Initiation of Transcription of Ribosomal Deoxyribonucleic Acid Sequences in Isolated Nuclei of *Physarum polycephalum*: Studies Using Nucleoside 5′-[γ-S]Triphosphates and Labeled Precursors†

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ABSTRACT: Nuclei of the plasmodial stage of the lower eucaryote *Physarum polycephalum* contain 100–200 copies of an extrachromosomal, linear, palindromic rDNA molecule of 61 kilobase pairs (kb), which includes duplicate coding regions for 5.8S, 19S, and 26S rRNA species. We have used incorporation of nucleoside 5′-[γ-S]triphosphates and nucleoside 5′-[α-^{32}P]triphosphates to study initiation of ribosomal gene transcription in isolated nuclei. Sulfur-containing, labeled RNA, isolated by organomercurial column chromatography, contains newly initiated rDNA transcripts with sulfur-derivatized 5′-triphosphate termini. Following a 25-min pulse with sulfur-derivatized adenosine 5′-triphosphate (ATP) and ^{3}H-labeled uridine 5′-triphosphate in the presence of α-amanitin, 5.5 ± 2.8% of the incorporated ^{3}H label can be detected in newly initiated chains. Initiation of transcription of RNA complementary to the rDNA preferentially utilizes 5′-[γ-S]-ATP over 5′-[γ-S]GTP by a factor of nearly 15-fold, suggesting that pppA– is the primary intiating group in rDNA transcription. Sequences comprising the rDNA transcription unit (positioned from about 17 to 5 kb from the ends of the palindrome) are selectively transcribed while a segment of at least 27 kb in the central spacer is not transcribed, as determined by hybridizing labeled nuclear transcripts to rDNA restriction fragments. Most rDNA transcripts in nuclei initiate near an *Xho*I cleavage site at 17.0 kb as revealed by hybridization of newly initiated transcripts to restriction fragments. These results are in accord with those obtained using in vivo labeled nuclear rRNA precursor. A significant percentage of rDNA transcripts initiated in nuclei elongates to a size greater than 11 kb.

The ribosomal genes of *Physarum polycephalum* are located on an extrachromosomal, nucleolar satellite DNA molecule of ~61 kilobase pairs (kb)[1] of DNA (Braun & Evans, 1969; Newlon et al., 1973; Bradbury et al., 1973; Molgaard et al., 1976; Vogt & Braun, 1976; Grainger & Ogle, 1978). The rDNA molecule is a palindrome with the rRNA coding regions arranged symmetrically about a large central spacer (Molgaard et al., 1976; Campbell et al., 1979). Positions of the 5.8S, 19S, and 26S rRNA coding regions have recently been mapped relative to cleavage sites of several different restriction enzymes, and the locations of two intervening sequences in the 26S gene have been established (Campbell et al., 1979). In microplasmodia the rDNA constitutes 1 to 2% of the total nuclear DNA (Hall & Braun, 1977). Quantitative changes in α-amanitin-insensitive RNA polymerase activity have been observed in nuclei isolated at different stages in the nuclear proliferative cycle (Mittermayer et al., 1965; Grant, 1972). Davies & Walker (1977) have reported incorporation of labeled nucleotides into rRNA precursors of 34 and 44 S in isolated *Physarum* nuclei. Recently, Seebeck et al. (1978) have described the isolation of rDNA-containing chromatin which possesses endogenous RNA polymerase activity. Electron microscopic observations of Grainger & Ogle (1978) have established that transcription proceeds away from the central spacer of the rDNA molecule toward the ends and that a large central region of the palindrome does not appear to be transcribed. Sizing of rRNA precursors from *Physarum* suggests that the primary rDNA transcript is a molecule of 40–44 S (Jacobson & Holt, 1973; Melera & Rusch, 1973).

In the present study we report that transcription of rDNA in isolated *Physarum* nuclei initiates with a high degree of site specificity and that a fraction of newly initiated chains elongates through the length of the rDNA transcription unit. We have employed a method (Reeve et al., 1977; Smith et al., 1978a,b) in which nuclear RNA is labeled with nucleoside 5′-[α-^{32}P]triphosphates in the presence of purine nucleoside 5′-[γ-S]triphosphates, and labeled RNA with a 5′-terminal sulfur is isolated on organomercurial columns. Our results indicate that this system provides sensitivity in detecting newly initiated chains in isolated nuclei and that it will serve as a useful tool for sequence analysis of newly initiated eucaryotic rRNA.

Materials and Methods

Incorporation of Labeled Nucleoside Triphosphates by Isolated Physarum Nuclei. Nuclei were isolated from 3-day-old *Physarum* microplasmodia or from synchronized plasmodium in the G-2 phase (3 h before mitosis III) as described by Mohberg & Rusch (1971). Nuclei were incubated in a solution (0.25 mL) containing 0.06 M Tris-HCl (pH 7.6), 0.08 M KCl, 4 mM $MgCl_2$, 8 mM $MnCl_2$, 10% glycerol, 0.4 mM CTP, 0.4 mM UTP, 0.8 mM ATP, 4.3 μM [^{3}H]GTP (specific activity 1430 cpm/pmol) or [α-^{32}P]GTP (specific activity 3.8 × 10^5 cpm/pmol), and (1–2) × 10^6 nuclei. Incubations were carried out at 26 °C with or without α-amanitin at a concentration of 4 μg/mL. (*Physarum* microplasmodia grow at 26 °C.) After incubation for 10 min, the reaction was terminated by the addition of 1 mL of cold 10% trichloroacetic acid (Cl_3AcOH) in the presence of 50 μg of yeast tRNA as carrier. The precipitate was collected on a Whatman GF/A filter and washed 3 times with 10 volumes of cold 5% Cl_3AcOH. The filters were dried, and radioactivity was assayed by scintillation spectrometry.

Isolation of Nuclear RNA Pulse Labeled in Vivo. Individual plasmodia (2 × 10^8 nuclei) were pulsed in the G-2 phase for 20 min with 20 mCi of $NaH_2{}^{32}PO_4$ in medium depleted

† From The Rockefeller University, New York, New York 10021. *Received September 20, 1978.* Supported by the National Science Foundation (PCM 76-19926), the American Cancer Society (NP 228 H), and the U.S. Public Health Service (GM 17383 and GM 26174).

[1] Abbreviations used: HS-nRNA, sulfur-containing RNA synthesized in isolated nuclei in the presence of nucleoside 5′-[γ-S]triphosphates; kb, kilobase pairs; nRNA, RNA synthesized in isolated nuclei; PEI, polyethyleniminecellulose; rDNA, DNA comprising the ribosomal genes as well as associated transcribed and nontranscribed spacers.

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of inorganic phosphate (Johnson et al., 1978a,b). RNA was extracted from nuclei essentially as described by Jacobson & Holt (1973). For sucrose gradient analysis, ethanol-precipitated nuclear RNA was resuspended in 10 mM Tris-HCl and 80% formamide (pH 7.6). The solution was heated at 70 °C for 1 min before being diluted with 3 volumes of H_2O, layered onto a 15–30% sucrose gradient containing 0.1% sodium dodecyl sulfate ($NaDodSO_4$), 0.1 M NaCl, 5 mM EDTA, and 5 mM Tris-HCl, pH 7.6, and centrifuged for 20 h at 24000 rpm in a SW 41 rotor. Purified 4S, 18S, and 28S rRNA markers, as well as a 35S Rous-associated virus 2 (RAV-2) RNA marker, were centrifuged in parallel. Fractions of 0.44 mL were collected, and radioactivity was assayed after aliquots of each fraction were diluted with 2 volumes of H_2O and precipitated with cold 10% Cl_3AcOH in the presence of 50 μg of yeast tRNA. Under these conditions a prominent peak of labeled RNA was detected at 40–44 S.

Isolation and Characterization of RNA Labeled with Nucleoside 5′-[γ-S]Triphosphates. Nuclei [(1–2) × 10^6] were incubated with [α-^{32}P]UTP, [α-^{32}P]GTP, [α-^{32}P]CTP, [α-^{32}P]ATP (all from New England Nuclear, with a final specific activity of 2.7 × 10^6 cpm/pmol of nucleotides), 6 μM unlabeled CTP and UTP, 0.1 mM 5′-[γ-S]ATP or 5′-[γ-S]GTP, 0.08 M KCl, 4 μg/mL α-amanitin, 4 mM $MgCl_2$, 8 mM $MnCl_2$, 10% glycerol, and 0.06 M Tris-HCl, pH 7.6, in a final volume of 0.25 mL. After 10 min of incubation at 26 °C, the RNA was extracted as described above. Prior to affinity chromatography on an organomercurial column, RNA was passed through a Sephadex G-25 column (1 × 6 cm) preequilibrated in 10 mM Tris-HCl (pH 7.9), 0.1 M NaCl, 10 mM EDTA, and 0.1% $NaDodSO_4$ (TNES) to remove unincorporated nucleotides. All fractions containing ^{32}P were pooled and directly applied to an Hg–Sepharose column (1 × 9 cm) equilibrated with TNES buffer. [Hg–Sepharose was prepared essentially as described by Ruiz-Carrillo & Allfrey (1973) as modified by Reeve et al. (1977).] The unbound RNA was eluted with TNES buffer. The mercury-bound RNA (HS-nRNA) was then eluted with TNES containing 10 mM DTT. Fractions containing ^{32}P radioactivity were pooled, and the solution was adjusted to pH 5.5 with 20% sodium acetate. Both unbound and mercury-bound RNA were recovered by ethanol precipitation.

For identification of 5′-terminal tetraphosphates, aliquots of concentrated HS-nRNA were brought to 0.3 M NaOH and incubated for 16 h at 37 °C. The hydrolysis reaction was terminated by neutralizing with HCl. The samples were adjusted to 0.1 M ammonium acetate and 1 mM EDTA and chromatographed on a second Hg–Sepharose column equilibrated with 0.1 M ammonium acetate and 1 mM EDTA. After elution of nonbound radioactive nucleotides, the sulfur-containing nucleotides were eluted with the same buffer containing 10 mM DTT. These sulfur-containing nucleotides were analyzed by thin-layer chromatography on polyethyleniminecellulose (PEI) plates developed in 0.75 M potassium phosphate buffer, pH 3.5, as described by Goody & Eckstein (1971) and Reeve et al. (1977).

In order to determine the net negative charge of the sulfur-containing hydrolysis products, we mixed aliquots of sulfur-containing nucleotides from the second Hg–Sepharose column, in 1 mL of 7 M urea, 1 mM mercaptoethanol, and 50 mM Tris-HCl, pH 8.0, with oligonucleotide charge markers (to produce markers, we incubated 1 mg of yeast tRNA and 0.13 μg of RNase A in 0.5 mL of 0.02 M Tris-HCl, pH 7.8, for 18 h at 37 °C) and applied the mixture to a Whatman DE-52 cellulose column (1 × 20 cm) equilibrated with the same buffer (Tener, 1976; Dottin et al., 1976). The nucleotides were eluted with a linear gradient of 0.05–0.3 M NaCl. Radioactivity in each fraction was determined by scintillation spectrometry.

Purification of rDNA and Separation of Restriction Fragments. Nuclei were isolated from microplasmodia according to the procedure of Mohberg & Rusch (1971) as modified by Bradbury et al. (1973). Nucleoli were isolated by centrifugation through a cushion of 1.0 M sucrose, 1 mM $CaCl_2$, and 10 mM Tris-HCl, pH 7.4 (Mohberg & Rusch, 1971), following disruption of nuclei by passing through a French pressure cell at 8000 psi at a concentration of 2.5 × 10^8 nuclei/mL in 0.25 M sucrose, 2.0 mM EDTA, and 10 mM Tris-HCl, pH 7.4, at 4 °C. rDNA was purified from nucleoli as previously described (Bradbury et al., 1973; Johnson et al., 1978b). Restriction endonuclease digestion of purified rDNA was carried out as previously described (Campbell et al., 1979). Electrophoresis was performed for 4.5 h at 100 V on 1.4% agarose gels containing 20 mM sodium acetate, 1.0 mM EDTA, and 40 mM Tris–acetate, pH 8.2. Gels were stained with ethidium bromide (1 μg/mL) for photography. rDNA bands were transferred to Millipore HA (0.45 μm) membrane filters by the procedure of Southern (1975) for hybridization. Individual restriction fragments were eluted from agarose gels with an Isco electrophoretic concentrator.

Hybridization of ^{32}P-Labeled RNA to rDNA Restriction Fragments. Hybridization was performed by wetting membrane filter strips containing rDNA fragments with a solution containing 0.1% $NaDodSO_4$, 1.0 mM EDTA, 0.3 M NaCl, 50% formamide, 25 mM Tes buffer, pH 7.0, and labeled RNA (20000–100000 cpm), wrapping them in Saran Wrap, and incubating for 16 h at 50 °C. Following hybridization, filter strips were treated with RNase A (500 units/mL; Worthington) and RNase T1 (5 units/mL; Calbiochem) in 20 mL of 2 × SSC, washed 3 times for 20 min with 50 mL of 2 × SSC, and dried. Autoradiography was performed by using Du Pont Cronex film and a Du Pont Hi-plus intensifying screen.

Labeling of rDNA Restriction Fragments by Nick Translation. Purified restriction fragments (1–5 μg) were labeled by nick translation using *Escherichia coli* DNA polymerase I (2 units; Boehringer) and [α-^{32}P]dATP and -dCTP (each at 1.8 μM; 350 Ci/mmol) as described by Maniatis et al. (1975). Incubation was for 30 min at 18 °C. DNase I was not used. Under these conditions specific activities of greater than 10^7 cpm/μg were obtained while degradation of restriction fragments was limited, as evident by bands obtained upon reelectrophoresis of nick-translated fragments. Prior to hybridization, labeled fragments were heated to 90 °C in 0.5 M NaOH for 5 min. Hybridization of nick-translated fragments with nuclear RNA fractions was carried out by using DNA (20000 cpm) and RNA in 50 μL of 80% formamide, 0.1% $NaDodSO_4$, 1.0 mM EDTA, 0.3 M NaCl, and 24 mM Tes buffer, pH 7.0. After incubation for 18 h at 50 °C, samples were added to 1.0 mL of 30 mM sodium acetate buffer, pH 4.6, containing 1.0 mM $ZnCl_2$, 50 mM NaCl, 5% glycerol, and 1.0 unit of nuclease S_1 (Calbiochem). After incubation for 1 h at 37 °C, samples were precipitated with 10% Cl_3AcOH, collected on Millipore HA filters, washed 3 times with 20 mL of 5% Cl_3AcOH, and assayed for radioactivity.

Results

Synthesis of rRNA in Isolated Nuclei. Isolated *Physarum* nuclei incorporate ^{3}H-labeled nucleoside triphosphates during incubation at 26 °C for periods in excess of 1 h under the

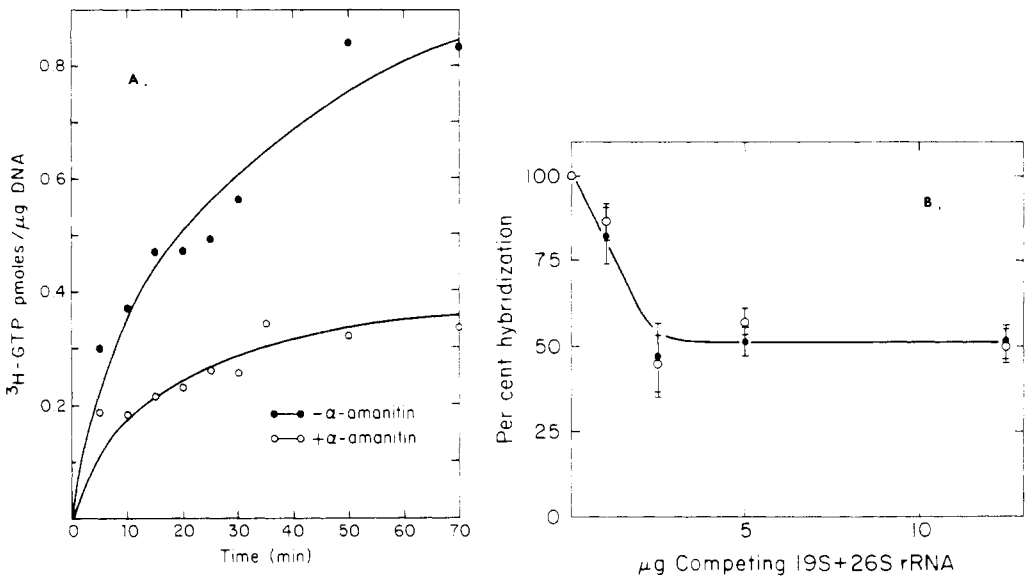

FIGURE 1: (A) Incorporation of [^{3}H]GTP by isolated nuclei. Nuclei were prepared as described and incubated (2×10^6/point) with [^{3}H]GTP for various times at 26 °C as described under Materials and Methods. α-Amanitin was employed at 4 μg/mL. Note that incorporation continues for as long as 60 min. (B) Hybridization of rDNA with labeled nuclear RNA in the presence of competing 19S + 26S RNA. Nuclear RNA was prepared in the presence (●) or absence (○) of α-amanitin as described, following labeling for 10 min with all four of the nucleoside [α-^{32}P]triphosphates to a specific activity of 6.2×10^4 cpm/μg. Nuclear RNA (5.0 μg) was hybridized with sheared purified rDNA (0.9 μg) in solution as described. In competition reactions, annealing in the presence of various concentrations of unlabeled 19S + 26S RNA was allowed to proceed for 4 h prior to addition of labeled nuclear RNA. Note that α-amanitin has no effect upon the extent to which RNA hybridizing to rDNA is labeled. Also note that unlabeled 19S + 26S RNA inhibits hybridization to the rDNA by ~50%.

conditions described (Figure 1A). Incorporation into RNA is indicated by the alkali lability of the labeled product and by the requirement for all four nucleoside triphosphates in the incubation mixture. The agent α-amanitin, used as indicated, inhibits total incorporation by ~50%. It can be seen that in the presence of α-amanitin 0.19 pmol of GTP is incorporated per μg of nuclear DNA in a 10-min pulse with labeled precursor. This translates into a rate of ~750 nucleotides s^{-1} nucleus^{-1} [2 to 3 nucleotides s^{-1} (transcription unit)$^{-1}$]. Rates were not enhanced by inclusion of cytoplasmic extracts during incubation or by varying the KCl concentration from 0.08 to 0.6 M. About 50% of incorporation in the presence of α-amanitin is into rDNA transcription products, as determined by hybridization of labeled nuclear RNA to saturation with purified rDNA. When labeled nuclear RNA is hybridized to purified rDNA in a competition assay following prehybridization of the rDNA with increasing amounts of unlabeled 19S + 26S RNA, one obtains a measure of the percentage of the transcribed region of the rDNA molecule which consists of 19S and 26S coding regions (Figure 1B). In experiments involving hybridization of labeled nuclear RNA, results can be influenced by the presence of unlabeled endogenous RNA in nuclei before incubation. However, in the experiments summarized in Figure 1B, hybridization was carried out in rDNA excess over nuclear RNA and would thus be expected to reveal virtually all labeled RNA sequences complementary to the rDNA. On the assumption of even incorporation of label throughout transcribed rDNA sequences, the result shown indicates that ~50% of the rDNA transcribed consists of sequences external to the 19S + 26S coding regions. Since each of the two 19S + 26S coding regions accounts for 5.8 kb of the rDNA (Campbell et al., 1979), our results indicate nuclear transcription in each half of the palindrome of a region of ~12 kb, consistent with previous measurements of the rDNA transcription unit (Grainger & Ogle, 1978; Melera & Rusch, 1973). It can be seen that α-amanitin has no effect upon the extent to which 19S and 26S rRNA sequences are represented in rDNA transcripts (Figure 1B). This result suggests that all of the rDNA transcription involves an α-amanitin-insensitive RNA polymerase, most likely the nucleolar RNA polymerase previously isolated from *Physarum* (Burgess & Burgess, 1974; Gornicki et al., 1976), comparable to RNA polymerase I of mammalian systems [cf. Roeder (1976)].

RNA Synthesis in the Presence of Purine Nucleoside 5′-[γ-S]Triphosphates. Under conditions similar to those described for Figure 1, isolated nuclei were pulsed with radioactively labeled nucleoside 5′-triphosphates in the presence of either 5′-[γ-S]ATP or 5′-[γ-S]GTP. In numerous experiments we have determined that substitution of the sulfur-derivatized nucleotides for unmodified nucleoside triphosphates does not impair labeling of RNA in isolated nuclei. Incorporation in the presence of sulfur-derivatized triphosphates was always equal to, and in some cases slightly higher than, incorporation in the presence of unmodified triphosphates alone. Following pulse labeling of isolated nuclei, RNA chains bearing a sulfur-derivatized 5′-triphosphate terminus (termed HS-nRNA) were isolated by subjecting purified nuclear RNA to chromatography on Hg–Sepharose organomercurial columns and eluting with dithiothreitol (DTT). Following a 10-min pulse with the triphosphate precursors, the chromatographic profile obtained upon organomercurial affinity chromatography is shown in Figure 2. The results of labeling nuclei in the presence of 5′-[γ-S]ATP or 5′-[γ-S]GTP for 10 and 25 min in the presence of α-amanitin are summarized in Table I. It can be seen in Figure 2 and Table I that the incorporation of ^{32}P-labeled precursors in the presence of 5′-[γ-S]ATP, expressed as the percent of input radioactivity, is more than twice as high as that in the presence of 5′-[γ-S]GTP at each incubation time. If nucleoside 5′-[γ-S]triphosphates are omitted from the nuclear incubation, no radioactivity is detected upon elution with DTT. It can be seen in Table I that, following a 10-min pulse with 5′-[γ-S]ATP, ~3% of recovered label is detected in HS-nRNA. The actual proportion of newly initiated chains is likely to be higher than this value since the peak of unbound

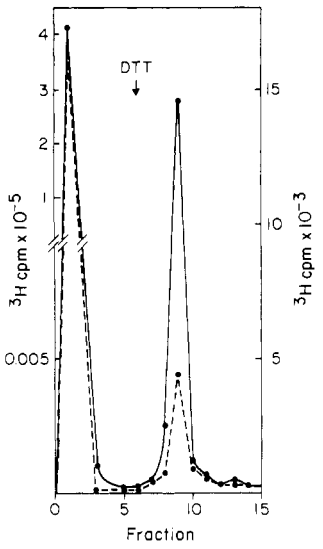

FIGURE 2: Organomercurial column chromatography of RNA synthesized in the presence of purine nucleoside 5′-[γ-S]triphosphates. Nuclei were pulse labeled for 10 min with [^{3}H]UTP in the presence of either 5′-[γ-S]ATP (—) or 5′-[γ-S]GTP (---), and RNA was purified and subjected to Hg–Sepharose column chromatography as described. Fractions 1–6: 9 mL/fraction. Fractions 7–15: 1 mL/fraction, eluted in the presence of 10 mM DTT. Radioactivity of each fraction was assayed by Cl_3AcOH precipitation as described under Materials and Methods. The scale on the left of the figure refers to the initial peak of radioactivity, while the scale on the right refers to the radioactivity eluted after addition of DTT.

Table I: Incorporation of 5′-[γ-S]ATP and 5′-[γ-S]GTP into Nuclear RNA[a]

	organomercurial column chromatography			
	incubn time (min)	RNA recovd in effluent (cpm)	RNA recovd in eluate as HS-nRNA (cpm)	% RNA recovd as HS-nRNA
5′-[γ-S]ATP	10	45 000	1530	3.3[b]
	25	107 000	7490	6.5[b]
5′-[γ-S]GTP	10	41 000	536	1.3
	25	67 000	878	1.3

[a] Nuclei (3×10^7) were incubated with nucleoside 5′-[γ-S]triphosphates (0.1 mM) and [^{3}H]UTP (specific activity 8×10^5 cpm/pmol) at 26 °C in the presence of α-amanitin as described under Materials and Methods. Total nuclear RNA was then extracted and subjected to affinity chromatography on an Hg–Sepharose column as described. Radioactivities were determined following precipitation of RNA with cold 10% Cl_3AcOH in the presence of 60 μg of carrier RNA. Precipitates were collected on Millipore HA membrane filters, washed twice with 10 mL of 5% Cl_3HcOH, and assayed by scintillation spectrometry. [b] Results presented are from a typical experiment. Averages for 5′-[γ-S]-ATP at 10 and 25 min of incubation are 3.1 ± 0.8 and 5.5 ± 2.8%, respectively.

labeled nuclear RNA may also include chains initiated with underivatized ATP, as well as some acid-soluble labeled material, e.g., unincorporated free nucleotides and short oligonucleotides. The percent of acid-precipitable RNA recovered as HS-nRNA in Table I is ~6.5% after a 25-min pulse. Over many experiments, the average recovery was 5.5 ± 2.8%.

Identification of RNA 5′-Termini Bearing [γ-S]Triphosphate Groups. Following incubation of isolated nuclei for 10 min with 5′-[γ-S]ATP or 5′-[γ-S]GTP and all four of the nucleoside 5′-[α-^{32}P]triphosphates, HS-nRNA was isolated by affinity chromatography on an Hg–Sepharose column as described for Figure 2. The HS-nRNA was then subjected to alkaline hydrolysis and rechromatographed on an Hg–Sepharose column. The nucleotide fraction retained on the column and eluted with DTT was then chromatographed on a DEAE-cellulose column in the presence of added unlabeled oligonucleotide charge markers (derived from RNase A digestion of yeast tRNA). It can be seen in Figure 3 that less than 10% of the recovered radioactive nucleotides from *Physarum* nuclear transcripts containing a 5′-terminal sulfur label elutes at the position of nucleoside monophosphates, which possess a net charge of −2 under the elution conditions. The bulk of ^{32}P-labeled hydrolysis products elutes at the position of nucleoside tetraphosphates, which possess a net charge of −6 (Figure 3). A similar result, but of lower magnitude, is obtained following hydrolysis of RNA labeled in the presence of 5′-[γ-S]GTP. It can be seen in Figure 3 that alkaline hydrolysis also yields a secondary nucleotide with a net charge of −5 in addition to the nucleotide with a charge of −6. This product with a charge of −5 may be the result of partial degradation of the thiophosphate analogue or cyclization of the tetraphosphate. When 5′-[γ-S]ATP is chromatographed on the DEAE column, it elutes with a predominant net charge of −4 (Figure 3). Thus, following incorporation into nuclear RNA and alkaline hydrolysis, the sulfur-containing nucleotide has acquired ^{32}P radioactivity and two more negative charges. The results obtained using either 5′-[γ-S]ATP or 5′-[γ-S]GTP are consistent with hydrolysis of labeled HS-nRNA to yield the product S-pppN*p, which occurs at 5′ termini. Little or no incorporation of sulfur-derivatized nucleotides occurs internally in the nuclear RNA chains, as indicated by the lack of sulfur-containing monophosphate hydrolysis products. Following alkaline hydrolysis of the HS-nRNA and rechromatography on the Hg–Sepharose column as described above, the ^{32}P-labeled sulfur-containing hydrolysis products were also subjected to chromatography on thin-layer PEI plates along with unlabeled standards: purine nucleoside 5′-[γ-S]triphosphates, ATP, GTP, ADP, GDP, AMP, and GMP. After being labeled in the presence of 5′-[γ-S]ATP, the sulfur-containing ^{32}P-labeled hydrolysis

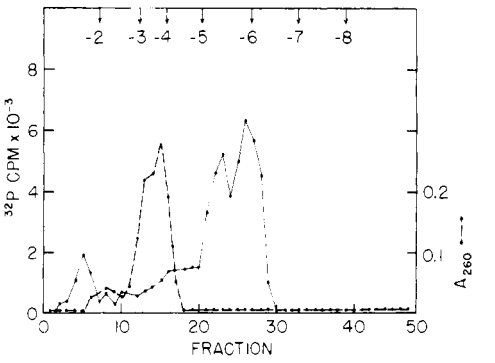

FIGURE 3: Determination of net negative charge on sulfur-bearing nucleotides obtained following alkaline hydrolysis of HS-nRNA. HS-nRNA was obtained by pulse labeling nuclei in the presence of all four of the nucleoside [α-^{32}P]triphosphates and 5′-[γ-S]ATP for 10 min and isolating the sulfur-containing RNA on an Hg–Sepharose column as described. HS-nRNA was then subjected to alkaline hydrolysis, and sulfur-bearing nucleotides were isolated by chromatography on another Hg–Sepharose column. These labeled sulfur-bearing nucleotides were then subjected to DE-52 cellulose column chromatography in the presence of added 5′-[γ-S]ATP and in parallel with an RNase A digest of yeast tRNA to serve as charge markers as described. Absorbance at 260 nm reveals the presence of added 5′-[γ-S]ATP, and ^{32}P cpm reveals the presence of labeled sulfur-bearing nucleotides. Note that the bulk of sulfur-bearing hydrolysis products elutes with a net charge of −6 while the 5′-[γ-S]ATP has a net charge of −4.

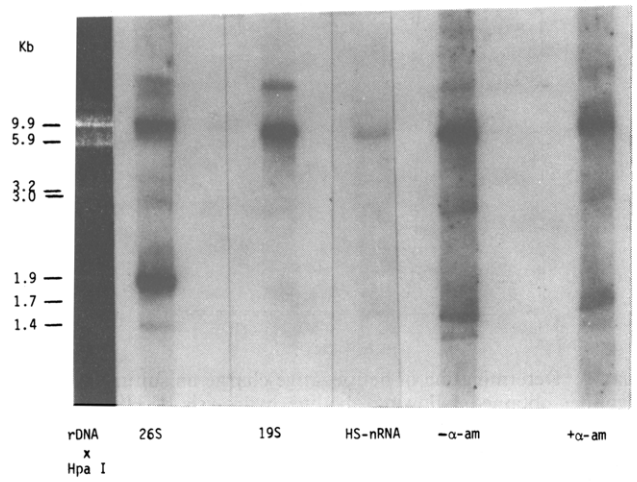

FIGURE 4: Hybridization of ^{32}P-labeled nuclear RNA to rDNA fragments generated by restriction endonuclease *Hpa*I. Restriction fragments were prepared as described, separated on a 1.4% agarose gel, and transferred to a member filter as described. (Note that the most central *Hpa*I fragment is too small to be recovered from this gel.) At the left is a strip of the gel showing rDNA bands stained with ethidium bromide. Hybridization was carried out as described using either ^{32}P-labeled 26S RNA, ^{32}P-labeled 19S RNA, ^{32}P-labeled HS-nRNA synthesized as described using 5′-[γ-S]ATP (HS-nRNA), nuclear RNA pulse labeled for 10 min with [α-^{32}P]GTP in the absence of α-amanitin (−α-am), or nuclear RNA pulse labeled for 10 min with [α-^{32}P]GTP in the presence of α-amanitin (4 μg/mL) (+α-am). Approximately 10^5 cpm was used for each hybridization.

product had the chromatographic mobility of a tetraphosphate, as determined by autoradiography of the thin-layer plates and comparison with the mobilities of the standards (data not shown). These results indicate that 5′-[γ-S]ATP is incorporated virtually exclusively at the 5′ termini of newly initiated RNA chains in isolated nuclei. Similar results were also obtained by using 5′-[γ-S]GTP, although in this case sulfur-containing ^{32}P-labeled hydrolysis products included the tetraphosphate and a minor but significant percentage of material migrating at the positions of GTP and GMP.

The nucleotide group initiating transcription on the rDNA molecule was studied by hybridizing HS-nRNA to saturation with *Physarum* DNA. HS-nRNA was isolated following pulse labeling of nuclei for 10 min, in the presence of α-amanitin, with either 5′-[γ-S]ATP or 5′-[γ-S]GTP and all four of the nucleoside 5′-[α-^{32}P]triphosphates. The recovered HS-nRNA was hybridized with either purified rDNA or total *Physarum* DNA (to C_0t = 10). Approximately 84% of the hybridized radioactivity was detected reannealed to rDNA when 5′-[γ-S]ATP was used and ~16% when 5′-[γ-S]GTP was used. This nearly 5-fold difference in hybridization to rDNA, taken together with the nearly 3-fold difference in rates of isotope incorporation in the presence of 5′-[γ-S]ATP or 5′-[γ-S]GTP, indicates that initiation of transcription of the rDNA template prefers 5′-[γ-S]ATP to 5′-[γ-S]GTP by a factor of about 15-fold.

Location of Initiation Sites on the rDNA Molecule in Isolated Nuclei. We wished to determine whether initiation on the rDNA molecule in isolated nuclei occurs at specific sites. Total nuclear RNA pulse labeled for 10 min with nucleoside 5′-[α-^{32}P]triphosphates as described was purified, hybridized to rDNA restriction fragments, separated by agarose gel electrophoresis, and transferred to membrane filters by the method of Southern (1975) in order to localize rDNA sequences transcribed in nuclei. Detailed restriction mapping of the ribosomal genes in *Physarum* has been presented elsewhere (Molgaard et al., 1976; Campbell et al., 1979). (A summary of the map of several restriction cleavage sites is shown in Figure 7.) In Figure 4 and Table II hybridization of HS-nRNA is compared with hybridization of total nuclear RNA labeled in vitro. Electron microscopic studies have previously demonstrated the exclusion of central spacer sequences from the rDNA transcription unit (Grainger & Ogle, 1978). The studies presented here demonstrate the fidelity of in vitro transcription with regard to omission of these sequences. Figure 4 shows hybridization of the ^{32}P-labeled RNA to fragments of purified rDNA generated by the restriction endonuclease *Hpa*I. This enzyme is known to give two fragments located entirely within the central spacer of the rDNA molecule (bands at 5.9 and 3.2 kb). *Hpa*I also cleaves at three points near the distal end of the 26S gene. In the autoradiograph shown, hybridization of the rDNA fragments to total labeled nuclear RNA is compared with hybridization to purified ^{32}P-labeled 19S and 26S species. The labeled nuclear RNA hybridizes most extensively to the largest of the *Hpa*I rDNA fragments (Figure 4), which contains sequences coding for both 19S and 26S RNA's. Hybridization to a *Hpa*I band of ~3 kb is also detected. This gel region contains a

Table II: Hybridization of Newly Synthesized Nuclear RNA to rDNA Restriction Fragments

enzyme treatment, size (kb)	map coordinates (kb from end)	hybridization to: labeled nuclear RNA[a] (% detected)	hybridization to: HS-nRNA[b] (% detected)
*Hpa*I			
3.0	0–3.0	<10	0
1.7	3.0–4.7	30	<10
1.4	4.7–6.1	<10	0
1.9	6.1–8.0	<10	0
9.9	8.0–17.9	62	92
3.3[c]	19.9–21.2	0	0
5.9	21.2–27.1	0	<10
3.2	27.1–30.3[d]	0	0
*Hin*dIII			
8.0	0–8.0	34	21
5.0	8.0–13.0	36	<10
35.2	13.0–48.2	30	79
*Pst*I			
3.0	0–3.0	0	<10
2.3	3.0–5.3	<10	<10
10.6	5.3–15.9	90	39
29.3	15.9–45.2	10	51
*Bam*H-I			
2.7	0–2.7	22	19
21.2	2.7–23.9	78	81
13.4	23.9–37.3	0	0

[a] Nuclei were pulse labeled with [α-^{32}P]GTP for 10 min in the presence of α-amanitin, and total RNA was isolated as described. Hybridization to rDNA restriction fragments on membrane filters was conducted as described previously. All data for this table have been calculated from densitometry scans of autoradiographs. Results of representative experiments are presented. [b] Following labeling of nuclei for 10 min with [α-^{32}P]GTP in the presence of 5′-[γ-S]ATP, RNA was extracted and HS-nRNA was isolated by affinity chromatography on an Hg–Sepharose column as described. Hybridization was conducted as described under Materials and Methods. [c] The two *Hpa*I fragments migrate as a doublet on agarose gels. Band 3.0 kb is diffuse due to length heterogeneity and is distinguished from band 3.2–3.3 kb by the narrow bandwidth of the latter (Campbell et al., 1979). [d] The cleavage at ~30 kb from the end of the molecule leaves a central fragment of ~0.6 kb, too small to recover on 1.4% agarose gels. At this point, the order of the three most central *Hpa*I fragments is not precisely known. However, none of these fragments hybridize significantly with nuclear RNA.

heterogeneous band of 3.0 kb (average) containing the ends of the rDNA palindrome (Campbell et al., 1979). The three smallest detected *Hpa*I bands represent fragments near the distal end of the 26S gene. Two of these bands (1.9 and 1.4 kb) hybridize to 26S RNA (Figure 4 and Table II), and these bands also hybridize to labeled nuclear RNA. The third of these bands (1.7 kb) also hybridizes significantly with the labeled nuclear transcripts. This third band is located distal to the 3′ terminus of the 26S gene. The rDNA fragment of 5.9 kb does not hybridize with either 19S or 26S RNA and is located entirely within the central spacer. This rDNA fragment does not hybridize with the labeled nuclear RNA, indicating that these spacer sequences are not transcribed. In addition, a *Hpa*I band at 3.2 kb does not hybridize significantly with labeled nuclear RNA. This band contains sequences located entirely within the central spacer (Campbell et al., 1979). Although the possibility of interference with hybridization by unlabeled endogenous RNA cannot be discounted, this is considered unlikely here since it would presuppose a large pool of nuclear RNA complementary to central spacer sequences. All fragments generated by *Hin*dIII hybridize to the labeled nuclear RNA (Table II). This is expected since all of these fragments contain rDNA coding sequences. The restriction enzyme *Pst*I cleaves the rDNA into four distinct segments, including one center segment which does not contain 19S or 26S rRNA coding regions. It is notable that this center segment hybridizes to some nuclear RNA transcripts (Table II), since this indicates that transcription of the rRNA precursor may occur well into the central spacer. The bulk of hybridization to *Pst*I bands is detected in the fragment of 10.6 kb which contains most of the 19S and 26S rRNA coding regions.

The results of hybridizing labeled HS-nRNA to *Hpa*I fragments are presented in Figure 4 and Table II. The hybridization autoradiograph shown in Figure 4 is with HS-nRNA synthesized by using 5′-[γ-S]ATP. (The HS-nRNA synthesized by using 5′-[γ-S]GTP gives negligible hybridization.) The predominant band observed on the autoradiograph (accounting for more than 90% of the density of exposure) is that of the largest *Hpa*I fragment. This fragment includes all of the 19S gene and part of the adjacent central spacer. No hybridization is detected to the fragment which is located at 3.2 kb, indicating that no initiation occurs in the 3.2-kb fragment derived from the central spacer. In the case of both *Hin*dIII and *Pst*I, the most prominent hybridizing fragment is one which contains central spacer sequences within 3 kb of the 19S gene (Table II). It is clear from hybridization of HS-nRNA to *Bam*H-I fragments that no initiation occurs in the central 13.4 kb of the rDNA (Table II).

Transcription of the rDNA molecule begins central to the 19S gene and proceeds outward toward the ends (Grainger & Ogle, 1978). On the basis of the measurements of the rDNA transcription unit (Grainger & Ogle, 1978) and the rRNA 40–44S precursor (Melera & Rusch, 1973; Jacobson & Holt, 1973), the ends of the rDNA molecule are not likely to be transcribed. However, the termini of the rDNA palindrome contain selectively located single-strand gaps (E. M. Johnson, unpublished experiments), and some RNA chain initiation may occur at those sites in isolated nuclei. This probably accounts for the small but variable amount of hybridization of HS-nRNA to restriction fragments which map in the terminal regions of the palindrome (Table II). In no case does this amount to more than 20% of the hybridization of HS-nRNA to rDNA restriction fragments containing putative in vivo initiation sites. Our results indicate that the bulk of initiation in the isolated nuclei occurs selectively on the rDNA molecule.

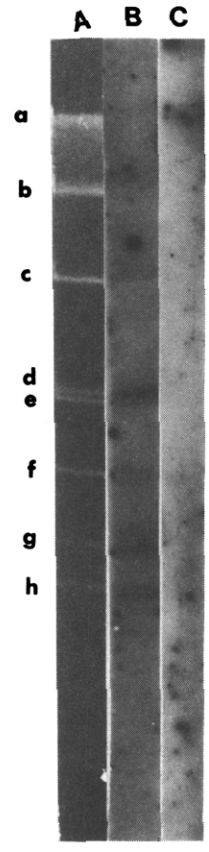

FIGURE 5: Hybridization of rDNA *Xho*I fragments with nuclear ribosomal precursor RNA pulse labeled in vivo and with HS-nRNA pulse labeled in isolated nuclei. *Physarum* microplasmodia were pulse labeled with $NaH_2{}^{32}PO_4$ for 20 min, and isolated nuclei were pulse labeled with α-^{32}P-labeled nucleoside triphosphates and 5′-[γ-S]ATP for 5 min as described under Materials and Methods. Filter hybridizations were conducted by using 3×10^5 cpm/strip. (A) Stained 1.4% agarose gel pattern of rDNA treated with *Xho*I. (B) Hybridization of the *Xho* fragments with nuclear ribosomal precursor RNA labeled in vivo. (C) Hybridization of the *Xho* fragments with HS-nRNA labeled in isolated nuclei. Note that the nuclear precursor hybridizes prominently to bands g and h and slightly to band a. The HS-nRNA, containing predominantly chains 100–200 nucleotides long, hybridizes prominently to band a.

Initiation of the rDNA Transcription Unit. The results in Figure 4 and Table II are consistent with the location of a primary initiation site on the rDNA central to the *Pst*I cleavage site at 16.0 kb. Further experiments were undertaken to localize more precisely the in vitro initiation site and to compare this with the site homologous to the 5′ end of the 12.3-kb rRNA precursor previously described by Melera & Rusch (1973). Nuclear RNA, pulse-labeled in vivo as described under Materials and Methods, was hybridized to *Xho*I rDNA fragments as shown in Figure 5. It can be seen that *Xho* fragments g and h hybridize extensively to the precursor RNA. These adjacent fragments are located central to the 19S gene, from 14.7 to 17.0 kb, and do not hybridize with 19S or 26S rRNA (Campbell et al., 1979). Only slight hybridization could be detected to the *Xho* a band, central to the cleavage site at 17.0 kb and adjacent to *Xho* g. These results indicate that the rDNA transcription unit extends approximately to the *Xho*I site at 17.0 kb. At this point it is not known whether the 12.3-kb rRNA precursor as isolated still contains an intact 5′-triphosphate terminus, and thus the precise location of the initiation point cannot be demonstrated by hybridization with this RNA alone. However, hybridization of the *Xho*I

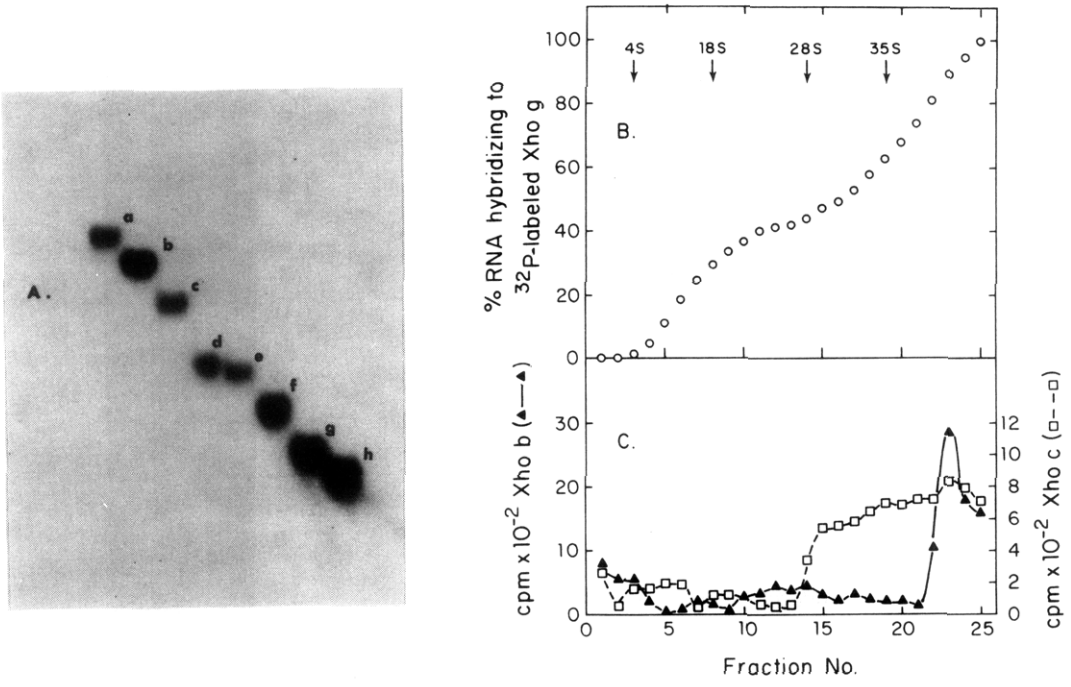

FIGURE 6: Hybridization of nick-translated rDNA *Xho*I fragments with HS-nRNA chains of different lengths. (A) *Xho*I fragments were separated by 1.4% agarose gel electrophoresis and isolated by electrophoretic elution. The isolated restriction fragments were labeled with ^{32}P by nick translation as described to a specific activity of 6×10^7 cpm/μg. Aliquots of the labeled fragments were then subjected again to electrophoresis on a 1.4% agarose gel to test for purity, with the result shown. (B) Aliquots of the ^{32}P-labeled rDNA *Xho* g fragment (20 000 cpm) were hybridized to HS-nRNA chains separated according to size by sucrose gradient sedimentation. HS-nRNA was prepared from nuclei (2×10^7) as described under Materials and Methods following a 20-min pulse with 5′-[γ-S]ATP and [^{3}H]UTP along with other unlabeled triphosphates. HS-nRNA eluted from the organomercurial column with DTT, and a total of 7.7×10^4 cpm was sedimented on a 15–30% sucrose gradient with RNA size markers as described under Materials and Methods. RNA for each fraction was precipitated with ethanol and annealed with the ^{32}P-labeled rDNA segment in solution as described. ^{32}P radioactivity was assayed as described under Materials and Methods. Note that hybridization to the *Xho* g fragment begins after the HS-nRNA chains reach a size of greater than 4 S and that nearly 40% of detected hybridization is to HS-nRNA chains greater than 35 S. (C) Hybridization of the ^{32}P-labeled *Xho* b and *Xho* c fragments to HS-nRNA chains separated by sucrose gradient sedimentation. Sucrose gradient sedimentation and hybridization were conducted as described for panel B. In panel C, hybridized ^{32}P cpm values are presented along the length of the gradient. Note that the *Xho* c fragment hybridizes to shorter HS-nRNA chains than does the *Xho* b fragment. The latter fragment contains the terminus of the rDNA transcription unit.

fragments with rapidly labeled HS-nRNA is consistent with initiation in isolated nuclei near the *Xho*I site at 17.0 kb. As prepared, most HS-nRNA chains average ~100–200 nucleotides in length, as determined by gel electrophoresis. It can be seen (Figure 5) that they hybridize almost exclusively with the *Xho* a fragment. These results, taken together with hybridization of the 12.3-kb precursor, indicate that initiation on the rDNA transcription unit occurs ~200 base pairs central to the *Xho*I site at 17.0 kb.

Elongation of Newly Initiated rDNA Transcripts. Experiments were designed to test whether HS-nRNA chains are elongated from a site near 17.0 kb from the rDNA termini. If initiation occurs on *Xho* a near 17.0 kb and elongation is allowed to proceed, then short HS-nRNA chains should hybridize selectively to fragments near this site while longer chains should hybridize to additional fragments located more distally in the transcription unit. This was found to be the case. HS-nRNA was isolated following a 20-min pulse with 5′-[γ-S]ATP and subjected to sucrose gradient sedimentation. Fractions containing HS-nRNA chains of different lengths were annealed with *Xho*I rDNA fragments labeled to high specific activity with ^{32}P by nick translation and purified as shown in Figure 6, panel A. It can be seen in Figure 6, panel B, that HS-nRNA ranging in size from ~4 to >35 S hybridizes with the *Xho* g fragment. This rDNA fragment is located central to the 19S gene at 15.8–17.0 kb (Campbell et al., 1979) (Figure 7). The result shown indicates that a significant percentage of HS-nRNA chains is elongated through the length of the rDNA transcription unit. More than 40% of hybridization to *Xho* g is detected with HS-nRNA

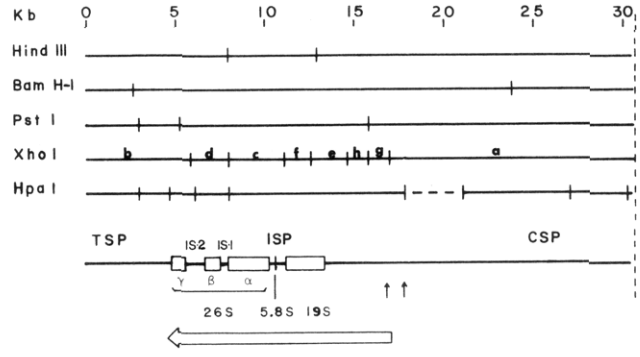

FIGURE 7: Restriction map showing *Physarum* rDNA coding regions and the approximate position of the ribosomal gene transcription unit. The restriction enzymes shown, those discussed in the text, have previously been mapped by Campbell et al. (1979). One-half of the rDNA palindrome is depicted. Rapidly pulsed HS-nRNA hybridizes to *Hpa* a (Figure 4) and to *Xho* a (Figure 5), indicating limits for initiation points as shown by small vertical arrows. The direction of transcription and the length of the transcription unit are as indicated by the horizontal arrow at the bottom.

chains of size greater than 35 S (Figure 6, panel B). Significant hybridization to *Xho* g is also detected with chains of ~5 S, but virtually no hybridization is seen with chains less than 4 S (Figure 6, panel B). (Approximately 80% of HS-nRNA is of size 4–8 S as measured by gel electrophoresis, while ~3–5% is of size greater than 35 S). With *Xho* b, shown in Figure 6, panel C, significant hybridization is detected only with HS-nRNA chains greater than 35 S. The *Xho* b fragment is located from 0 to 5.9 kb and includes the 3′

terminus of the 26S gene (Campbell et al., 1979). This result indicates that only newly initiated chains which have elongated to great size can hybridize with fragments at the 3′ terminus of the transcription unit. Consistent with this notion are the results obtained with *Xho* c. This fragment is located from 8.0 to 11.1 kb and hybridizes significantly only with fragments greater than 18 S. These results are consistent with initiation of HS-nRNA near 17.0 kb followed by elongation through the rDNA transcription unit.

Discussion

Our results indicate that nucleoside 5′-[γ-S]triphosphates are useful tools in the analysis of initiation of ribosomal gene transcription in vitro. Isolated *Physarum* nuclei incorporate radioactive nucleoside triphosphates into RNA in the presence of sulfur-derivatized nucleotides, and the resulting sulfur-containing RNA chains (HS-nRNA) can be isolated by chromatography on Hg–Sepharose columns (Figure 2 and Table I). Analysis of the sulfur-bearing group in HS-nRNA by DE-52 cellulose column chromatography (Figure 3) following alkaline hydrolysis indicates that the SH moiety is present on a nucleoside tetraphosphate and is thus present at the 5′ termini of the RNA chains. Reeve et al. (1977) and Smith et al. (1978a) have previously shown that *E. coli* RNA polymerase utilizes nucleoside 5′-[γ-S]triphosphates for incorporation at RNA 5′ termini in transcribing various templates. They have also demonstrated the effectiveness of 5′-[γ-S]GTP in initiating 5S RNA synthesis in mouse myeloma nuclei (Smith et al., 1978b). In their studies the sulfur-derivatized nucleoside triphosphates were incorporated at the same rate as the unsubstituted nucleoside triphosphates. Our results extend these findings to demonstrate that these sulfur-derivatized nucleotides can be utilized in isolated nuclei of eucaryotes to initiate ribosomal RNA synthesis.

In *Physarum* nuclei, initiation proceeds at a rate sufficiently high to allow recovery and analysis of newly initiated chains. Following a 25-min pulse with 5′-[γ-S]ATP and radioactive nucleoside triphosphates in the presence of α-amanitin, HS-nRNA averages ~6% of the recovered labeled RNA (Table I). The incorporation of ^{32}P-labeled nucleoside triphosphates into HS-nRNA allows improved sensitivity in detecting newly initiated chains.

RNA synthesis in isolated *Physarum* nuclei maintains a high degree of fidelity with regard to transcription of rDNA coding regions and exclusion of most of the central spacer region. Using a variety of criteria for evaluation, several workers have reported the fidelity of rDNA transcription in the nuclei of several organisms (Zylber & Penman, 1971; Reeder & Roeder, 1972; Ballal et al., 1977). Upon hybridizing either total labeled nuclear RNA or HS-nRNA to *Hpa*I restriction fragments (Figure 4), it can be seen that fragments of 5.9 and 3.2 kb, located within the central spacer, are not transcribed. Similarly, a *Bam*H-I fragment of 13.4 kb, located entirely within the central spacer, is not transcribed (Table II). These results, together with hybridization of HS-nRNA to *Xho*I fragments (Figures 5 and 6), indicate that a segment of ~27 kb, comprising the center of the rDNA palindrome, is not transcribed in isolated nuclei.

Hybridization of rapidly labeled HS-nRNA to purified rDNA restriction fragments allows identification of those fragments containing initiation sites for rDNA transcription in isolated nuclei (Figures 4–6 and Table II). Following a 10-min pulse with sulfur-derivatized ATP, the majority of HS-nRNA chains initiated on rDNA hybridize to restriction fragments likely to contain proper in vivo initiation sites. Upon hybridization of HS-nRNA to fragments generated by either *Hpa*I, *Hin*dIII, *Bam*H-I or *Xho*I, 80–90% of the detected label is hybridized to a single restriction fragment in each case (Figures 4 and 5 and Table II), and in each case this fragment contains putative regulatory sequences proximal to the 19S gene. Hybridization of labeled HS-nRNA to the *Xho*I rDNA fragments (Figure 6) indicates that initiation occurs in isolated nuclei as far as 17 kb from the rDNA termini and as far as 3 kb from the 5′ end of the 19S gene (see Figure 7).

The data presented suggest common starting points for initiation both in isolated nuclei and in vivo. On use of the in vivo labeled rDNA precursor, *Xho* a hybridizes slightly while *Xho* g and h hybridize prominently, suggesting that the precursor is homologous to rDNA sequences extending ~17.0 kb from the termini. The rRNA precursor hybridizes to *Xho* b, suggesting that termination occurs in vivo near the 3′ terminus of the 26S gene. HS-nRNA chains ~100–200 nucleotides long hybridize to the *Xho* a band (Figure 5), suggesting that initiation occurs central to the 17.0-kb cleavage site. Since elongation beyond ~100–200 nucleotides results in hybridization to the *Xho* g band (Figure 6), it is likely that initiation occurs very near the *Xho*I cleavage site at 17.0 kb. Our results are consistent with those of Melera & Rusch (1973), who observed a 12.3-kb rRNA precursor, and with those of Grainger & Ogle (1978), who observed a *Physarum* rDNA transcription unit of 4.0–4.2 μm, corresponding to 12.0–12.6 kb of extended DNA. At present we cannot rule out the possibility that there is more than one starting site for ribosomal gene transcription in vivo or in isolated nuclei. Recent reports indicate that two transcription starting sites are present on each of certain *E. coli* rRNA operons (Gilbert et al., 1979; Young & Steitz, 1979). Preliminary fingerprints following RNase T1 digestion of HS-nRNA hybridizing to rDNA suggest heterogeneity, although further work is required to assess the reasons for this.

It is apparent that not all initiation on the rDNA molecule in isolated nuclei involves the region of rDNA adjacent to the 19S coding sequences. A minor percentage of HS-nRNA chains initiated on rDNA hybridizes to fragments containing the rDNA termini, which are not likely to contain proper in vivo starting sites. This phenomenon is particularly evident in the *Hin*dIII and *Bam*H-I experiments presented in Table II. In each of these cases, ~20% of hybridizing HS-nRNA is associated with rDNA terminal fragments. This may represent a "background" of erroneous initiation at nicks since it is known that eucaryotic RNA polymerases will initiate preferentially at nicks [cf. Chambon (1975)] and since single-stranded nicks or gaps have been found at the *Physarum* rDNA termini (E. M. Johnson, unpublished experiments). Selective initiation predominates over this background by a factor of greater than fourfold, but reduction of erroneous initiations will aid in further sequence analysis of newly initiated RNA.

Initiation of transcription on the rDNA molecule in isolated nuclei preferentially utilizes 5′-[γ-S]ATP over 5′-[γ-S]GTP (Table I) by an overall factor of approximately 15-fold (including incorporation and hybridization), suggesting that pppA– is the primary initiating group in the rDNA transcription unit. It remains to be determined whether pppA– is the 5′-terminal nucleotide group in the rDNA precursor primary transcript as isolated from intact *Physarum* plasmodia. Recently, Batts-Young & Lodish (1978) have identified pppA– as a possible initiating group in rRNA precursor synthesis in the cellular slime mold *Dictyostelium*. In addition, Reeder et al. (1978) found that pppA– is the 5′-terminal nucleotide of the *Xenopus* rRNA precursor. The

relatively small utilization of 5′-[γ-S]GTP may represent erroneous initiation events, but the possibility also exists that a minor fraction of rDNA transcription initiates with pppG–. Although most eucaroytic ribosomal gene transcriptions studied thus far initiate with ATP, initiation with CTP has recently been observed on bacterial ribosomal genes (Gilbert et al., 1979; Young & Steitz, 1979). The extent to which pyrimidine nucleotides function as initiating groups in eucaryotes has not been fully ascertained.

Newly initiated rDNA transcripts can elongate to a size of greater than 35 S. This is evident by selective hybridization of the *Xho* b rDNA fragment to HS-nRNA chains greater than 35 S (Figure 6). This result also implies that chains initiated near 17.0 kb are elongated through the length of the transcription unit. In separate experiments, not presented, we have hybridized HS-nRNA pulse labeled with ^{32}P for various lengths of time with *Hin*dIII and *Xho*I rDNA fragments to estimate the rate of elongation. We find that HS-nRNA chains are elongated at an average rate of about five to six nucleotides per second. This is somewhat higher than the rate of incorporation of label into total nuclear RNA in the presence of α-amanitin (about two to three nucleotides per second) (Figure 1). In the present study, following any labeling period, only ~20% of HS-nRNA is detected in chains greater than 200 nucleotides long. This may reflect the fact that there is always a detectable pool of recently initiated chains which have not elongated very far. Nonetheless, there is significant nucleotide incorporation into HS-nRNA chains greater than 35 S. It is these longer chains (comprising only ~2% of the total HS-nRNA) which account for greater than 40% of the detectable hybridization to the *Xho* g rDNA fragment (Figure 6, panel B). An interesting aspect of Figure 6, panel B, is that HS-nRNA chains which hybridize to *Xho* g are distributed unevenly throughout the size range of the gradient. It appears that many of the newly initiated chains elongate to a size of ~2 kb (18 S) and proceed no further. The bulk of those which do proceed elongates to a size of greater than 35 S. The possibility cannot be ruled out that, following initiation and elongation of a short segment, some additional regulatory factor, missing in isolated nuclei, is required for further efficient elongation. [RNA chain initiation without extensive elongation has been observed in adenovirus 2 infected cells (Fraser et al., 1978) and in HeLa cells (Tamm, 1977) treated with the inhibitor 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole.] It remains to be determined whether any attenuation process, as suggested by those results, is a factor in ribosomal gene transcription in eucaryotes.

Acknowledgments

We thank Dimitrios Drivas for excellent technical assistance. We are indebted to Gerald R. Campbell for assistance in mapping rDNA restriction fragments. Dr. William S. Hayward of the Rockefeller University kindly provided labeled RAV-2 35S RNA.

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