Isolation of a High Specific Activity 35S Ribosomal RNA Precursor from *Tetrahymena pyriformis* and Identification of Its 5′ Terminus, pppAp†

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ABSTRACT: RNA isolated from starved and refed *Tetrahymena pyriformis* GL, pulse labeled with $H_3{}^{32}PO_4$, contains an RNA molecule of 2.4×10^6 molecular weight which can be identified by polyacrylamide gel electrophoresis. This RNA has been shown to be a precursor of the mature 25S rRNA and 17S rRNA by a kinetic analysis of its synthesis, by hybridization competition with pure 25S rRNA and 17S rRNA, and by two-dimensional fingerprint analysis. The 5′-terminal nucleotide was isolated from the total digestion products of the 35S rRNA precursor and shown to be pppAp by analysis of the products of snake venom phosphodiesterase digestion. Low, but significant yields of the 5′ terminus pppAp were also isolated from the 17S rRNA demonstrating that the primary transcript of the rRNA gene in *T. pyriformis* contains the 17S rRNA at its 5′ end.

A primary site for the regulation of gene expression is at the level of transcription. In addition, each RNA synthesized is "processed" in one or more enzymatic steps to form the mature active RNA (Perry, 1976), and any of these processing steps might also serve as a site of regulation of the rate of maturation of a specific RNA.

In eukaryotes, we know little about the recognition events which occur during specific initiation and termination of transcription or in site or sequence-specific processing steps. Questions related to transcription and processing could be more easily approached in vitro if we were able to prepare reasonable amounts of a high specific activity in vivo primary transcription product. Knowledge of the base sequences of the 5′- and 3′-terminal oligonucleotides of the in vivo synthesized primary transcript and the processing intermediates would provide an unambiguous assay for specific initiation and termination of transcription and processing endonuclease activity, in vitro.

Transcription of the *T. pyriformis* rRNA gene and processing of the rRNA precursor is an ideal model system for these studies. The *T. pyriformis* rRNA gene has been isolated and shown to be an extrachromosomal palindrome of 12.6×10^6 molecular weight containing two rRNA transcription units (Gall, 1974; Karrer & Gall, 1976; Engberg et al., 1976). The rDNA can also be prepared in a nucleoprotein fraction whose nucleosomal substructure is identical with that of total macronuclear chromatin (Mathis & Gorovsky, 1976; Piper et al., 1976).

This report describes a procedure for obtaining a high specific activity precursor of the mature 25S, 17S, and 5.8S rRNA. In addition, this precursor is shown to be a primary transcription product, at least at the 5′ end, by the isolation of the nucleoside tetraphosphate, pppAp. Furthermore, since the precursor to 17S rRNA contains a low, but significant level of pppAp, the 17S rRNA must be located at the left end of the primary transcript.

A preliminary report of this work has been presented (Niles, 1977a).

Materials and Methods

Tetrahymena pyriformis GL was obtained from Dr. Kathy Karrer and grown and maintained as described by Gorovsky et al. (1975).

Labeling of T. pyriformis RNA. T. pyriformis was grown at 29 °C in a shaking water bath in proteose peptone medium (Gorovsky et al., 1975) to a concentration of $1-3 \times 10^5$ cells/mL. Cells, $2-4 \times 10^7$, were harvested by centrifugation at 600*g* and gently washed in 50 mL of 0.01 M Tris-Cl, pH 8, containing 0.1 mL of antibiotic, antimycotic mix (Gibco, 5246). The cells were again collected by centrifugation and resuspended at 2×10^5 cells/mL in 0.01 M Tris-Cl, pH 8, antibiotic antimycotic mix and starved for 18–24 h (Mathis & Gorovsky, 1976). At the end of the starvation period the cells were diluted with 1 volume of a refeeding mixture described by Mathis & Gorovsky (1976), except that the potasium phosphate was omitted and the proteose peptone concentration was decreased to 40 mg/L. Three h subsequent to the initiation of refeeding, the cells were collected by centrifugation and resuspended at 10^7 cells/mL in refeeding medium diluted 1:1 with water. The cells were used immediately for labeling.

$H_3{}^{32}PO_4$, from NEN, was dried by vacuum in a 25-mL Erlenmeyer flask. At the initiation of the pulse labeling, 2 mL of cells, at 10^7 cells/mL, were added to the flask and shaken at 29 °C. In kinetic experiments, $H_3{}^{32}PO_4$ (1 mCi) was used and 0.1 mL of cells was removed and disrupted by heating at 65 °C in lysing buffer: 0.1 M Tris-Cl, pH 8, 1% $NaDodSO_4$[1], 40 mM EDTA. The lysate was extracted once with 1 mL of phenol:chloroform:isoamyl alcohol (50:50:1) and the nucleic acid precipitated at −20 °C by the addition of 2.5 volumes of 95% ethanol. In preparative labeling experiments, 50 mCi of $H_3{}^{32}PO_4$ was used and the cells were pulsed labeled for 45 min. The cells were collected by centrifugation at 10 000*g* for 1 min and resuspended in 10 mL of lysing buffer. Cell disruption was assisted by heating the extract at 65 °C for 5 min. The lysate was extracted three times with phenol:chloroform:isoamyl alcohol, 50:50:1, and the nucleic acid precipitated

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; p25S rRNA and p17S rRNA, immediate precursors to the 25S rRNA and 17S rRNA, respectively.

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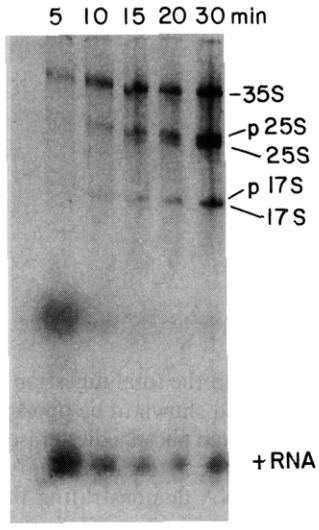

FIGURE 1: *T. pyriformis* GL was pulse labeled for various lengths of time with $H_3{}^{32}PO_4$, and a 100-μL aliquot of cells was removed and the RNA isolated. The pulse times and the number of counts per minute of ^{32}P rRNA applied are as follows: 5 min, 1000; 10 min, 2200; 15 min, 2100; 20 min, 1700; 30 min, 2800. After electrophoresis for 4 h at 4 V/cm, the gel was dried and the positions of the radioactive RNA species were determined by autoradiography.

by the addition of 2.5 volumes of 95% ethanol and stored at −20 °C for 2 h.

The optimal time for refeeding the cells prior to labeling is 2.5 to 3 h. Between 0 and 30 min after adding the refeeding mixture, the rate of labeling of rRNA is less than 10% the rate observed between 2.5 and 3 h. There is a linear relationship between the cell density and the amount of ^{32}P incorporated into rRNA up to 10^7 cells/mL. At this cell density the sample is highly packed and about 30% cells by volume.

The nucleic acid was collected by centrifugation at 10 000 rpm in a Sorvall SS34 rotor, and the pellet dried by vacuum. The pellet was resuspended in 0.1 M Tris-Cl, pH 8, 1 mM EDTA for 1 h at room temperature. The samples for kinetic studies were resuspended in 0.2 mL; the preparative samples were resuspended in 2.0 mL. The DNA was removed by the addition of $MgCl_2$ to 5 mM and 50 μg/mL of DNase I (Worthington RNase free and checked for RNase prior to use). After 30 min at room temperature, $NaDodSO_4$ was added to 1% and EDTA was added to a final concentration of 10 mM. The samples were heated at 65 °C for 10 min. To the samples was added 20% by volume of 50% glycerol, containing bromothymol blue tracking dye.

Gel Electrophoresis. The analytical RNA samples were resolved by gel electrophoresis in 20 × 20 × 0.15 cm, 2.5% acrylamide, 0.5% agarose gels (Summers, 1969). The samples were electrophoresed for 4 h at 4 V/cm. The gels were dried by vacuum and the positions of the radioactive RNA were detected by autoradiography.

Since 2×10^7 cells contain about 4 mg of RNA (Conner & Koroly, 1973), the preparative samples were divided into four aliquots. The RNA in each aliquot was separated by electrophoresis in a 20 × 40 × 0.15 cm, 2–4% acrylamide gradient gel (Niles & Condit, 1975). After 16 h at 3.5 V/cm, the top plate was removed and the gel covered with Saran Wrap. The positions of the RNA were determined by autoradiography of the wet gel. The regions of the gel containing the RNA bands of interest were cut out and the RNA was electroeluted for 24 h at 4 °C, as described by Galibert et al. (1974). The RNA was removed from the dialysis bag, phenol extracted, and precipitated with 2.5 volumes of 95% ethanol. The yield of each RNA species from the gel was greater than 90%. A sample of each RNA fraction was routinely analyzed by analytical gel electrophoresis in order to test contamination which was less than 5% in each case.

The 35S rRNA was routinely prepared in 10-μg amounts with a specific activity of 1.0 to 5×10^6 dpm/μg.

The apparent molecular weights of the 35S rRNA and the p17S rRNA and p25S rRNA processing intermediates were determined by analytical gel electrophoresis in a 2.5% polyacrylamide, 0.5% agarose gel. The *E. coli* 30S, 23S rRNA, 16S rRNA, 5S rRNA, and tRNA species, isolated from *E. coli* BL 311 (Studier, 1975) were used as molecular weight markers.

Hybridization. The ^{32}P-labeled rRNA precursor was hybridized to S and SB6 filters which contained 10 μg of denatured total macronuclear DNA, according to the procedure described by Gall (1974). In competition experiments, cold 25S rRNA and 17S rRNA was prepared by phenol extraction of ribosomes isolated by the methods of Hallberg & Bruns (1976). Saturating levels of 35S rRNA were employed in these experiments.

RNA Sequencing. Fingerprints of the 35S rRNA precursor and 25S rRNA and 17S rRNA were carried out as described by Barrell (1971). Homo C, 3%, 30 min, and 20 × 40 cm glass backed TLC plates (Analtech) were employed in the second dimension.

Total digestion of the rRNA samples was carried out with a mixture of pancreatic RNase, RNase T1, and RNase T2 as described by Barrell (1971). The total digestion products were separated in one dimension on DEAE-cellulose in pH 4 citrate buffer, as described by Kramer et al. (1974). The digestion products of snake venom phosphodiesterase (Worthington) and alkaline phosphatase (purified according to Torriani, 1966) were separated on DEAE-cellulose at pH 3.5. Unlabeled marker nucleotides were purchased from Sigma. pppGp was prepared by RNase T1 digestion of [γ-^{32}P]GTP-labeled T7 late in vitro synthesized mRNA (Golomb & Chamberlin, 1974). $^{32}PP_i$ was prepared as a product of this in vitro transcription reaction.

Results

In Vivo Labeling of rRNA. In pulse-labeled *T. pyriformis*, several groups have identified a 35S RNA molecule whose kinetics of labeling suggested that it was the precursor of the mature 25S rRNA and 17S rRNA (Kumar, 1970; Prescott et al., 1971). The question addressed in this report asks if this is indeed the rRNA precursor, and, in addition, if this is a primary transcript containing an identifiable 5′ nucleoside tetraphosphate. In order to approach these questions, a protocol was developed which permits the preparation of the 35S rRNA with a specific activity of 10^6 dpm/μg or greater.

The procedure is based on the observation that, after starved *T. pyriformis* are refed, ribosome biosynthesis is carried on at a high rate (Hallberg & Bruns, 1976). After pulse labeling the starved and refed cells, which had been packed to 10^7 cells/mL, the RNA is extracted and separated by polyacrylamide gel electrophoresis.

Initially, pulse-labeling experiments were carried out on an analytical scale. In Figure 1, a large RNA, approximately 2.4×10^6 molecular weight, is labeled within 5 min. As the length of the labeling period increased to 10 min, p25S and mature 25S rRNA appear. The p17S rRNA and mature 17S rRNA are poorly resolved in this figure, but they can be identified 10 min after the pulse has begun. If the cells are chased with 100 mM potassium phosphate, pH 7.0, at any time during the pulse, within 15 min, the 35S RNA is no longer labeled.

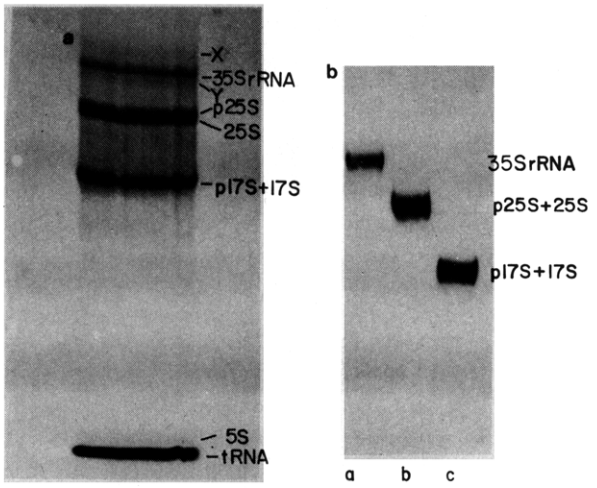

FIGURE 2: (a) An autoradiograph of a preparative 2–4% acrylamide gradient gel (20 × 40 cm) is presented. RNA was prepared from a 1/4 aliquot of a 45-min pulse-labeling mixture containing 50 mCi of $H_3{}^{32}PO_4$ and 2 mL of 10^7 cells/mL. The RNA was isolated and separated by electrophoresis at 3.5 V/cm for 16 h, and the position of the RNA in the wet gel was determined by autoradiography. (b) The gel fractions containing RNA were cut out and the RNA was isolated by electroelution (Galibert et al., 1974). A portion of the isolated RNA species was analyzed by analytical gel electrophoresis as described in Figure 1. Samples a, b, and c are the 35S rRNA precursor, p25S rRNA plus 25S rRNA, and p17S rRNA plus 17S rRNA, respectively.

Therefore, the kinetics of pulse labeling the 35S RNA is consistent with the supposition that it is a precursor of the 25S rRNA and 17S rRNA.

The molecular weights of the major *T. pyriformis* RNA species identified in Figure 1 were estimated by comparing their migration distances with those of the *E. coli* 30S, 23S, 16S, and 5S rRNA, and 4S tRNA isolated from pulse-labeled *E. coli* BL 311 (Studier, 1975). Table I shows that the 35S RNA is approximately equal in size to the sum of the molecular weights of the p25S and p17S rRNA species. The difference of 0.1×10^6 can be attributed to the experimental uncertainty in the molecular weight determination. RNA samples X and Y in Figure 2 are not separated on an analytical gel so their molecular weights have not been determined.

Preparation of large quantities of the 35S RNA from 2×10^7 cells requires separation on four polyacrylamide gradient gels. Figure 2a is an autoradiograph of a preparative gel. In this figure, the 35S RNA is well resolved from other RNA species.The p25S rRNA and 25S rRNA are also separated and can be easily identified. In a 45-min pulse sample, 17S rRNA fraction is largely either mature 17S rRNA or a mixture of the mature 17S rRNA plus one or more processing intermediates. The 5.8S rRNA, 5S rRNA, and tRNA can also be identified in the autoradiograph.

In longer exposures of the film, one can identify several other RNA species. An RNA (Y) which is slightly smaller than the 35S RNA can be observed in low and variable amounts and may be identical with the 30S rRNA reported by Kumar (1970). A RNA band (X), which migrates more slowly than the 35S rRNA, can also be identified in preparative gels. This RNA may either represent a conformational isomer of the 35S rRNA (Slack et al., 1975) or it may be a precursor of the 35S rRNA. Only low levels of X and Y can be isolated at this time; most experiments were carried out on the 35S rRNA.

The RNA fractions can be isolated from the gel by electroelution (Galibert et al., 1974). The homogeneity of the rRNA fractions were routinely tested by an analytical gel electrophoresis of the isolated samples as shown in Figure 2b.

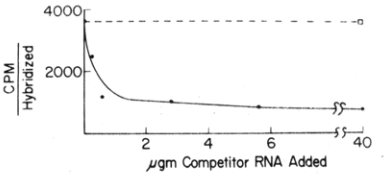

FIGURE 3: Hybridization of the rRNA precursor to total *T. pyriformis* DNA was competed by increasing amounts of purified 25S rRNA and 17S rRNA (●—●). Competition by *E. coli* rRNA was measured as a control (□—□). The hybridization efficiency in the absence of competitor was calculated to be 20% assuming that the rDNA palindrome is 1% of the total *T. pyriformis* DNA (Yao et al., 1974). The specific activity of the 35S rRNA was 4.7×10^5 cpm/μg.

TABLE I: *T. pyriformis* rRNA Molecular Weights.[a]

RNA	mol wt × 10^{-6}
35S rRNA	2.4
p25S rRNA	1.5
25S rRNA[b]	1.3
p17S rRNA	0.8
17S rRNA[b]	0.69

[a] The molecular weights were determined by electrophoresis of ^{32}P-pulse-labeled *T. pyriformis* RNA in a 2.5% acrylamide–0.5% agarose gel. [^{32}P]RNA from pulse-labeled *E. coli* BL311 (Studier, 1975) was employed as a molecular weight standard. The weights represent an average of three determinations carried out on separate preparations of *T. pyriformis* RNA. The values for the molecular weights of the *E. coli* BL311 RNA, used in constructing a standard curve, were 2.1×10^6, 1.1×10^6, 0.55×10^6, 4.2×10^4, and 2.5×10^4 for the 30S, 23S, 16S, 5S, and 4S RNAs. [b] In agreement with the values reported by Loening (1968).

The purified RNA fractions are less than 5% contaminated with any other RNA species.

Is the 35S rRNA Chemically Related to the Mature 25S rRNA and 17S rRNA? Although the size of the 35S RNA and kinetics of labeling the 35S RNA are consistent with that of RNAs role as a precursor of the 25S rRNA and 17S rRNA, chemical proof was required.

The presence of shared sequences was tested by hybridization competition. The 35S RNA was hybridized to nitrocellulose filters containing 10 μg of denatured total *T. pyriformis* macronuclear DNA, in the presence of increasing amounts of pure 25S rRNA and 17S rRNA. The 10 μg of total DNA should contain 0.1–0.2 μg of rDNA (Yao et al., 1974). In Figure 3, it is apparent that the mature 25S rRNA and 17S rRNA serve as very efficient competitors, to a maximum competition level of 85% found in three separate measurements. This maximum competition is consistent with the ratio of the sum of the molecular weights of the 17S rRNA and 25S rRNA (Table I, Loening, 1968) to the apparent molecular weight of the 35S RNA precursor, $1.99 \times 10^6/2.4 \times 10^6$ or 84%. The similarity of these values demonstrates the isotopic purity of the 35S rRNA precursor preparation. *E. coli* rRNA does not compete with the rRNA precursor (Figure 3). Both RNA species X and Y in Figure 2a are competed with 25S and 17S rRNA demonstrating that they are also related to the mature 17S and 25S rRNA (data not presented).

As a final proof that this 35S RNA is the precursor to the mature 25S rRNA and 17S rRNA, pancreatic RNase digestion products of each RNA were separated by two-dimensional fingerprints and compared (Figures 4a–d). The oligonucleotides in the high molecular weight region of the chromatograms were removed and their RNase T1 digestion

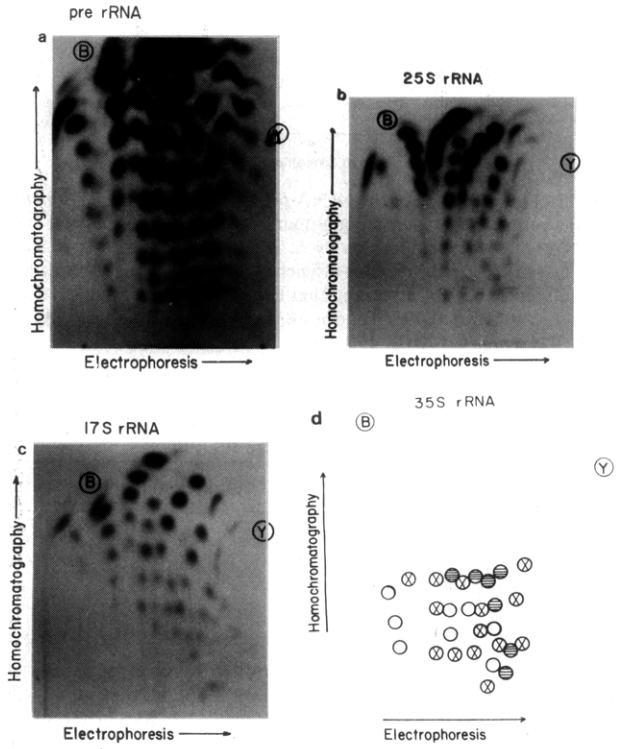

FIGURE 4: The 35S rRNA precursor (a) was isolated from a polyacrylamide gel as described in Figures 2a and 2b. The 25S rRNA (b) and 17S rRNA (c) were isolated from ^{32}P-labeled ribosomes as described by Niles (1977b). d is a tracing of the lower region of the 35S rRNA fingerprint. ⊗ and ⊖ represent oligonucleotides which contain the same RNase T1 digestion products as the 25S rRNA and 17S rRNA, respectively. o represents oligonucleotides which are unique to the 35S rRNA. Two-dimensional fingerprints of the pancreatic RNase digestion products were carried out as described by Barrell (1971). Y and B mark the position of the methyl orange and xylene cyanol FF marker dye.

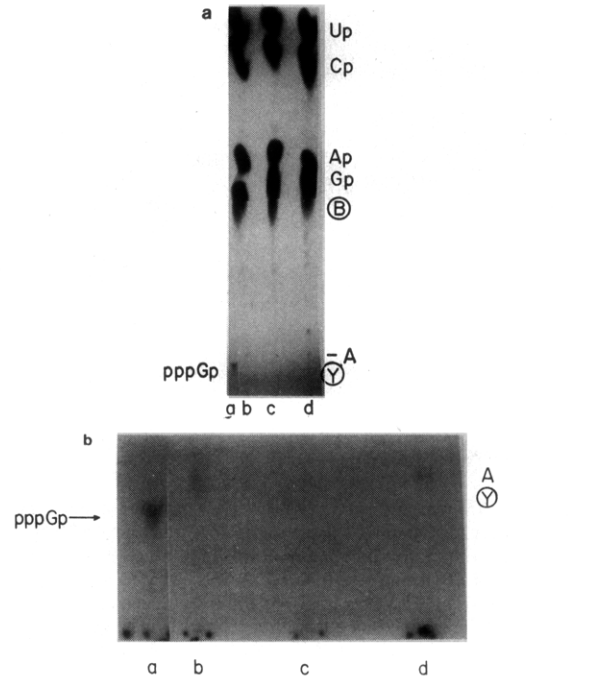

FIGURE 5: (a) The 35S rRNA, the p25S rRNA plus 25S rRNA pool, and the p17S plus 17S rRNA pool isolated from cells pulsed for 45 min were digested to completion according to Barrell (1971) and the products separated on DEAE-cellulose at pH 4 in sodium citrate (Kramer et al., 1974). The pppGp marker was obtained from T7 late mRNA transcribed in vitro and labeled with [γ-^{32}P]GTP. This sample was electrophoresed on a different paper, under identical conditions, and is presented to mark the expected position for pppGp. The yields of pppAp in samples b, c, and d were 82%, <0.1%, and 11%, respectively. The Y and B mark the positions of the methyl orange and xylene cyanol FF marker dye. (b) The lower region of the autoradiograph presented in a has been expanded in order to allow easy identification of spot A.

products compared. Each of the large oligonucleotides present in the mature 25S rRNA and 17S rRNA could be identified in the 35S rRNA precursor.

Is the 35S rRNA Precursor a Primary Transcript? There is no reason to assume that this 35S rRNA is the primary transcript since it could be, itself, a processing product. In order to test this, attempts were made at identifying the 5′ terminus of the 35S rRNA.

The 35S rRNA, p25S rRNA, and 25S rRNA mixture and p17S rRNA and 17S rRNA mixture were prepared from a 45-min pulse labeling. The rRNA samples were digested to mononucleotides and the products were separated according to charge on DEAE-cellulose in sodium citrate at pH 4.0. Figure 5 is an example of this analysis. Adjacent to the *T. pyriformis* rRNA samples is a marker pppGp derived from the 5′ end of the T7 in vitro synthesized mRNA (a). In the 35S rRNA precursor (b), a single spot, A, migrating ahead of the pppGp marker, could be identified which might correspond to a nucleoside tetraphosphate. In the 25S rRNA pool there was no evidence of a nucleoside tetraphosphate (c), while in the 17S pool (d), a spot, comigrating with A, was present. ^{32}P-containing regions of the paper were cut out and counted. Regions above and below the possible nucleoside tetraphosphate were also cut out and counted and these blank values were subtracted from the counts present in the possible 5′ terminus. The stoichiometry of this apparent pppXp was determined by assuming that it has four phosphates and that there is a total of 8000 phosphates in the rRNA precursor, 4000 phosphates in the 25S rRNA, and 2000 phosphates in the 17S rRNA. In ten preparations of rRNA, the yield of this apparent nucleoside tetraphosphate was 50–95% in the 35S rRNA, <0.1–5% in the 25S rRNA fraction, and 5–20% in the 17S rRNA fraction.

The chemical structure of this molecule was determined by digestion with either bacterial alkaline phosphatase or snake venom phosphodiesterase (Figures 6a,b). Alkaline phosphatase digestion yields only inorganic phosphate as a product (Figure 6a) as would be predicted for a substrate which contains only external phosphates. This eliminates the possibility that the spot is a RNase-resistant tri- or tetranucleotide. Snake venom phosphodiesterase digestion yields three products which comigrate with P_i, PP_i, and pAp (Figure 6b), as would be predicted for a nucleoside tetraphosphate with the structure pppAp. The P_i results from contamination of the venom phosphodiesterase with a phosphatase activity.

The presence of pppAp in low but significant yields in the 17S rRNA fraction from 45-min pulse-labeled cells shows that the p17S rRNA contains the same 5′ terminus as the 35S rRNA precursor. The yield of pppAp in the 17S rRNA pool is low because this fraction contains both the p17S rRNA and the mature 17S rRNA, and the 5′ region of the p17S rRNA is removed in the maturation of the 17S rRNA. Consistent with this interpretation, in 10- and 20-min pulse-labeled samples, the yield of pppAp in the 17S rRNA fraction is 55% and 25%, respectively (data not presented). Therefore, the p17S rRNA must be located at the left end of the 35S rRNA precursor and the initial endonucleolytic processing event must occur between the 17S rRNA and 25S rRNA regions of the 35S rRNA molecule a large percentage of the time.

Discussion

A procedure has been presented which permits the preparation of high specific activity *T. pyriformis* rRNA and resolution of this rRNA into a primary transcript plus processing intermediates and mature rRNA species (Figures 2a,b). The 35S rRNA was shown to be a precursor of the mature 25S rRNA and 17S rRNA by a kinetic analysis of its synthesis (Figure 1), by hybridization competition with mature 25S rRNA and 17S rRNA (Figure 3), and by two-dimensional fingerprint analysis (Figures 4a–d). From the 35S rRNA precursor and the 17S rRNA fraction (Figure 5), but not the p25S rRNA and 25S rRNA fraction, was isolated a nucleoside tetraphosphate which was shown by snake venom phosphodiesterase digestion (Figure 6b) to be pppAp.

Several conclusions about the transcription of the rRNA gene and processing of the rRNA precursor in *T. pyriformis* can be drawn from these results.

The 35S rRNA precursor is a primary transcript, at least at the 5′ end since the nucleoside tetraphosphate pppAp can be isolated in high yields. pppAp has also been demonstrated recently to be the initial nucleotide in the 40S rRNA precursor of *X. laevis* (Reeder et al., 1977) and the p17S rRNA precursor in *D. discoideum* (Batts-Young, B., & Lodish, H. F., 1978). The *E. coli* 30S rRNA precursor had previously been shown to initiate with pppAp (Ginsburg & Steitz, 1975).

In the case of the eukaryotic rRNA transcripts, the conservation of the 5′ pppAp may signify a common initiation requirement for the form I RNA polymerases which transcribe the rRNA gene in vivo (Roeder, 1976). A comparison of the subunit compositions of the form I RNA polymerase purified from several organisms shows a high degree of conservation of the constituent subunits (Roeder, 1976). Therefore, it might be reasonable to expect that the information necessary for proper initiation of transcription of the rRNA genes will be similar in different organisms.

The fact that the p17S rRNA contains low levels of pppAp during the 45-min pulse period, while there is no trace of pppAp in the p25S rRNA and 25S rRNA fraction demonstrates that the 17S rRNA region lies at the left end of the 35S rRNA molecule. This polarity is found in all other prokaryotic (Ginsburg & Steitz, 1975) and eukaryotic species studied (Dawid & Wellauer, 1976; Reeder et al., 1976; Schibler et al., 1976; Batts-Young & Lodish, 1978).

In *T. pyriformis* and *D. discoideum* (Batts-Young & Lodish, 1978), since a 5′-tetraphosphate can be isolated from the 17S rRNA precursor, one of the first endonucleolytic cleavages must occur between the 17S rRNA and the 25S rRNA regions in the primary transcript. In most higher eukaryotes (Perry, 1976), however, a portion of the primary transcript containing the 5′ terminus is removed first yielding a processing intermediate which contains both the 18S and the 28S rRNA. Since cleavage of the rRNA precursor both 5′ and 3′ to the 17S rRNA must occur during maturation, the pathway selected will depend on the relative rates of cleavage at each site.

In *T. pyriformis,* there is evidence that both processing pathways are being followed in vivo. Since the p17S rRNA and 17S rRNA are 0.8 and 0.69 × 10^6 molecular weight, respectively, (Table I), a rRNA precursor which contains both the 17S rRNA and 25S rRNA would be maximally only 0.11 × 10^6 molecular weight smaller than the 35S rRNA, and might not easily be separated from the 35S rRNA by gel electrophoresis.

In some RNA preparations (Figure 2a), a minor RNA species, Y, which is 5–50% the amount of the 35S rRNA, and smaller in size than the 35S rRNA, is observed. After electroelution from a preparative gel, this RNA is stable to heating at 65 °C for 10 min suggesting that it is not an aggregate. The RNA contains both the 17S rRNA and 25S rRNA sequences since both RNA competitors must be present to reach a maximum competition of greater than 90%. Also, it lacks the 5′ terminus pppAp and, therefore, cannot be a conformational isomer of the 35S rRNA (Slack et al., 1975). The evidence strongly suggests that it is a processing intermediate which lacks the 5′ terminal region of the 35S rRNA.

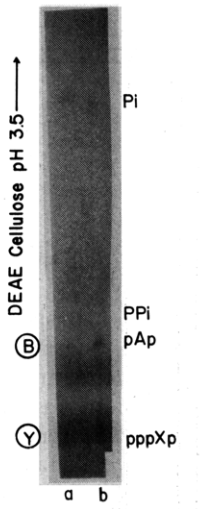

FIGURE 6: (a) The apparent nucleoside tetraphosphate, A (Figures 5a,b), was digested with bacterial alkaline phosphatase, 300 μg/mL in 0.1 M Tris-Cl, pH 8, for 4 h at 37 °C. (b) The apparent nucleoside tetraphosphate, A (Figures 5a,b), was digested with 20 μL of snake venom phosphodiesterase, 4 mg/mL for 4 h at 37 °C. The products were separated by electrophoresis on DEAE-cellulose for 90 min at 30 V/cm. Y and B mark the positions of the methyl orange and xylene cyanol FF marker dye. The migration positions of pAp and PP_i are presented in the figure.

This second processing pathway may explain the low and variable yields of pppAp in the 35S rRNA and 17S rRNA samples. If the 35S rRNA is contaminated with this intermediate, which lacks the 5′ terminus, the yield of pppAp will be lowered depending upon the relative amounts of the 35S rRNA and this processing intermediate. Since a 17S rRNA will be released from this intermediate which will lack the 5′ terminus, this 17S rRNA will contribute to the total 17S rRNA pool and thus lower the yield of pppAp.

The other minor RNA fraction, X (Figure 2a), is also related to the 25S and 17S rRNA since it can be competed to a maximum of 80% with 25S rRNA and 17S rRNA. Because of the low yield, we do not yet know if this RNA contains a 5′-triphosphate end. This RNA may either be a precursor to the 35S rRNA or it may be a conformational isomer of the 35S rRNA (Slack et al., 1975). The determination of the precise location of the 5′ and 3′ ends of the 35S rRNA, RNA X, and RNA Y in the physical map of the rRNA gene will answer these questions.

A model for the transcription of the rRNA gene, based on these data and the physical studies carried out by Karrer & Gall (1976) and Engberg et al. (1976), is presented in Figure 7. Transcription initiation takes place near the center of the DNA palindrome and proceeds to a point near the end of the linear DNA. From calculations of the apparent molecular weight of the 35S rRNA precursor of 2.4 × 10^6 and the molecular weight of the rDNA, 12.6 × 10^6 (Gall, 1974), the nontranscribed spacer region accounts for only approximately 24% of the rRNA gene. The precise location of the transcrip-

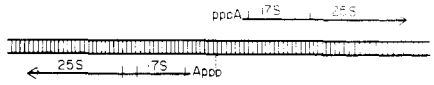

FIGURE 7: A model of the physical map of the rDNA palindrome is presented which is based on the studies of Karrer & Gall (1976) and Engberg et al. (1976).

tion initiation and termination sites are now being determined.

With the availability of a high specific activity rRNA primary transcript, the 5′ and 3′-terminal oligonucleotides will be identified and will serve as an unambiguous assay for transcription initiation and termination in vitro. Furthermore, the [^{32}P]rRNA precursor will serve as a substrate for the identification of processing endonucleases and a comparison of the terminal oligonucleotides of the in vitro generated processing products with those generated in vivo will serve as a means of demonstrating in vitro processing specificity.

Note Added in Proof

Electron microscopic observation of an actively transcribing rRNA gene has also demonstrated that transcription is initiated near the center of the rDNA palindrome (Gall et al., 1977).

Acknowledgments

I would like to thank Larry Rubin for his expert technical assistance, and Daniel Kosman, Rakesh Jain, and Ron Aloysius for their critical reading of this manuscript.

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