were isolated according to Mohberg and Rusch (1971) except that 0.1% Nonidet P40 (Shell Chemical Ltd., Australia) was used as the detergent. The isolated nuclei were suspended in 20–40 volumes of either 2 × SSC–5 mM EDTA (pH 7.2) or lysis medium (Holt and Gurney, 1969) and treated with 20 μg/ml of ribonuclease for 30 min. Sodium dodecyl sulfate (0.2–0.5%) and Pronase (1 mg/ml, preincubated at 80° for 10 min) were added and incubation at 37° was continued for 12–20 hr. The solution was shaken with liquified phenol and centrifuged in a clinical centrifuge to separate the phases, and the aqueous phase was further extracted two or three times with chloroform–isoamyl alcohol (24:1). The DNA was precipitated with ethanol, wound on a glass rod, dissolved in 0.1 × SSC, and stored at 4° over a few drops of chloroform. The recovery of DNA by this procedure was 60–80%.

In certain experiments (noted in the text), DNA was further purified on MAK columns by the method of Mandell and Hershey (1960) or by banding on 5-ml ethidium bromide–CsCl gradients containing 200–300 μg of DNA, 100–200 μg/ml of ethidium bromide (a gift of Boots Pure Drug Co., Ltd., Nottingham, England), and CsCl to a refractive index (25°) of 1.3890. The gradients were centrifuged at 22° for 2 hr at 15,000–25,000 rpm and for an additional 12 hr at 45,000 rpm in a Spinco SW-50L or SW-50.1 rotor. Ethidium bromide was removed by extraction with isoamyl alcohol and CsCl was removed by dialysis against SSC.

Phage ϕe DNA was prepared by phenol extraction of purified phage and was a gift of K. Loveday (Massachusetts

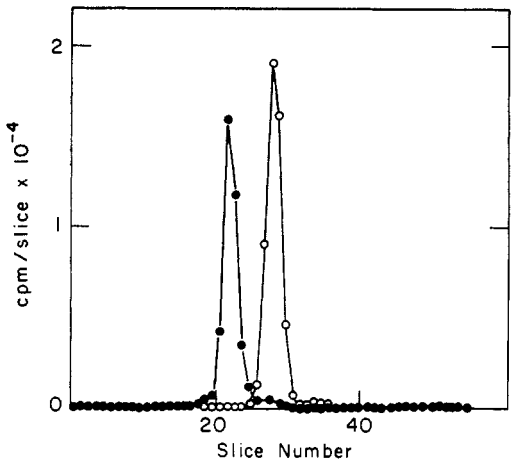

FIGURE 2: Electrophoresis of 4S and 5S RNA on acrylamide gels. 4 RNA (○) and 5S RNA (●) were run on separate gels.

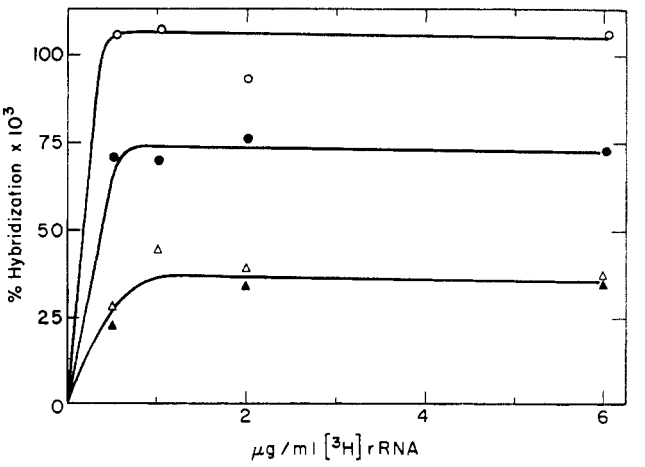

FIGURE 3: Hybridization of 19S and 25S rRNA with nuclear DNA. Filters containing 10 μg of [^{14}C]DNA (20 cpm/μg) were incubated in vials containing various concentrations of [^{3}H]rRNA (5.41 × 10^5 cpm/μg) for 20 hr at 70°. Each vial contained two filters (one DNA and one blank) and 1 ml of RNA in 4 × SSC. Samples on the plateau containined 2000–6000 cpm. Blank filters contained less than 150 cpm: (○) 1:2 mixture of tritiated 19S + tritiated 25S rRNA; (●) tritiated25S rRNA alone; (△) tritiated 19S rRNA alone; (▲) tritiated 19S rRNA + 5 μg/ml of nonradioactive 25S rRNA.

Institute of Technology). *Bacillus subtilis* DNA was a gift of Dr. A. L. Sonenshein.

DNA–RNA Hybridization. For hybridization on filters, a modification of the procedure of Gillespie and Spiegelman (1965) was used. DNA denaturation was carried out in 0.5 N NaOH for 1 hr at room temperature to eliminate any contaminating RNA. Denatured DNA was diluted to a concentration of 0.5–2 μg/ml with ice-cold 4 × SSC containing enough HCl to neutralize the alkali. (CsCl gradient fractions, 0.2 ml, were diluted at least 1:3 with 0.1 × SSC, denatured, neutralized, and diluted to a volume of 10 ml with 4 × SSC.) Before use, filters (B-6 Bac-T-Flex, Schleicher and Schuell, Keene, N. H.) were boiled for 5 min in 4 × SSC and washed with 10 ml of 4 × SSC to reduce nonspecific RNA binding (Westphal and Dulbecco, 1968). Annealing was carried out at 69–70° in 4 × SSC or at 37° in 70% 4 × SSC–30% formamide (Bonner *et al.*, 1967). The retention of DNA on filters throughout the procedure was greater than 95%. The radioactivity on blank filters was subtracted from that on DNA filters in all cases. (Radioactive rRNA bound to blank filters at the same level as to filters bearing 10 μg of *B. subtilis* DNA).

For hybridization of DNA in solution, DNA was denatured in 0.5 N KOH at 37° for 2 hr and dialyzed against 4 × SSC. The DNA concentration was adjusted to 10 μg/ml with 4 × SSC. The DNA was then incubated with 9 μg/ml of rRNA for 4 hr at 69–71°, diluted with an equal volume of water, and treated with 0.1 μg/ml of T_1 ribonuclease and 20 μg/ml of pancreatic ribonuclease for 1–2 hr at room temperature. Solid CsCl and marker DNA were added, and the solution was adjusted to a refractive index of 1.4024 and centrifuged at 20° for 36 hr at 35,000 rpm in a Spinco SW-50.1 rotor.

Density Shift Experiments. To density label DNA, cultures were incubated in the dark in medium containing 25 μg/ml of iododeoxyuridine (IdU). Analytical CsCl gradients were performed according to Meselson *et al.* (1957), using 1 μg of *Physarum* DNA and 0.5 μg of φe DNA per cell. Densities were calculated using the equation of Sueoka (1961) and assuming a density of 1.742 g/cm^3 for φe DNA (Szybalski, 1968). Preparative CsCl gradient centrifugation was performed in a Spinco 50 rotor as previously described (Horwitz and Holt, 1971) using 8–25 μg of DNA per gradient. Gradients of density labeled DNA were adjusted to a refractive index (25°) of 1.4003–1.4008 before centrifugation. In order to assign densities to particular gradient fractions we first determined a standard empirical curve relating density to fraction number. One-drop samples were collected under mineral oil at three fraction (one fraction = six drops) intervals and the density of the one-drop samples was determined by refractive index measurement (Anderson and Anderson, 1968). A total of 15 gradients, centrifuged at four different times, were studied in this manner. The density profiles obtained could always be made to coincide by translation of the curve along the density axis. The density of each fraction of a gradient was determined by assigning the density of the peak of principal nuclear DNA measured on analytical gradients to the OD peak of principal nuclear DNA on the preparative gradients and reading the densities of the other fractions off the density profile curve.

Analytic Procedures. RNA was analyzed in polyacrylamide gels by the method of Weinberg *et al.* (1967). DNA was fractionated in CsCl gradients as described by Horwitz and Holt (1971); the refractive index of the solution was adjusted to 1.3993–1.3998 and centrifugation was for 48 hr at 22° in a Spinco 50 rotor. Six-drop fractions were collected and diluted with 0.2–0.4 ml of 0.1 × SSC for measurement of optical density. Aliquots of gradient and column fractions were either precipitated on filter paper disks (Holt and Gurney, 1969) and counted in Omnifluor–toluene scintillation fluid (New England Nuclear) or counted directly in toluene–methoxyethanol–Omnifluor (600 ml:400 ml:4 g). Hydrolyzed gel slices were counted in 10 ml of the latter scintillation fluid. Hybridization filters were counted in toluene–Omnifluor. Chemical determinations of DNA, RNA, protein, and polysaccharide were carried out by conventional methods (Burton, 1956; Dische, 1955; Lowry *et al.*, 1951; Smith and Montgomery, 1956) using as standards 2-deoxyribose, 5′-AMP, bovine serum albumin, and glucose, respectively.

Results

Preliminary Characterization of RNA and DNA Used for Hybridization. The 4S RNA, 5S RNA, and rRNA preparations contained no detectable DNA or protein under conditions where 1% contamination would have been detected.

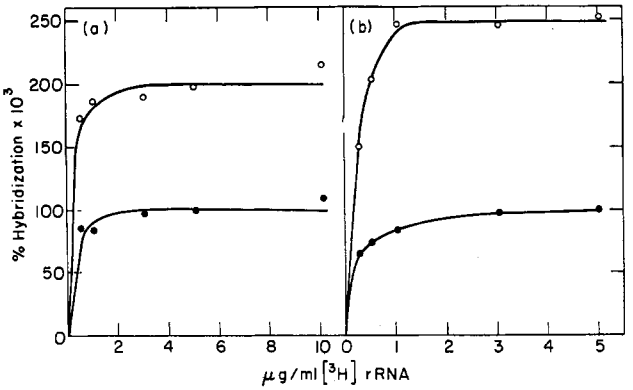

FIGURE 4: Hybridization of rRNA with DNA before and after purification of DNA on ethidium bromide–CsCl gradients and MAK columns. (a) Filters containing either 12 μg of [^{14}C]DNA purified by the standard procedure or 5 μg of the same DNA further purified on ethidium bromide–CsCl gradients were incubated in vials containing various concentrations of [^{3}H]rRNA (6.64 × 10^{4} cpm/μg) in 4 × SSC for 18 hr at 70°. Samples on the plateau contained approximately 1000 cpm. Blank filters contained less than 100 cpm: (○) ethidium bromide–CsCl purified DNA; (●) standard DNA. (b) Filters containing 15 μg of [^{14}C]DNA purified by the standard procedure or 15 μg of the same DNA further purified by MAK column chromatography were incubated as described in a above. Samples on the plateau contained about 1000 or 2500 cpm. Blank filters contained less than 100 cpm: (○) MAK-purified DNA, (●) standard DNA.

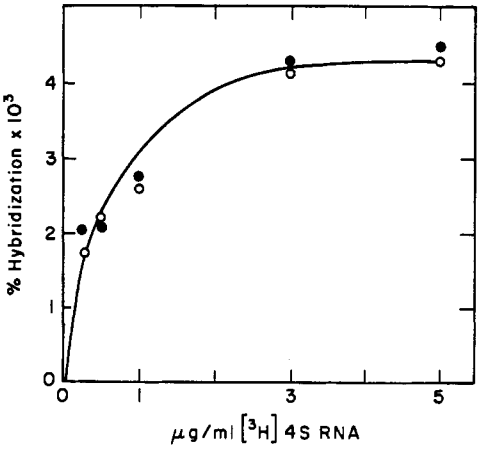

FIGURE 5: Hybridization of 4S RNA with DNA before and after purification of DNA on MAK columns. Filters containing 15 μg of the same DNA preparations used in Figure 4b were preincubated at 70° for 7 hr with 10 μg/ml of nonradioactive rRNA in 4 × SSC, rinsed, and then further incubated with various concentrations of tritiated 4S RNA (6.63 × 10^{4} cpm/μg) for 20 hr at 70°. The filters were processed as described in Materials and Methods. Samples on the plateau contained about 70 cpm. Blank filters contained less than 15 cpm: (○) MAK-purified; (●) standard DNA.

The amount of sugar in the preparations could be accounted for by ribose in the RNA. More than 95% of the acid-precipitable radioactivity was made acid soluble by pancreatic ribonuclease and more than 99.5% of the radioactivity was made acid soluble by 0.5 N NaOH. Thus, the RNA preparations are pure RNA by the usual criteria.

The most retarded peak from the Sephadex columns was identified as 4S RNA by coelectrophoresis with an excess of unlabeled 4S RNA from yeast. The 4S RNA preparations contained less than 1% 5S RNA (Figure 2). Evidence on the purity of the rRNA preparations is given below.

Nuclear DNA preparations contained no detectable protein or RNA and their ratio of absorbance at 260 mμ to that at 280 mμ was 1.96:2.00. The usual DNA preparations contained between one and four times as much polysaccharide as DNA by weight. Preparations further purified on MAK columns or CsCl–ethidium bromide gradients were free of polysaccharide.

Fraction of the Genome Coding for rRNA. The 25S and 19S components of the rRNA were hybridized separately to nuclear DNA (Figure 3); the ratio of the saturation values was 2.0. The 19S rRNA preparation was not contaminated with 25S rRNA since the 19S species alone reached saturation and since cohybridization of tritiated 19S rRNA with nonradioactive 25S rRNA did not decrease the saturation value. The observed ratio of saturation values is essentially the same as the ratio (1.9–1.93) of molecular weights of the two RNA species (Jacobson, 1971; Malera *et al.*, 1970). This indicates that no extraneous hybridizing RNA species are included in the rRNA preparation.

The saturation value for rRNA was about 0.1% (Figure 3), which corresponds to 300 rRNA genes per diploid nucleus. Five out of a total of six preparations of nuclear DNA tested gave values of 0.10–0.11%. Several different rRNA preparations were used and all gave the same results. The sixth DNA preparation gave a value of 0.06%. The saturation values were the same whether the incubation was carried out at 70° or in formamide at 37°.

Saturation curves were also determined with DNA that was prepared in the usual way and then freed of polysaccharides by chromatography on MAK columns or by centrifugation on ethidium bromide–CsCl gradients. The saturation values were 2–2.5 times higher than those above (Figure 4). For several reasons, we believe that the increase in per cent saturation is not due to interference of polysaccharide with the hybridization reaction. (1) Polysaccharide concentration was measured before and after passage of DNA solutions through the hybridization filters. The concentration was unchanged, showing that little or no polysaccharide is bound to the filters. (2) The saturation value for 4S RNA was not affected by removal of polysaccharide (Figure 5). (3) A partial separation of polysaccharide and DNA is achieved on CsCl gradients (see, *e.g.*, Holt and Gurney, 1969). Saturation values calculated for nuclear DNA purified on CsCl gradients were the same as those for the DNA before gradient centrifugation. The increase in per cent saturation may be explained by selective loss of principal nuclear DNA during the final purification step. About 40% of the DNA applied to MAK columns and 5–50% of the DNA centrifuged in ethidium bromide–CsCl gradients was recovered. Postulation of preferential loss of principal nuclear DNA is reasonable in view of the fact that it is separable from nuclear satellite DNA by physical treatments (see Discussion).

Density of Ribosomal DNA. Autoradiographs of plasmodia exposed to [^{3}H]thymidine during the G2 phase (when nuclear satellite DNA but not principal nuclear DNA is replicated) show that the radioactivity incorporated into nuclei is localized in the nucleoli (Guttes and Guttes, 1969). This, and the relatively high density of nuclear satellite DNA, suggested to us that this DNA might code for rRNA. To determine whether rDNA has the same buoyant density as nuclear satellite DNA, [^{14}C]nDNA was banded on a CsCl gradient and DNA from each fraction of the gradient was hybridized with [^{3}H]rRNA (Figure 6). The peak of hybridized rRNA was located at the position expected for nuclear satellite DNA (Holt and Gurney,

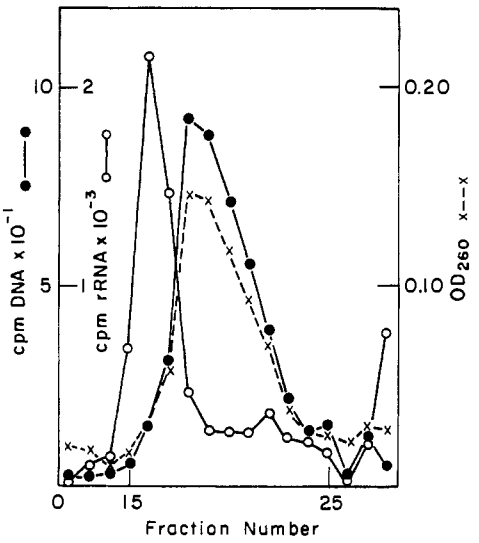

FIGURE 6: Density distribution of rDNA. Thirty micrograms of [^{14}C]DNA (28 cpm/μg) was centrifuged in a CsCl gradient as described in Materials and Methods. DNA from fractions 13–28 was immobilized on filters and incubated together with blank filters at 70° for 18 hr in 3 ml of 5 μg/ml of [^{3}H]rRNA (4.87 $\times$ 10^5 cpm/μg). Filters were processed as described in Materials and Methods. Blank filters contained an average of 209 cpm. The blank value was subtracted from the data shown: (●) cpm of DNA on filters; (○) cpm of rRNA hybridized; (×) OD_{260} of gradient fractions.

1969). About 80% of the rDNA banded in the nuclear satellite region. The remainder of the rDNA appeared in a shoulder extending into the principal nuclear DNA region (Figure 6). The hybridization conditions used in this experiment lead to saturation of DNA with rRNA. The per cent of DNA homologous to rRNA (calculated from the total [^{14}C]DNA recovered and the total [^{3}H]rRNA hybridized) is 0.094%, a value close to the saturation value of total nuclear DNA without prior CsCl fractionation (see above).

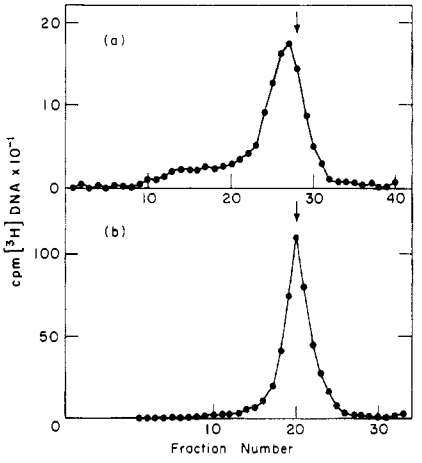

FIGURE 7: Hybridization of nuclear satellite DNA and rRNA. (a) A plasmodium was labeled with [^{3}H]thymidine (16.5 mCi/μmol; 80 μCi/ml) for 3 hr during the G2 period and nuclear DNA was extracted. The DNA was banded on CsCl and fractions containing the nuclear satellite DNA were combined. The nuclear satellite DNA was denatured, hydridized with rRNA in solution, mixed with ^{14}C marker DNA, and centrifuged in CsCl. (b) As a control, nuclear DNA isolated from a culture labeled for 1.5 doublings in [^{3}H]thymidine (0.7 mCi/μmol; 14 μCi/ml) was denatured, hybridized and centrifuged as in a. The marker DNA is alkaline-denatured nuclear DNA isolated from a culture labeled for four doublings in [^{14}C]thymidine (1 μCi/ml). Arrows indicate the position of the ^{14}C marker DNA peaks.

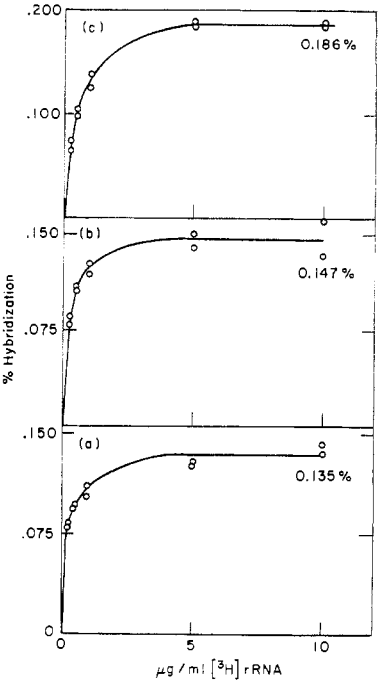

FIGURE 8: Hybridization of rRNA with DNA isolated from synchronous cultures. Fifteen synchronous cultures were incubated on medium containing [^{14}C]thymidine (0.5 nCi/μg; 2 μg/ml) from the time of preparation. At MII + 3.5 hr, MII + 7 hr, and MII + 11.5 hr (prophase of MIII), five cultures were harvested, and nuclear DNA was isolated. Filters containing 7–10 μg of DNA were incubated in vials containing varying amounts of [^{3}H]RNA (1.36 $\times$ 10^5 cpm/μg) at 70° for 19 hr and processed as described in Materials and Methods. Samples on the plateau contained 1400–1700 cpm and blank filters about 100 cpm: (a) MII + 3.5 hr; (b) MII + 7 hr; (c) MII + 11.5 hr.

In determining the buoyant density of rDNA, it was important to centrifuge no more than 30 μg of DNA on a gradient. If increasing amounts of DNA were centrifuged, an increasing fraction of the rDNA appeared on the light side of the principal DNA peak. When rDNA from the light side was recentrifuged, in CsCl, the bulk of it now banded at the density of nuclear satellite.

Hybridization of Labeled Nuclear Satellite DNA with rRNA. To determine more directly whether the nuclear satellite DNA codes for rRNA, plasmodia were labeled during G2, thereby preferentially labeling the nuclear satellite DNA (Holt and Gurney, 1969). The nuclear satellite DNA was further purified on a CsCl gradient, denatured, hybridized with rRNA in solution, and rerun on CsCl. The profile of the hybridized DNA (Figure 7a) displays a shoulder at a density higher than that of denatured DNA and in the region expected for DNA–RNA hybrids (Brown and Weber, 1968). In various experiments of this type, the shoulder contained between 10 and 25% of the radioactivity recovered from the gradient. The specificity of the hybrid formation was shown by doing the same experiment with DNA that was isolated from cultures incubated with [^{3}H]thymidine for 1.5 generations. With this DNA, only a very small fraction of the radioactivity appeared in the DNA–RNA hybrid region of the gradient (Figure 7b). Thus, these experiments show that DNA synthesized during the G2 phase of the mitotic cycle codes

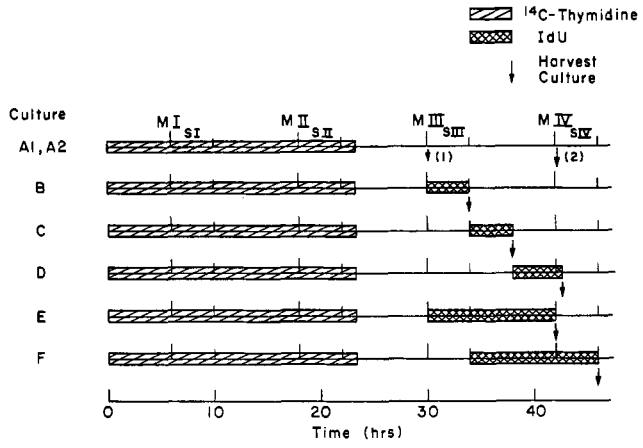

FIGURE 9: Schedule for rDNA density shift experiment. At $t = 0$, 28 synchronous cultures were prepared from the same asynchronous culture. Groups of four cultures, labeled A, B, etc., were incubated on medium containing [^{14}C]thymidine (0.002 μCi/μg; 2 μg/ml) and on medium containing IdU (25 μg/ml) according to the scheme illustrated. At the times indicated by arrows, cultures were harvested, nuclei were isolated, and DNA was prepared from the nuclei.

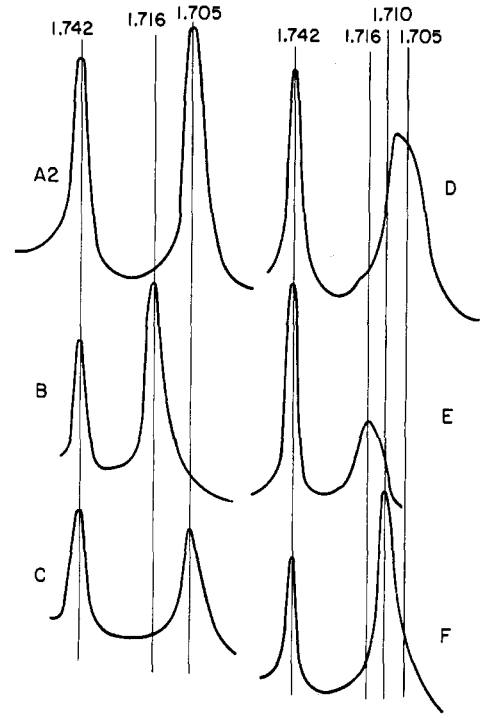

FIGURE 10: Analytical CsCl gradient profiles of IdU-labeled DNA. Samples from the cultures described in Figure 9 were centrifuged to equilibrium in a CsCl solution in a Spinco Model E ultracentrifuge equipped with uv optics. The photographs were scanned on a modified Gilford recording spectrophotometer. The peak at 1.742 g/cm^3 is marker ϕe DNA.

for rRNA. The possible labeled contaminants of our preparations of nuclear satellite DNA are principal nuclear DNA and mitochondrial DNA. Mitochondrial DNA does not hybridize appreciably with cytoplasmic rRNA (Sonenshein, G. E., unpublished results). Furthermore, the mitochondrial DNA and almost all of the small quantity of principal nuclear DNA labeled in this preparation were eliminated in the initial CsCl gradient. Therefore, the experiments also strongly indicate that the G2-labeled DNA that hybridizes with rRNA is the nuclear satellite DNA.

rRNA Saturation Values of DNA Isolated from Synchronous Cultures. To estimate the fraction of rDNA replicated in different periods of the mitotic cycle, rRNA saturation values were determined for nuclear DNA isolated from synchronous cultures at different times during the mitotic cycle. DNA was isolated from cultures 3.5 hr after MII (just after S), 7 hr after MII (midway through G2), and 11.5 hr after MII (prophase of MIII). The saturation value of DNA isolated just after S was 0.135% (Figure 8). Approximately halfway through G2, the saturation value had increased to 0.147%, and by the end of G2 the saturation value had further increased to 0.186%. In this experiment, the ratio of the rRNA saturation value at the beginning of G2 to the saturation value at the end of G2 is 1.4. In a similar experiment, this ratio was 1.6. These results suggest that about 50% of the total rDNA is synthesized during G2.

Time of Synthesis by Density Labeling. DNA was density labeled with iododeoxyuridine (IdU) applied to plasmodia at different times in the cell cycle. The schedule for a density labeling experiment is given in Figure 9. Cultures were prelabeled with a low level of [^{14}C]thymidine, chased with cold thymidine, and, at the times shown, pulsed with IdU and harvested. Data on the synthesis of principal nuclear DNA in this experiment are given in Table I. The ^{14}C specific activity of the bulk DNA was 50 cpm/μg prior to the application of IdU (culture A1). DNA from cultures harvested after the passage of one S period had one-half this specific activity, whether IdU was present (cultures B–E) or not (culture A2). The DNA from a culture (F) that was harvested after the passage of two S periods had one-quarter the specific activity. Thus, principal nuclear DNA doubled normally in the presence of IdU.

Figure 10 shows the density profiles of DNA samples from the above experiment. Unsubstituted principal nuclear DNA banded at 1.705 g/cm^3. The same DNA from culture B, which was exposed to IdU for one S period only, banded at a density greater by 0.011 g/cm^3. The density profile of the substituted DNA had about the same width and shape as the profile of unsubstituted DNA, and none of the DNA remained at the 1.705 g/cm^3 position. Thus, substitution with IdU began as soon as the IdU was added, and the percentage substitution did not vary markedly throughout the S period. Culture C, which was exposed to IdU during early G2, gave only unshifted DNA, as expected (Figure 10). Culture D was exposed

TABLE I: Specific Activity of DNA Isolated from Synchronous Cultures.[a]

Culture	Time Harvested	Sp Act. (cpm/μg)
A1	MIII	50
A2	MIV	24
B	MIII + 4 hr	28
C	MIII + 8 hr	25
D	MIV + 20 min	23
E	MIV	24
F	MIV + 4 hr	14

[a] Same experiment as described in Figure 9.

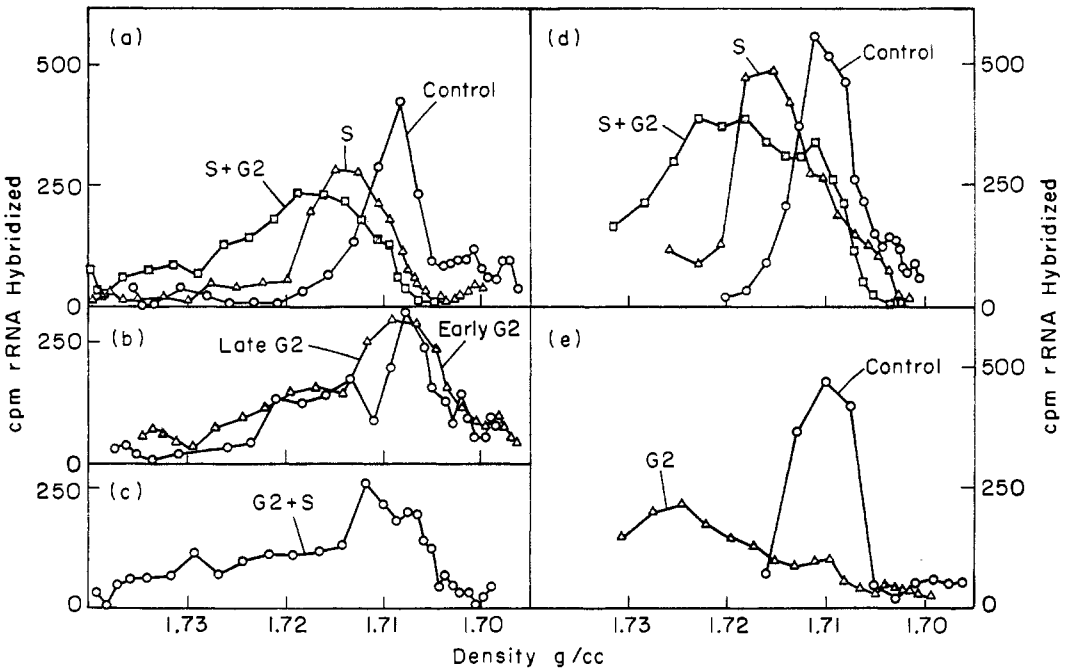

FIGURE 11: Density distribution of IdU-labeled rDNA. DNA was centrifuged in CsCl gradients in a Spinco 50 rotor, and the DNA from each fraction was immobilized on filters and hydridized with rRNA as described in Materials and Methods. The density of each gradient fraction was determined as described in Materials and Methods. (a–c) DNA was prepared from cultures described in Figure 9. Hybridization was for 21 hr at 70° in 5 μg/ml of [^{3}H]rRNA (1.35 $\times$ 10^5 cpm/μg). Blank filters contained an average of 34 cpm. The data for each gradient shown were multiplied by a factor between 1.00 and 1.96 to normalize the data to a constant amount of DNA per gradient (0.206 OD_{260}): control, A2; S, B; S + G2, E; early G2, C; late G2, D; G2 + S, F. (d,e) DNA was prepared from additional cultures prepared essentially as described above. Hybridization was for 20 hr at 70° in 10 μg/ml of [^{3}H]rRNA (1.35 $\times$ 10^5 cpm/μg). Blank filters contained an average of 60 cpm. The data for each gradient shown were multiplied by a factor between 1.0 and 2.0 to normalize to a constant amount of DNA per gradient (0.264 OD_{260}): control no IdU, culture harvested at MIII; S, culture was incubated with IdU from MII to MII + 4 hr; S + G2, culture was incubated with IdU from MII to MIII; G2, culture was incubated with IdU from MII + 4 hr to MIII.

to IdU during the second half of G2 and harvested 20–30 min after MIV. It is clear from the tracing of the photographic plate (Figure 10) that the DNA was partially replicated in the presence of IdU. Thus, when plasmodia are incubated with IdU, the principal nuclear DNA becomes denser, but only during S phase. Although cultures E and F were exposed to the same concentration of IdU for one full cycle, the density of the DNA from culture E (1.716 g) was greater than that from culture F (1.710 g). Exposure to IdU began at the beginning of S phase for culture E and at the beginning of G2 phase for culture F. Thus, preincubation with IdU prior to S phase reduced the per cent substitution.

Incubation of plasmodia with IdU had no effect on the per cent of nuclear DNA that hybridized with rRNA. Cultures A2 and E were harvested at the same time in the mitotic cycle. Culture E had been exposed to IdU for one generation and culture A2 served as a control without IdU. Identical rRNA saturation curves, plateauing at 0.08%, were obtained for the DNA from the two cultures. In addition, culture A1, which was harvested one cell cycle earlier, gave DNA with a saturation value of 0.08%.

Figure 11 shows the density profiles of rDNA from cultures exposed to IdU for various periods of the cell cycle. Each point on the curves represents the total hybridization of rRNA to the DNA in a single fraction from a CsCl gradient. The points are plotted on a linear density axis and are not evenly distributed on the axis due to the nonlinearity of angle head CsCl gradients. The figure includes data from the above experiment as well as data from other similar experiments. For all times of exposure to IdU, some fraction of the rDNA acquired a buoyant density greater than that of unsubstituted rDNA. Therefore, rDNA is synthesized during S phase and throughout G2 phase.

Individual density species are not apparent in the profiles shown in Figure 11. For example, after exposure to IdU during a 4-hr period at the beginning or at the end of the G2 period, the rDNA profiles display broad shoulders extending to densities as great as 1.73 g/cm^3. Exposure for a 4-hr interval encompassing the S period resulted in a broad rDNA peak including the unsubstituted control density and extending to about 1.72 g/cm^3. The heterogeneity in density presumably arises because of varying per cent of substitution with IdU.

Discussion

The results presented in this paper demonstrate that rDNA in growing *Physarum* plasmodia is replicated throughout most of the mitotic cycle rather than at a particular time. The replication of rDNA is not conservative. Application of a DNA density label for a full cell cycle causes the bulk of the rDNA to increase in density (Figure 11) and causes the nuclear satellite DNA observable on analytical CsCl gradients of whole plasmodial DNA to disappear, presumably by gaining a density equal to that of other species in the same gradient (Zellweger *et al.*, 1972). Thus, the data are compatible with a hypothesis that rDNA replicates semiconservatively. If the bulk of the rDNA is a population of independently replicating molecules, one might expect that the amount of this DNA would increase exponentially with time (Brown and Blacker, 1972). The observed replication during G2 phase is nonlinear with time (Figure 8). Additional data will be required to determine whether the increase is indeed exponential and, if so,

whether the model of independently replicating molecules is correct.

The bulk of the rDNA in *Physarum* appears to be identical with the nuclear satellite DNA observed by G2 labeling (Holt and Gurney, 1969), in isolated nucleoli (Zellweger *et al.*, 1972), and sometimes as a small shoulder or peak on analytical CsCl gradients of nuclear DNA (Braun *et al.*, 1965). About 80% of the rDNA has the same buoyant density as the nuclear satellite (Figure 6). The rDNA synthesis that occurs during G2 and the replication of nuclear satellite DNA that occurs in G2 are the same event (Figure 7). Zellweger *et al.* (1972) have isolated amounts of nuclear satellite adequate to account for the rRNA saturation values they observe.

The nuclear satellite rDNA is distinguishable from the bulk of the chromosomal DNA not only in its time of synthesis but in other features as well. (1) On sucrose gradients of crude plasmodial lysates, the satellite DNA sediments much more slowly than the bulk nuclear DNA (Sonenshein and Holt, 1968). (2) When a selective extraction procedure based on salt and sodium dodecyl sulfate precipitation (Hirt, 1967) is applied to plasmodial lysates, the nuclear satellite is separated from the bulk nuclear DNA (Braun and Evans, 1969). (3) When the onset of mitosis and chromosomal DNA synthesis is delayed, nucleolar DNA synthesis continues (Guttes and Telatnyk, 1971). It is conceivable that the nuclear satellite is in fact extrachromosomal, as in the case of the bulk of the rDNA in oocytes (Gall, 1968).

Although our conclusions are generally in agreement with those from Braun's laboratory, we find saturation of nuclear DNA with rRNA at 0.1% of the DNA whereas they report saturation at 1.3% (Zellweger and Braun, 1971). Their curve of rRNA hybridized *vs.* rRNA concentration did not saturate at the highest concentration (about 60 μg/ml) used, which suggests that their preparation of rRNA contained labeled species other than rRNA. The presence of such labeled species might account for part of the difference between 0.1 and 1.3%. It is possible that some of the difference arose from the somewhat different growth conditions used: we obtain a 12-hr doubling time whereas they obtain an 8–9 hr doubling time. The possibility that the different levels of rDNA reflect a physiological adaptation is intriguing but untested.

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