Enzymic Polyadenylation of 5S Ribosomal Ribonucleic Acid and Synthesis of a Complementary Deoxyribonucleic Acid†

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ABSTRACT: The 5S ribosomal RNA has been isolated, pure and intact, from rat liver (5 mg of 5S RNA from 150 g of liver). The 5S RNA serves as a primer for calf thymus poly(A) polymerase with 20% of the efficiency of $(Ap)_3A$. Bacterial 5S RNA and transfer RNA also serve as primers; rat liver 18S and 28S ribosomal RNAs support poly(A) synthesis poorly. Neither the 5S RNA primer nor the appended poly(A) tract is nicked or degraded by poly(A) polymerase, and initiation of poly(A) tracts on 5S RNA primers continues throughout the reaction period. The rate of initiation is dependent on the enzyme concentration; the ATP concentration affects the rate of elongation. The polyadenylated material increases in size over time, with the largest material reaching a size of 6.8 S in 5 h, corresponding to an appended poly(A) tract of 140 nucleotides. Using polyadenylated 5S RNA, oligo(dT) as primer, and avian myeloblastosis virus reverse transcriptase, we synthesized DNA complementary to 5S RNA. The complementary DNA has an apparent molecular weight (in alkaline sucrose gradients) of 4.3×10^4. Base composition analysis and nearest-neighbor analysis of the DNA are as expected for a complement of 5S RNA, indicating that the entire 5S sequence is copied. The complementary DNA hybridizes to 5S RNA with a $R_0t_{1/2}$ of 8.9×10^{-4} mol·s·L^{-1}. No hybrid is formed with *Escherichia coli* 16S and 23S ribosomal RNA, *E. coli* 5S ribosomal RNA, yeast transfer RNA, rat liver transfer RNA, or rat liver 18S and 28S ribosomal RNA. The T_m of the 5S RNA:5S DNA hybrid in 15 mM NaCl containing 1.5 mM sodium citrate is 74 °C, 2.5 °C below the theoretical melting temperature of a DNA duplex of 60% G + C. Analysis of the hybrid in buoyant density gradients also indicates that hybridization is both specific and precise. The complementary DNA anneals to calf thymus, rat liver, and salmon sperm DNAs but not to *E. coli* DNA. Annealing of 5S cDNA to calf thymus DNA with a $C_0t_{1/2}$ of 2.1 suggests that there are several thousand 5S RNA genes in the calf thymus genome (haploid). At least that number of 5S RNA genes is present in the salmon sperm genome.

Eucaryotic ribosomes contain four species of RNA and multiple proteins. One species of RNA, 5S ribosomal RNA (5S RNA),[1] is transcribed from a set of genes located at a site different from that of the other ribosomal RNA species (Wimber & Wimber, 1977; Pilone et al., 1974). The 5S RNA is transcribed by RNA polymerase III, in contrast to the 5.8S, 18S, and 28S ribosomal RNA molecules which are transcribed by RNA polymerase I (Marzluff & Huang, 1975; Reeder & Roeder, 1972; Weinmann & Roeder, 1974).

In order to extend our studies on transcription of DNA and chromatin by RNA polymerase III (Keshgegian & Furth, 1972; Keshgegian et al., 1973, 1975a,b; Henner et al., 1975; Atikkan & Furth, 1977; Chiu et al., 1977), we decided to use as a probe DNA complementary to 5S RNA. To obtain such a probe, it is necessary to obtain pure 5S RNA, polyadenylate it, and transcribe it with reverse transcriptase.

Here we report a method developed to obtain milligram quantities of pure and intact 5S RNA from rat liver, polyadenylation of this RNA by calf thymus poly(A) polymerase, and the synthesis of a DNA copy of 5S RNA. With this cDNA copy, the number of 5S genes in calf thymus DNA has been determined. By hybridization of the cDNA to enzymically synthesized RNA, it has been observed that RNA polymerase III preferentially transcribes 5S RNA from both native and denatured DNA (Ackerman et al., 1978; Ackerman & Furth, 1979).

Materials and Methods

Sucrose (Schwarz/Mann UltraPure) had little, if any, RNase activity. $NaDodSO_4$ and dextran sulfate (av mol wt 500 000) were from Sigma. $NaDodSO_4$ was recrystallized from 100% ethanol to remove RNase. Heparin, purchased from Sigma (grade A sodium salt) or from Reiker, was used at 200 μg/mL unless otherwise indicated. Diethyl pyrocarbonate, *m*-cresol, and 8-hydroxyquinoline were purchased from Aldrich; 8-hydroxyquinoline was recrystallized from 100% water. *E. coli* 5S RNA and 16S + 23S rRNA were obtained from Miles. Poly(vinyl sulfate) was from Eastman Organic. $(Ap)_3A$ was from Collaborative Research. Phosphocellulose (P1) and DEAE-cellulose (DE32) were obtained from Whatman, hydroxylapatite from Bio-Rad, and Sephadex G-25, G-50, and G-100 from Pharmacia.

Bovine catalase (2× crystallized) and actinomycin D (resuspended at 2 mg/mL in 95% ethanol) were obtained from Sigma Chemical Co. CsCl and Cs_2SO_4 (optical grade) were from Harshaw. Formamide (spectrograde), obtained from Matheson Coleman and Bell, was further purified by passage through activated charcoal (Norit A), membrane filtration (through 0.45-μm filters), treatment with Amberlite MB-1 (2 g/50 mL of formamide) for 2 h at room temperature (Pinder et al., 1974), and filtration through 0.45-μm membrane filters. Formamide was stored at −20 °C. *Haemophilus influenza*

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[1] Abbreviations used: 18S + 28S RNA, 18S + 28S ribosomal RNA; 5S RNA, 5S ribosomal RNA; tRNA, transfer RNA; poly(A) polymerase, ATP:polynucleotide adenylyltransferase (EC 2.7.7.19); DEPE, 1% diethyl pyrocarbonate in 95% ethanol; TKMSH, 0.05 M Tris-Cl (pH 7.4), 0.025 M KCl, 0.005 M $MgCl_2$, 0.25 M sucrose, 200 μg/mL heparin; $NaDodSO_4$, sodium dodecyl sulfate; Temed, *N,N,N',N'*-tetramethylethylenediamine; PCA, perchloric acid; hnRNA, heterogeneous nuclear RNA; HAP, hydroxylapatite; NEH, 0.1 M sodium acetate containing 2 mM EDTA (pH 5.1) and 200 μg/mL heparin; cDNA, complementary DNA; 5S cDNA, cDNA complementary to 5S rRNA; C_0t, annealing of DNA to DNA, where C_0 is the initial concentration of DNA (in moles of nucleotide/liter) and t is time (in seconds); R_0t, hybridization of RNA to DNA with RNA in vast excess, where R_0 is the initial concentration of RNA and t is the time (in seconds); SSC, 0.15 M NaCl containing 0.015 M sodium citrate; AMV, avian myeloblastosis virus.

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[^{3}H]DNA (8–10000 counts min^{-1} μg^{-1}) prepared as described by Goodgal (1968) was a gift from Dr. S. Goodgal. SV40 [^{3}H]DNA was prepared by published procedures (Hirt, 1967; Radloff et al., 1967). [^{3}H]DNA from KB cells was prepared by the procedure of Marmur (1961), somewhat modified. Oligo(dT_{12-18}) and poly(A) were from Collaborative Research. *Hin*dIII restriction nuclease was obtained from Bethesda Research Laboratory. AMV reverse transcriptase was obtained from the National Cancer Institute (see Acknowledgments).

Isolation of Ribosomes and rRNA. Glassware was soaked in 1% DEPE for 15 min and baked at 200 °C for 6 h. Equipment and lab benches were washed with DEPE; solutions were treated with DEPE for 15 min and brought to a boil, and CO_2 was removed by bubbling N_2 through the solution and then degassed in vacuo. Plastic gloves were worn when handling glassware, dialysis tubing, etc. Rats of various strains and sexes, but mainly Wistar-Furth, 300–500 g, were mixed and fasted for 24 h and sacrificed by decapitation. Livers were excised into ice-cold TKMSH, rinsed in the same buffer, frozen in a dry ice–alcohol bath, and stored at −90 °C.

Ribosomes were prepared by a modification of the procedure of Muramatsu (1973). Liver (150 g) was partially thawed and minced in 5 volumes (750 mL) of TKMSH containing dextran sulfate (20 μg/mL) and poly(vinyl sulfate) (20 μg/mL)[2] and triturated in a Potter homogenizer with a tight-fitting Teflon pestle by ten up and down strokes (4 °C). The homogenate was filtered through cheesecloth and centrifuged at 500g for 15 min (4 °C) to pellet the nuclei. The nuclei were then homogenized as above in 100 mL of 0.3 M sucrose containing 10 mM Tris-Cl (pH 8.0), 3 mM $CaCl_2$, 5 mM magnesium acetate, 5 mM dithiothreitol, 0.1% Triton X-100 (Henner et al., 1975), heparin, dextran sulfate (20 μg/mL), and poly(vinyl sulfate) (20 μg/mL) to remove ribosomes adhering to the endoplasmic reticulum associated with the nucleus. The washed nuclei and postnuclear supernatant were (separately) centrifuged at 18000g for 25 min. Supernatants were combined and adjusted to 1% sodium deoxycholate (freshly prepared), stirred for 15 min (0 °C), and centrifuged at 100000g for 90 min in the Beckman 50.1 fixed angle rotor.

Ribosome pellets were resuspended in 15 mL of NEH buffer with a Dounce homgenizer and an equal volume of 5% $NaDodSO_4$ was added. The volume was increased to 100 mL with NEH buffer and stirred for 10 min at room temperature.

The following steps were also performed at room temperature. After 10 min, 100 mL of phenol (redistilled):*m*-cresol:water:8-hydroxyquinoline (7:1:2:0.1) was added and stirring continued for 15 min. One hundred milliliters of chloroform:isoamyl alcohol (25:1) was added and stirring continued for 15 min.[3] The mixture was centrifuged at 1600g for 20 min and the aqueous phase removed. The organic phase plus interface were shaken with 100 mL of NEH and 5 mL of 1-octanol for 15 min. The mixture was centrifuged as above and the organic phase removed. The protein interface, which swells and forms a gel in the aqueous phase, was shaken with 100 mL of chloroform:isoamyl alcohol (25:1) and 1 mL of 1-octanol for 15 min. The organic phase was shaken with 100 mL of NEH and 5 mL of 1-octanol for 15 min. Each was centrifuged separately at 1400g for 40 min, the aqueous phases were removed, combined, and extracted with 200 mL of chloroform:isoamyl alcohol (50:1) for 15 min, and the RNA was precipitated with 2 volumes of 95% ethanol. The organic layers and interface were combined and mixed overnight (4 °C) with 100 mL of NEH, and the solution was centrifuged as above. Two volumes of ethanol was added and, after 15 h at −20 °C, RNA was recovered by centrifugation at 16000g. A typical preparation yielded 400 mg of rRNA from 150 g of liver.

Isolation of 5S RNA. Ribosomal RNA (400 mg) was dialyzed against 2 L of NEH overnight (4 °C) (to remove nucleotides) and applied, in two batches of 200 mg in 10 mL, to a 6.5 × 65 cm column of Sephadex G-100 over 6.5 × 5 cm of Sephadex G-25 at 16 °C. Three fractions were obtained and pooled: 18S + 28S RNA (pool I), 5S RNA (pool II), and transfer RNA (pool III) (Figure 1).

Pool II was concentrated by ethanol precipitation and resuspended in a minimal volume (5 mL of NEH buffer). Five milligrams (~1 mL) of this fraction was applied to a 1.5 × 210 cm Sephadex G-100 column over 1.5 × 5 cm Sephadex G-25. This procedure was repeated until all pool II material had been passed through the column.

The main peak (retarded material) (cf. Muramatsu, 1973, Figure 7) was concentrated by ethanol precipitation and taken up in a minimal volume of water. The RNA was then dialyzed against first 1 L of 0.1 M NaCl containing 10 mM sodium acetate (pH 5.1), 2 mM EDTA, and 50 μg/mL heparin overnight (0 °C) and then three changes of 30 mM Tris-Cl (pH 7.4) containing 1 mM $MgCl_2$ and 30 μg/mL heparin (8 h each time at 0 °C). The RNA was stored at −90 °C in 200-μL aliquots.

Preparation of Poly(A) Polymerase. Calf thymus poly(A) polymerase was purified as described by Keshgegian et al. (1975b). The fraction eluting from hydroxylapatite at 0.2 M phosphate was concentrated against solid sucrose and stored at −90 °C. This fraction has no DNase or RNase activity. RNA synthesized in vitro by calf thymus RNA polymerase III is not degraded; 5S RNA remains intact even after incubation with the enzyme for 5 h (Figure 3).

Polyadenylation of 5S RNA. The reaction mixture contained (0.3 mL): 100 mM Tris-Cl (pH 8.3), 2 mM $MnCl_2$, 4 mM 2-mercaptoethanol, 40 mM ammonium sulfate, 320 μM [^{14}C]ATP (7.3 counts min^{-1} pmol^{-1}), 5S RNA at 7–10 μM termini, and 25 or 88 units of enzyme. After incubation for 3 or 5 h at 37 °C, acid precipitability was determined on a 5-μL aliquot (Austin et al., 1973). Twenty microliters of 0.2 M EDTA (adjusted to pH 7.0) was added and RNA was separated from unincorporated nucleotides by Sephadex G-50 gel filtration in 0.2 mM EDTA containing 5 μg/mL heparin. Excluded fractions (RNA) were lyophilized to dryness and resuspended in water.

Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out at 21 °C with 8% polyacrylamide gels (5 × 75 mm) at 10 V/cm with 36 mM Tris, 30 mM NaH_2PO_4, and 1 mM EDTA (pH 7.8 solution). Bisacrylamide was 0.2%, Temed was 12.5 μL/g acrylamide, and ammonium persulfate (10% stock solution) was 125 μL/g acrylamide. Gels took 30–40 min to solidify, giving uniform polymerization (Gressel et al., 1975). Solutions were degassed and gels prepared as described by Loening (1967).

Reverse Transcriptase Assays. The complete reaction mixture contained (in 100 μL): 50 mM Tris-Cl (pH 8.3), 5 mM magnesium acetate, 25 mM KCl, 6 mM dithiothreitol, 10 μg of catalase, 10 μg of bovine serum albumin (crystallized

[2] Heparin, poly(vinyl sulfate), and dextran sulfate were used to inhibit RNase activity (Brawerman, 1973) that is present in rat liver homogenates (McIntosh & Rubin, 1975).

[3] The phenol solution was mixed with the ribosomes before the chloroform solution was added. If chloroform was added prior to phenol, hemoglobin and heme would not be extracted to the organic phase but would remain in the aqueous phase tightly bound to the RNA and be precipitated by ethanol.

fraction), 1–2.5 μg of oligo(dT_{12-18}), 5 μg of poly(A), 2 μg of actinomycin D, 60 μM [^{3}H]dTTP (90 counts min^{-1} pmol^{-1}), and 0.1 unit of enzyme. (One unit of enzyme activity is expressed as the incorporation of 1 nmol of dTTP into acid-insoluble product in 20 min at 37 °C (Kacian & Spiegelman, 1974).) After 60 min at 37 °C, acid-precipitable material was determined (Austin et al., 1973).

Reverse transcriptase activity was dependent upon poly(A) as template and oligo(dT) as primer. Maximal activity required the presence of either albumin or catalase; omission of either did not affect activity, although omission of both catalase and albumin reduced activity to 16% of control values. Omission of actinomycin D resulted in a 20% decrease in activity.

In assaying polyadenylated 5S RNA as template for reverse transcriptase, poly(A) was omitted and 5 μg of oligo(dT_{12-18}) added; dATP, dTTP, and dGTP were added at 100 μM; [^{3}H]dCTP was added at 60 μM (330 counts min^{-1} pmol^{-1}), and 1 unit of enzyme was used. The amount of polyadenylated 5S RNA used was 0.5–0.7 μg. Polyadenylated 5S RNA was not routinely separated from 5S RNA, which was present at 1.5–2.1 μg/mL. Concentrations of polyadenylated 5S RNA refer only to the 5S RNA primers that have had a poly(A) tract added and not to the total 5S RNA content (of which only 25% is polyadenylated). Incubation was for 60 min at 37 °C.

Preparation of cDNA to 5S RNA for Hybridization and Annealing Studies. Complementary DNA (cDNA) was synthesized by using a polyadenylated 5S RNA template as in the reverse transcriptase assay with the following modifications: (i) the reaction volume was 0.2 mL; (ii) 10 μg of oligo(dT_{12-18}), 2.8 μg of polyadenylated 5S RNA, and 5 units of reverse transcriptase were used; and (iii) the specific activity of [^{3}H]dCTP was 1×10^4 counts min^{-1} pmol^{-1}. All ingredients except catalase, polyadenylated 5S RNA, and reverse transcriptase were lyophilized to dryness. Catalase and RNA were added, the volume was increased to 0.19 mL, and the reaction mixture was cooled to 0 °C. Five units of reverse transcriptase was added (10 μL) and the reaction incubated for 60 min at 37 °C. Two microliters was then removed to determine the incorporation of dCTP into acid-insoluble product. The mixture was adjusted to 0.5 N NaOH and incubated for 16 h at 37 °C. The pH of the mixture was then adjusted to 7.5 with HCl and the DNA extracted with chloroform:isoamyl alcohol (100:1). The aqueous phase was reextracted with chloroform:isoamyl alcohol (100:1), the organic layers were back-extracted with aqueous buffer (50 mM Tris-Cl (pH 7.5), containing 5 mM NaCl), and the combined aqueous phases were lyophilized to dryness. The DNA was suspended in 0.2 mL of H_2O, and unincorporated nucleotides were removed by passage through a 1 × 35 cm Sephadex G-50 column. Fractions containing DNA were lyophilized to dryness, and Sephadex filtration was repeated. The DNA containing fractions were again lyophilized to dryness and resuspended in water at 800 counts min^{-1} μL^{-1} and stored in 200-μL aliquots at −90 °C. The specific activity of the cDNA, calculated from the specific activity of CTP and the base composition of 5S RNA, was 1×10^7 counts min^{-1} μg^{-1}.

Nuclease S_1 Assay. Enzyme (Miles) was resuspended at 1 mg/mL in 5 mM sodium succinate (pH 4.5) and stored in 20-μL aliquots at −20 °C. Enzyme retained activity for over 24 months. Reaction mixtures contained (0.1 mL) (Wiegand et al., 1975): 100 mM NaCl, 5 mM sodium succinate (pH 4.5), 1 mM $ZnCl_2$ (prepared from a solution of 100 mM $ZnCl_2$ in 5 mM HCl), nucleic acid, and enzyme. Eight units of enzyme was used for radioactive nucleic acid (0.1–1.0 μg); 200 units was used for unlabeled nucleic acid (90–200 μg).

Digestion of single-stranded nucleic acids (denatured *H. influenza* [^{3}H]DNA, denatured calf thymus DNA) by nuclease S_1 was completed in 10–30 min at 37 °C; 4% or less of double-stranded *H. influenza* [^{3}H]DNA, KB [^{3}H]DNA, or calf thymus DNA was solubilized in 60 min.

5S cDNA:RNA Hybridization. cDNA (500–1000 counts min^{-1}) was hybridized to RNA (0.1–10.0 μg/mL) in 50% formamide, 4 × SSC, 0.2 mM EDTA, and 0.01% $NaDodSO_4$). Aliquots (45–50 μL) were sealed in 100-μL Drummond microcapillary tubes (that had previously been boiled in 200 mM EDTA, rinsed with water, and dried). Samples were heated to 99 °C for 5 min and then immersed into a 37 °C water bath. After incubation for 16 s to 91 h (depending upon RNA concentration and R_0t value desired), the samples were diluted into 1.5 mL of 0.1 M NaCl containing 5 mM sodium succinate (pH 4.5). $ZnCl_2$ (to 1 mM) and nuclease S_1 (250 units) were then added, and the sample was incubated for 60 min at 37 °C. The reaction was terminated by the addition of 0.4 mL of 7% perchloric acid. One milligram of albumin was added and, after 10 min at 0 °C, the samples were centrifuged, and acid-precipitable and acid-soluble radioactivity was determined in Triton X-toluene scintillation fluid (Austin et al., 1973). The percentage of S_1-resistant material for each sample was calculated by dividing the acid-precipitable radioactivity by total radioactivity (acid-soluble plus acid-precipitable). Results are corrected for radioactivity (cDNA) not acid precipitable in control experiments in which samples were processed without nuclease S_1 treatment. Control experiments also demonstrated that >93% of cDNA is nuclease S_1 sensitive when heat-denatured after hybridization.

5S cDNA:DNA Annealing. Complementary DNA to 5S RNA was prepared and annealed to DNA as described for RNA hybridization. DNA concentration varied between 0.2 and 5.0 mg/mL. The DNA (calf thymus, rat liver, salmon sperm, and *E. coli*) had been extracted with phenol:chloroform:isoamyl alcohol (50:50:0.5), sonicated to 300 base pair lengths (as determined in alkaline sucrose gradients), incubated in 0.5 N NaOH overnight at 37 °C, dialyzed against water for 2 days, and stored at −20 °C. After incubation for 16 s to 91 h (depending upon the DNA concentration and the C_0t value desired) nuclease S_1 (360 units) was added and resistance determined as for cDNA:RNA hybridization.

T_m Analysis of cDNA:RNA Hybrids. After hybridization (at 10 μg of RNA/mL) to a R_0t of 10, the sample was dialyzed against four changes of 0.1 × SSC for 96 h at 0 °C. The sample was divided into ten aliquots (55–100 μL each). Each aliquot was heated at an indicated temperature for 5 min, diluted to 1.5 mL with 100 mM NaCl containing 5 mM sodium succinate (pH 4.5), and stored at −20 °C. Nuclease S_1 resistance was determined after incubation with 125 units of enzyme and 1 mM $ZnCl_2$ for 60 min at 37 °C.

Buoyant Density Analysis. The cDNA (4 ng) was hybridized to 1 μg of 5S RNA to a R_0t of 1 and then dialyzed against 10 mM Tris-Cl (pH 7.0) containing 0.1 M NaCl (control sample), 5 mM sodium succinate (pH 4.5) containing 0.1 M NaCl (nuclease S_1 treated sample) or 30 mM Tris-Cl (pH 7.7) containing 0.1 M NaCl (RNase-treated sample). Nuclease digestion was performed in 0.5–1.0-mL volumes with 4 ng/mL cDNA:RNA hybrid, 250 units/mL nuclease S_1 or 2 μg/mL RNase A, and 0.2 unit/mL RNase T_1 for 30 min at 37 °C. Yeast tRNA at 40 μg/mL was added prior to nuclease digestion.

After nuclease digestion, S_1-treated samples were extracted with chloroform:isoamyl alcohol, and RNase treated samples were extracted with phenol:*m*-cresol:8-hydroxyquinoline and chloroform:isoamyl alcohol. In sequential RNase and S_1 digestion, the RNase-treated sample was extracted with phenol, dialyzed, and digested with nuclease S_1 after the addition of yeast tRNA to 40 μg/mL and 5S RNA to 2.2 μg/mL. The hybrid samples were then dialyzed against 10 mM Tris-Cl (pH 7.0), 0.1 M NaCl and mixed with Cs_2SO_4 (in 50 mM NaCl containing 10 mM Tris-Cl (pH 7.5)) to a density of 1.550 g/cm^3 and a final volume of 5 mL. DNA samples were at a starting density of 1.450 g/cm^3 and RNA samples were at a starting density of 1.650 g/cm^3. Samples were placed in thick-walled polycarbonate tubes and overlayed with 0.2 mL of paraffin oil. Centrifugation was in the Beckman 50 fixed angle rotor at 37 500 rpm for 120 h at 25 °C. Gradients were fractionated as described for alkaline–sucrose gradients, except that 200-μL fractions were collected in glass tubes and 100 μL was weighed directly to determine density. Samples were then counted for radioactivity.

Centrifugation and analysis in CsCl were performed similarly. Samples were at a starting density of 1.750 g/cm^3.

Formamide–Sucrose Density Gradient Centrifugation. DNA was synthesized in a 0.2-mL reaction volume with [^{3}H]dTTP (90 counts min^{-1} pmol^{-1}) or [^{3}H]dCTP (330 counts min^{-1} pmol^{-1}) with or without unlabeled deoxynucleoside triphosphates. One microgram of polyadenylated 5S RNA and 2 units of reverse transcriptase were used as described in Reverse Transcriptase Assays. RNA was digested in 0.5 M NaOH for 16 h at 37 °C. DNA, with 0.1 mg of albumin as carrier, was precipitated with perchloric acid, washed two times with 1% perchloric acid, and resuspended in 50 mM Tris-Cl (pH 8.8). The material was extracted with chloroform:isoamyl alcohol (100:1) and the aqueous phase removed. The organic phase was back-extracted four times. Aqueous phases were combined, lyophilized, resuspended in 0.2 mL of water, and applied to 5–30% sucrose gradients containing 70% formamide, 0.1 M sodium acetate (pH 5.1), 2 mM EDTA, and 0.01% $NaDodSO_4$. After centrifugation in the Beckman SW41 rotor at 39 000 rpm for 48 h at 20 °C, fractions were collected directly into vials containing Triton–toluene scintillant.

Alkaline–Sucrose Density Gradient Centrifugation. cDNA, prepared as described for formamide–sucrose centrifugation, was applied to 5–30% sucrose gradients containing 0.9 M NaCl, 0.1 M NaOH, and 0.01 M EDTA. After centrifugation for 24 h at 20 °C in the Beckman SW 56 rotor at 50 000 rpm, samples were pumped from the bottom of the gradient through a 20-μL capillary tube directly into vials containing Triton–toluene scintillant. One milliliter of 0.1 M HCl was added and radioactivity content determined.

DNA markers were prepared by cleaving SV 40 [^{3}H]DNA (60 counts min^{-1} ng^{-1}) with *Hin*dIII restriction nuclease by using reaction conditions described by Bethesda Research Laboratory. Samples were stored at −20 °C.

Base Composition Analysis of Complementary DNA. Composition of the synthesized cDNA was determined by measuring the rate of incorporation of each base into cDNA by reverse transcriptase, with one of the four deoxynucleotides labeled with tritium, at the following concentrations (μM) and specific activities (counts min^{-1} pmol^{-1}): dGTP 100, 420; dATP 100, 76; dTTP 60, 90; dCTP 60, 330.

Nearest-Neighbor Analysis of Complementary DNA. cDNA was synthesized with [α-^{32}P]dCTP (120 counts min^{-1} pmol^{-1}) at 60 μM. The ^{32}P-labeled cDNA was applied to alkaline–sucrose gradients as described above. Fractions containing the bulk of the radioactivity (peak area) were combined, dialyzed against water, and lyophilized to dryness. Nearest-neighbor frequency was analyzed as described by Josse et al. (1961) modified slightly (Wang et al., 1976).

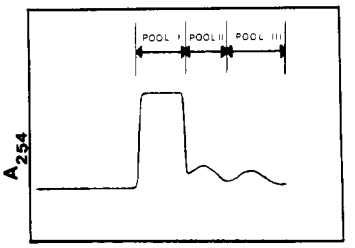

FIGURE 1: Fractionation of ribosomal RNA by gel filtration on Sephadex G-100. Ribosomal RNA (200 mg) was applied at 20 mg/mL to a 6.5 × 65 cm column of Sephadex G-100 over 6.5 × 5 cm of Sephadex G-25. The column was run at 16 °C in NEH buffer. UV absorbance was monitored at 254 nm, and 4 mL fractions were collected over 5 h.

Results

Preparation of 5S RNA. Total ribosomal RNA (400 mg) is extracted from rat liver ribosomes, and then separated into the constituent RNA species by column chromatography as described under Materials and Methods. As shown in Figure 1, 18S + 28S RNA (pool I) are cleanly separated from 5S RNA and transfer RNA (pool III), although the 5S fraction (pool II) contains some 18S + 28S RNA and tRNA. After filtration through a 1.5 × 210 cm Sephadex G-100 column, a sharp symmetrical band is observed (results not shown; cf. Muramatsu, 1973, Figure 7) and, upon polyacrylamide gel electrophoresis (Figure 3b), the 5S RNA appears pure and homogeneous. Gel electrophoresis in urea under denaturing conditions also demonstrates a single band (N. Kallenbach, personal communication).

Five milligrams of 5S RNA is obtained from 400 mg of total ribosomal RNA. The RNA can be stored for at least 15 months at −90 °C without evidence of degradation.

Polyadenylation of 5S RNA. The kinetics of incorporation of AMP using various RNAs as primer is shown in Figure 2. Ribosomal RNA, 5S RNA, and tRNA all support poly(A) synthesis. 5S RNA (bacterial or mammalian) is approximately 20% as effective as an $(Ap)_3A$ primer, while rat 18S + 28S rRNA supports poly(A) synthesis only 3% as well as $(Ap)_3A$. The greater effectiveness of *E. coli* 16S + 23S rRNA as primers is due to nicks in these RNA preparations which increase the number of available 3′-OH termini, as demonstrated by polyacrylamide gel electrophoresis (data not presented). Yeast tRNA is approximately 25% as efficient a primer as $(Ap)_3A$. For all RNA species, the reaction proceeds for more than 4 h.

The conditions used by Keshgegian et al. (1975c) to assay poly(A) polymerase were found to be optimal; if the Mn^{2+} or the $(NH_4)_2SO_4$ concentrations are decreased (cf. Tsiapalis et al., 1973) from 2 mM to 0.5 mM Mn^{2+} or 40 mM to 0 mM $(NH_4)_2SO_4$, the activity is 33% of the control. Heparin (20 μg/mL) stimulates the reaction twofold, possibly due to an increased rate of elongation (cf. Coupar & Chesterton, 1977).

Prior to the use of polyadenylated 5S RNA as template for reverse transcriptase, the polyadenylation reaction was investigated in greater detail. The time course of polyadenylation of 5S RNA, as monitored by gel electrophoresis, is shown in Figure 3. The 5S RNA is not degraded in 5 h when ATP is omitted (Figure 3a). In the presence of substrate, after incubation for 1, 3, and 5 h, there is no degradation of the RNA and there is substantial incorporation of ATP (Figures 3c–e).

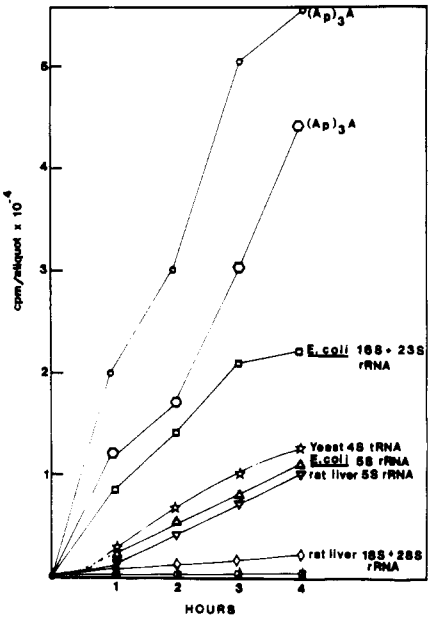

FIGURE 2: Kinetics of polyadenylation of various RNA species by calf thymus poly(A) polymerase. Standard reaction conditions (0.1 mL) with various RNAs as primers. At indicated times, 20-μL aliquots were removed and acid-precipitable radioactivity was determined (Bollum, 1959). Concentrations of RNA (expressed as 3'-OH termini) were $(Ap)_3A$, 4.2 and 2.1 μM; *E. coli* 16S + 23S RNA, 4 μM; yeast tRNA, 4.5 μM; *E. coli* 5S RNA, 4 μM; rat liver 5S RNA, 4 μM; rat liver 18S + 28S RNA, 1.9 μM. [^{3}H]ATP was at 19 counts min^{-1} pmol^{-1}.

Table I: Polyadenylation of 5S RNA by Calf Thymus Poly(A) Polymerase[a]

conditions		absorbance remaining in 5S RNA region (%)			
enzyme (units)	ATP (μM)	0 time	1 h	3 h	5 h
88	320	100	88	80	76
25	320	100	95	88	83
88	960	100	90	81	75

[a] Polyacrylamide gel analysis was performed as described under Materials and Methods and legend to Figure 3.

The incorporated nucleotides are covalently linked to the 5S RNA primer, increasing its size, while the amount of residual 5S RNA decreases. At all times the polyadenylated material has a peak at the leading edge with some material trailing off down to the 5S size. The *peaks* are at 6, 6.5, and 6.8 S in 1, 3, and 5 h, respectively (Figure 3). This corresponds to 60, 105, and 140 nucleotide-long poly(A) tracts, respectively, added onto the 120 nucleotide 5S RNA. The size profile is unchanged if the material is heated to 100 °C and rapidly cooled prior to electrophoresis. In formamide–sucrose gradients, the size profile is also unchanged (Figure 5).

By measuring the decrease in the area under the 5S peak, we estimate (in the experiment reported in Figure 3) that 12, 20, and 24% of the 5S RNA have been polyadenylated in 1, 3, and 5 h, respectively (Table I). Knowing the amount of ATP incorporated and the amount of 5S RNA polyadenylated, the *average* number of AMP moieties added to each polyadenylated 5S RNA can be estimated as 28, 34, and 48 molecules in 1, 3, and 5 h, respectively (Figure 3f). (In another experiment, averages of 29, 34, and 45 molecules of AMP per polyadenylated 5S RNA were added by the poly(A) polymerase and 13%, 20%, and 25% of the RNA was polyadenylated.) In parallel experiments at 5 h, *E. coli* 5S RNA has a polyadenylation peak at 6.6 S, and $(Ap)_3A$ has a broad peak between 5.8 and 6.2 S (results not shown). In all of these

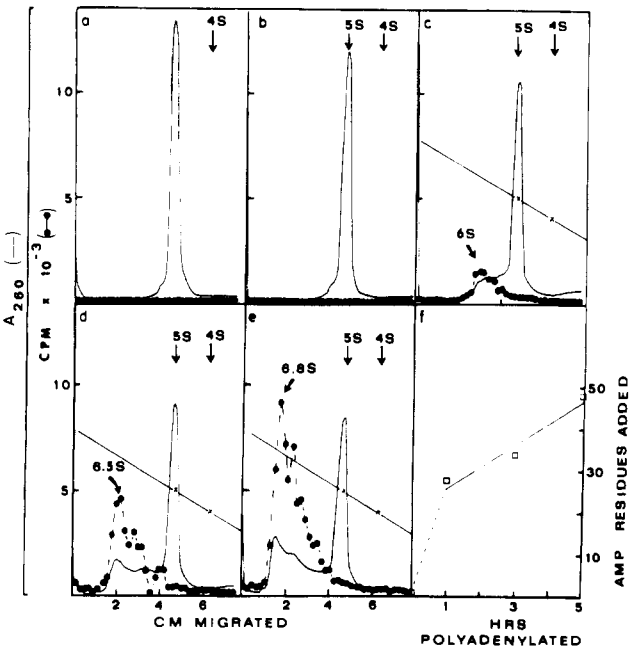

FIGURE 3: Polyadenylation of 5S RNA as a function of time. Aliquots (40 μL) were withdrawn from the standard reaction mixture (0.2 mL) with rat liver 5S RNA (7 μM 3'-OH termini), [^{3}H]ATP (19 counts min^{-1} pmol^{-1}), and 88 units of enzyme. To each aliquot, 2 μL of 0.2 M EDTA was added, and the aliquots were frozen at −90 °C. One sample (0.1 mL) was incubated for 5 h without ATP. Aliquots (40 μL) were electrophoresed in 8% acylamide gels until the bromophenol blue marker was at the end of the gel. The gels were scanned at 260 nm in a Gilford spectrophotometer (—). (To determine the amount of 5S RNA polyadenylated, the gel tracings were photocopied onto bond paper and the 5S area was cut out and weighed. Since degradation was not observed, the amount of 5S RNA polyadenylated could be determined from the decrease in weight of the 5S peak.) The gels were frozen and sliced into 1-mm segments. Two segments were added to a vial with 0.3 mL of 30% H_2O_2 and incubated at 50 °C for 4 h. Scintillation fluid was added plus 0.5 mL of 0.1 N HCl and radioactivity content determined (●) (Austin et al., 1973). (a) ATP omitted, 5 h; (b) 0 time; (c) 1 h; (d) 3 h; (e) 5 h; (f) *average* number of AMP added per polyadenylated molecule.

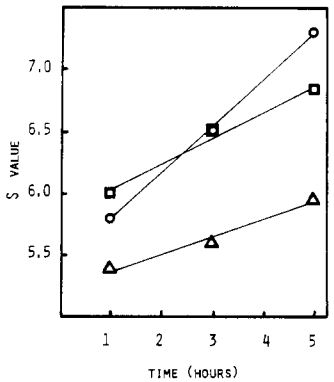

FIGURE 4: Rate of synthesis of poly(A) by poly(A) polymerase. The peak sizes (*s* values) of the polyadenylated material synthesized with various amounts of ATP and enzyme are plotted as a function of time. (□) Data of Figure 3; (△) 320 μM ATP, 25 units of enzyme; (○) 960 μM ATP, 88 units of enzyme.

incubations, there is no detectable breakdown of RNA.

In Figure 4 the peak *s* values of the polyadenylated material from the experiment of Figure 3 are replotted, together with similar data from experiments in which less enzyme or a higher concentration of ATP had been used. For all three conditions, the increase in size of the polyadenylated material is linear with time. However, at an increased ATP concentration, the slope of the line is greater (Figure 4), indicating a faster rate

Table II: Polyadenylated 5S RNA as a Template for AMV Reverse Transcriptase[a]

conditions	pmol incorp [^{3}H]-dCTP	[^{3}H]-dATP	[^{3}H]-dGTP	[^{3}H]-dTTP
complete	37	20	30	142
omit oligo(dT)	<2	<2	<2	<2
omit polyadenylated 5S RNA, add 5S RNA (70 μg)	<2			<2
omit polyadenylated 5S RNA, omit oligo(dT), add 5S RNA (70 μg)	<2			<2
omit polyadenylated 5S RNA, add poly(A) (5 μg)				177
omit polyadenylated 5S RNA, add poly(A) (5 μg), add 5S RNA (0.5 μg)				172
omit 3 unlabeled deoxynucleotides triphosphates	<2	<2	<2	187
omit 1 deoxynucleotide	<2	<2	<2	

[a] Assay as described under Materials and Methods with 0.56 μg of polyadenylated 5S RNA. The 5S RNA had been polyadenylated for 5 h. Unlabeled triphosphates were at 100 μM. The concentration (μM) and specific activity (counts min^{-1} pmol^{-1}) of the labeled triphosphates were: dATP, 100 and 76; dCTP, 60 and 330; dGTP, 100 and 420; dTTP, 60 and 90.

of nucleotide addition to the poly(A) tract. The rate of initiation of poly(A) tracts appears to be independent of ATP concentration, since the number of 5S RNA molecules polyadenylated is not affected by changing the concentration of ATP (Table I). In contrast, when the ATP concentration is held constant and a lower level of enzyme is used, the amount of 5S RNA polyadenylated decreases (Table I), but the rate of nucleotide addition to existing poly(A) tracts is similar to the rate at the higher enzyme level (Figure 4). This suggests that initiation of poly(A) tracts depends upon the enzyme concentration but that the rate of elongation is determined by the ATP concentration. Similar conclusions have been reached for *E. coli* poly(A) polymerase (Sano & Feix, 1976).

Polyadenylated 5S RNA as Template for Reverse Transcriptase. Polyadenylated 5S RNA is an effective template for AMV reverse transcriptase (Table II). Oligo(dT) is required for the reaction and 5S RNA, prior to polyadenylation, is inactive as a template. Since 5S RNA does not inhibit transcription of poly(A) (Table II), it is not necessary to separate polyadenylated 5S RNA from nonpolyadenylated 5S RNA prior to use as a template. Omission of the three (or only one) unlabeled deoxynucleoside triphosphates results in no incorporation of the labeled deoxynucleoside triphosphate except for [^{3}H]dTTP. Incorporation of [^{3}H]dTMP results from poly(dT) synthesis by using the poly(A) tail of polyadenylated 5S RNA as template.

With increased reaction times there is an increase in DNA synthesis. In a typical experiment, 6.2, 14.7, 22.3, and 25.9 pmol of [^{3}H]dCMP and 3.6, 7.3, 10.3, and 9.8 pmol of [^{3}H]dGMP were incorporated in 10, 30, 60, and 90 min, respectively.

Although actinomycin D is routinely added to the reaction to prevent reverse transcriptase duplicating the synthesized cDNA (Verma et al., 1974), its omission has no dramatic effect on the amount of dCMP incorporated. This is consistent with the absence of self-complementary regions at the 3′ terminus of the cDNA, although the addition of a few extra nucleotides might not be detected.

In order to avoid a large poly(dT) region covalently attached to the cDNA, a large excess of oligo(dT_{12-18}) is added to the reaction. If the large excess of oligo(dT) is not present, a long poly(dT) tract could precede the cDNA region (Devos et al., 1977; Falvey et al., 1976). When a large excess of oligo(dT) is present, the entire poly(A) region is saturated with oligo(dT) and only the one short oligo(dT) directly preceding the 3′ terminus of the RNA template is covalently attached to the synthesized cDNA. The first few nucleotides incorporated that are complementary to the RNA template anchor the primer in place (Devos et al., 1977) so that there is no slippage synthesis along the poly(A) region.

Table III: Base Composition of 5S Complementary DNA[a]

	dAMP	dTMP	dCMP	dGMP
obsd rel rate of incorp	0.64 ± 0.12		1.00	0.78 ± 0.05
theor composition of the complement of 5S RNA[b]	0.65	0.53	1.00	0.76

[a] Assay as described under Materials and Methods with 0.56 μg of polyadenylated 5S RNA. The concentration and specific activity of the labeled substrates were as described in the legend to Table II. These results are the average of 18 separate experiments and the standard deviation is expressed for the observed values. [b] The theoretical base composition of the cDNA was calculated from the base composition of 5S RNA (Knight & Darnell, 1967) by assuming that a complete DNA transcript would have the complementary composition of the 5S RNA. The percent dCMP (34) in the cDNA was set at 1.0.

Table IV: Nearest-Neighbor Analysis[a]

sample	frequency dCpdC	dApdC	dGpdC	dTpdC
(A) cDNA to 5S RNA[b]				
obsd	1.00	0.58 ± 0.01	0.60 ± 0.03	0.53 ± 0.01
theor	1.00	0.53	0.53	0.53
(B) replicated calf thymus DNA				
obsd[c]	0.052	0.053	0.048	0.061
theor[d]	0.054	0.055	0.047	0.064

[a] Analysis was performed as described under Materials and Methods. [b] Values were determined as follows. From the known base sequence of rat liver 5S RNA (Labrie & Sanger, 1969), the base sequence of the complementary strand (of opposite polarity) was constructed. This complementary strand contains, adjacent to dCMP (dNdC): 15 dCMP, 8 dAMP, 8 dGMP, and 8 dTMP. The relative ratio (dA:dC:dG:dT) with dCpdC = 1.00, is 1:0.53:0.53:0.53. For the experimental values, counts in each peak after electrophoresis were totaled, dCpdC set equal to 1.0, and the remaining values tabulated: 10 000 counts were subjected to electrophoresis; recovery was >95%. [c] Values were calculated after electrophoresis by totaling counts in each peak and determining the fraction of total counts each peak represented. Values were multiplied by the fraction of calf thymus RNA that is dCMP (0.215). Counts were subjected to electrophoresis; recovery was >95%. [d] From Josse et al., 1961.

Base Composition of 5S cDNA. The rate of incorporation of the various nucleoside triphosphates reflects the relative abundance of the complementary nucleotides in the template (Hurwitz et al., 1962). In experiments with a polyadenylated 5S RNA template, the observed rate of incorporation is similar to the theoretical relative base composition (Table III).

Further evidence concerning the composition of the cDNA synthesized was obtained by "nearest-neighbor" analysis by using [α-^{32}P]dCTP as the radioisotope. As shown in Table IV, there is close agreement between the observed and theoretical values. Control experiments using calf thymus DNA synthesized as described by Wang et al. (1976) give nearest-neighbor values similar to those described by Josse et al. (1961).

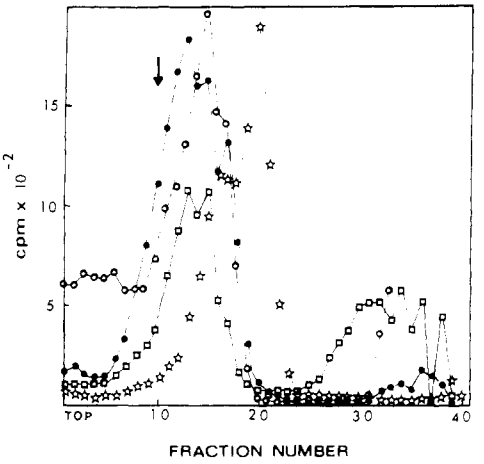

FIGURE 5: Formamide–sucrose sedimentation analysis of 5S cDNA. The 5S cDNA was synthesized and isolated as described under Materials and Methods. cDNA synthesized with: (□) [^{3}H]dCTP; (●) [^{3}H]dTTP; (○) [^{3}H]dTTP and unlabeled triphosphates omitted; (*) polyadenylated 5S RNA. Sedimentation of ^{3}H-labeled 5S RNA is indicated by the arrow.

Sucrose Sedimentation Analysis of 5S cDNA. In formamide–sucrose gradients (Figure 5), 5S cDNA sediments faster than 5S RNA but slower than polyadenylated 5S RNA. Polyadenylated 5S RNA appears as a sharp, homogeneous peak, as expected (Figure 3b). Complementary DNA synthesized with [^{3}H]dTTP or [^{3}H]dCTP has a similar profile. (The slight differences are ascribed to poly(dT) synthesis as measured by [^{3}H]dTTP.) Poly(dT), obtained by incubating polyadenylated 5S RNA with only [^{3}H]dTTP, is more heterogeneous in size with many small molecules. In contrast, there are fewer small molecules of 5S cDNA. The rapidly sedimenting material observed in cDNA synthesized with [^{3}H]dCTP as substrate is probably due to some aggregation (cf. Lehrach et al., 1977; Boedtker & Lehrach, 1976). Since the full RNA sequence of 39 000 daltons is transcribed (base composition and nearest-neighbor studies), and the synthesized cDNA is approximately 40 000 daltons, the amount of poly(dT) preceding the cDNA molecules must be negligible.

The molecular weight of the cDNA was determined directly by sedimentation in alkaline sucrose gradients (Figure 6) and comparison with markers obtained by *Hin*dIII digestion of SV 40 RF1 (Lai & Nathans, 1974), by using Studier's (1965) equation. The observed sedimentation value for the cDNA, 3.25 S, corresponds to an $s^0_{20,w}$ of 3.77 and a molecular weight of 43 100. The open arrow denotes where a molecule of 43 200 daltons (5S RNA–$(dT)_{12-18}$) would sediment. (The molecular weight of rat liver 5S RNA, calculated from the base composition, is 38 660 and the weight of the oligo(dT) primer is 4580.)

Hybridization of 5S cDNA to 5S RNA. The cDNA is almost completely sensitive (95%) to nuclease S_1 after removal of template RNA by alkaline hydrolysis. This indicates that the cDNA does not demonstrate intramolecular annealing. (In the reaction conditions used, double-stranded DNA is not degraded; Wiegand et al., 1975.) Since the 5S cDNA is sensitive to nuclease S_1, the formation of resistant material upon incubation with 5S RNA reflects the complementarity of the DNA to 5S RNA.

The 5S cDNA hybridizes to 5S RNA with a $R_0t_{1/2}$ of 8.9×10^{-4}, consistent with the known complexity of 5S RNA (120 nucleotides) (Figure 7). Double-reciprocal plot analysis (insert) indicates that a maximum of 88% hybridization would be observed at infinite R_0t. Other preparations of cDNA are 92–95% S_1 resistant at R_0t 0.1 after hybridization to 5S RNA.

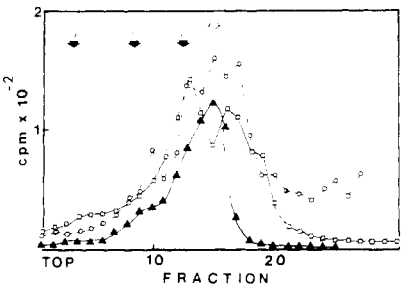

FIGURE 6: Alkaline–sucrose density gradient analysis of 5S cDNA. Procedure was as described under Materials and Methods. *Hin*dIII digests of SV40 RF1 were sedimented in a parallel gradient. Fragment sizes of 3700, 11 000–11 500 and 32 000 daltons are indicated by arrows a–c, respectively. The open arrow depicts the position where cDNA of the predicted size (43 200 daltons) would sediment. (○) Poly(dT) synthesized by using polyadenylated 5S RNA and only [^{3}H]dTTP; (□) cDNA synthesized by using polyadenylated 5S RNA, [^{3}H]dTTP, and unlabeled dATP, dCTP, and dGTP; (▲) cDNA synthesized by using polyadenylated 5S RNA, [^{3}H]dCTP, and unlabeled dATP, dGTP, and dTTP.

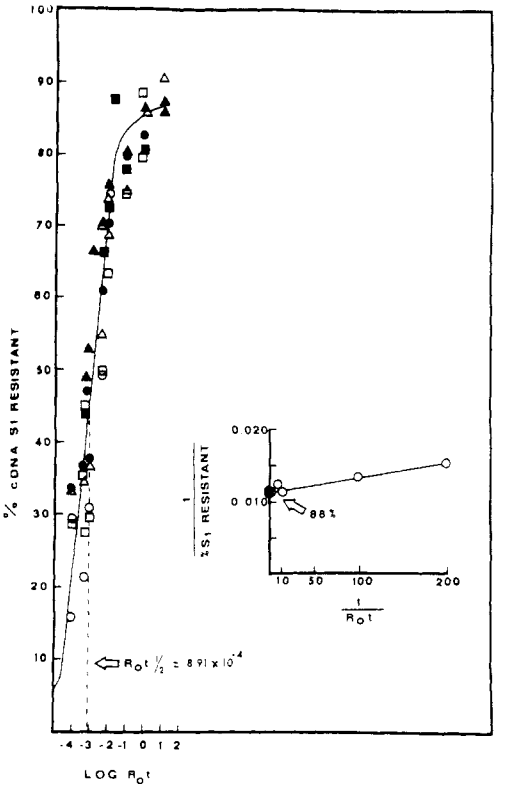

FIGURE 7: Hybridization of 5S cDNA to 5S RNA. Aliquots of cDNA (50–100 pg) were hybridized to 5S rRNA as described under Materials and Methods. Each point represents the average of four to seven determinations. The 5S RNA concentration varied from 0.1 to 10.0 μg/mL: (○) 0.1; (□) 0.5; (△) 1.0; (●) 2.0; (■) 5.0; (▲) 10.0. Four percent has been added to each value to correct for S_1 degradation of hybrid molecules. The insert is a double-reciprocal plot where the values of each R_0t were averaged and then plotted as a single point.

The R_0t curve is spread over >1.5 logs due to the presence of partial transcripts (Figure 6). These are cDNA molecules that were in the process of being synthesized when the incubation was terminated. The hybridization experiments are performed at 37 °C, although the rate of hybridization is two to three times faster at 55 °C (data not shown; Weber & Berger, 1976) in order to obtain points at lower R_0t values by using RNA concentrations of 1 μg/mL or higher.

Relevant to these studies is the work of Obinata et al. (1975) in which Qβ replicase was used to synthesize a cRNA to 5S RNA. Obinata et al. observed a $R_0t_{1/2}$ of 5×10^{-4} for hy-

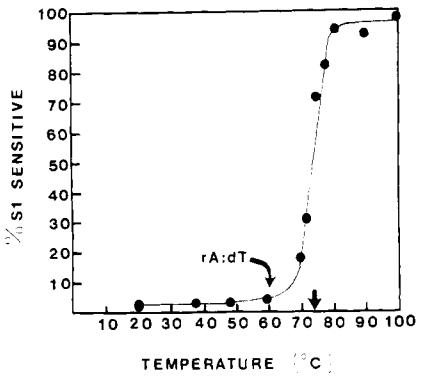

FIGURE 8: Thermal denaturation of the 5S RNA:5S cDNA hybrid. Procedure as described under Materials and Methods. Results are plotted as relative S_1 resistance. At 37 °C, 13% of the material was degraded; at 100 °C, 90% of the material was degraded. The T_m for poly(rA:dT) is indicated by an arrow. The T_m of the 5S RNA:5S cDNA hybrid (74 °C) is indicated by the unlabeled arrow.

Table V: Hybridization of 5S Complementary DNA to Various RNAs[a]

	% hybridization at R_0t value of		
	10^{-2}	10^{0}	10^{2}
yeast tRNA	6	8	6
rat tRNA	6	12	75
rat liver 18S + 28S rRNA	7	92	95
E. coli 5S RNA	8	6	2
E. coli 16S + 23S rRNA	6	2	3
rat liver 5S RNA	65	85	85

[a] Hybridization was performed as described under Materials and Methods. RNA concentrations were 10 μg/mL. Background values at zero time (4–7%) have not been subtracted.

bridization of the cRNA to 5S RNA at 50 °C. Since the rate of hybridization decreases as the temperature decreases with molecules of high GC content, the observed $R_0t_{1/2}$, determined at 37 °C, is as expected for a molecule of the complexity of 5S RNA.

The melting profile of the RNA:DNA hybrid is shown in Figure 8. The observed T_m is 74 °C, 2.5 °C below the theoretical T_m for a DNA:DNA molecule of 60% G + C content (Mandel & Marmur, 1968). In control experiments poly(rA):(dT), synthesized using poly(A) and reverse transcriptase, melted at the expected T_m of 60 °C (Kallenbach, 1968; Casey & Davidson, 1977).

In contrast to the hybridization observed with 5S RNA, the 5S cDNA does not form a hybrid with yeast tRNA, *E. coli* 5S rRNA, or *E. coli* 16S + 23S rRNA (Table V). Hybridization observed to rat tRNA and rat 18S + 28S rRNA at high R_0t values could be due to (i) sequences in tRNA and 18S + 28S rRNA identical with 5S RNA, (ii) cDNA containing sequences complementary to tRNA and/or 18S + 28S rRNA, or (iii) 5S RNA contaminating the tRNA and 18S + 28S rRNA preparations.

It is unlikely that identical sequences are present in the three RNAs since hybridization would have occurred at lower R_0t values than observed if even one species of tRNA (or a portion of 18S + 28S rRNA) possessed sequences identical with those of the 5S RNA. It is also unlikely that the cDNA contains sequences complementary to tRNA and/or the large ribosomal RNAs in view of the sharp Sephadex G-100 profile and polyacrylamide gel electrophoresis pattern of the 5S rRNA, and base composition of the cDNA (Table III). In addition, the absence of a biphasic R_0t curve on hybridization of 5S RNA to the cDNA (Figure 7) indicates the presence of only one species of RNA of the complexity of 5S RNA. (If, for example, the cDNA contained significant amounts of 18S + 28S rRNA sequences, a second component, with a $R_0t_{1/2}$ of 3×10^{-2}, would have been observed.) Furthermore, if the cDNA was contaminated with sequences complementary to rRNA or tRNA, then, when cDNA is hybridized to 18S + 28S RNA or tRNA, one would expect only a fraction of the cDNA to hybridize with a relatively low R_0t. Instead, all of the cDNA hybridizes but at high R_0t values, indicating the presence of a small amount of 5S RNA in the 18S + 28S RNA and tRNA preparations. Hybridization of rat tRNA to the 5S cDNA is observed with a $R_0t_{1/2}$ of about 10, while the $R_0t_{1/2}$ of 5S RNA hybridizing to the cDNA is 0.001 (Figure 7), indicating that less than 0.01% of the tRNA preparation is 5S RNA. Similarly, hybridization of rat 18S + 28S rRNA to the cDNA is observed with a $R_0t_{1/2}$ of about 0.1 giving a maximum value of <1% for contaminating 5S RNA species in the preparation used.

Buoyant Density Analysis. In Cs_2SO_4 gradients (Figure 9), KB 5S RNA bands at 1.630 g/cm^3, the expected density of RNA; native KB DNA bands at the expected density of 1.425 g/cm^3; the 5S cDNA bands at 1.455–1.460 g/cm^3, the expected value for single-stranded DNA molecules of 60% G + C content. The hybrid (5S RNA:5S cDNA) bands at 1.560 g/cm^3, as expected. After treatment with alkali, the remaining material, the 5S cDNA, bands at 1.470 g/cm^3. Treatment of the hybrid with nuclease S_1, RNases A and T_1 (by using conditions where only single-stranded RNA is degraded), and RNases A and T_1 and then nuclease S_1 all result in a hybrid banding at 1.530 g/cm^3, indicating that the hybrid has few, if any, single-stranded regions.

Similar results were obtained in CsCl gradients. The cDNA bands at 1.740 g/cm^3 as expected (Schildkraut et al., 1962) for a single-stranded DNA of 60% G + C and the hybrid bands at 1.786 g/cm^3. Treatment with nuclease S_1, RNases A and T_1, RNases A and T_1 and then nuclease S_1 does not change the density.

Annealing of 5S cDNA to Calf Thymus DNA and Salmon Sperm DNA. The 5S cDNA anneals to calf thymus DNA with a $C_0t_{1/2}$ of 2.1 (Figure 10) and to salmon DNA with a $C_0t_{1/2}$ of 0.7. No annealing to bacterial DNA is observed.

The number of 5S RNA genes in the calf thymus cell can be determined by comparing the $R_0t_{1/2}$ of 5S cDNA:5S RNA hybridization (Figure 7) with the $C_0t_{1/2}$ of 5S cDNA:calf thymus DNA annealing (Figure 10) taking into consideration differences in rate constants for hybridization and annealing in 50% formamide–4 × SSC, as well as the effect of the size of driver and tracer species (Wetmur & Davidson, 1968; Bishop, 1972; Muto, 1977; Hinnebusch et al., 1978), by comparing reannealing of *E. coli* DNA ($C_0t_{1/2}$ = 3.5; data not presented) to the annealing of cDNA to calf DNA (cf. Melli et al., 1971), and by titrating calf DNA to cDNA (cf. Muto, 1977) (manuscript in preparation). From these calculations, the number of 5S RNA genes is estimated to be between 1500 and 3000 per genome (haploid).

Discussion

A method has been developed to obtain large amounts of total ribosomal RNA and pure and intact 5S RNA. From 150 g of rat liver, 400 mg of ribosomal RNA is obtained, in contrast to the 1 mg of ribosomal RNA obtained by Muramatsu (1973) from 1 g of liver. From 400 mg of ribosomal RNA, 5 mg of 5S RNA is obtained. This represents a 70% yield of 5S RNA. The increased yields of total ribosomal RNA and 5S RNA are partially attributable to the use of heparin as an RNase inhibitor. The use of a buffer containing

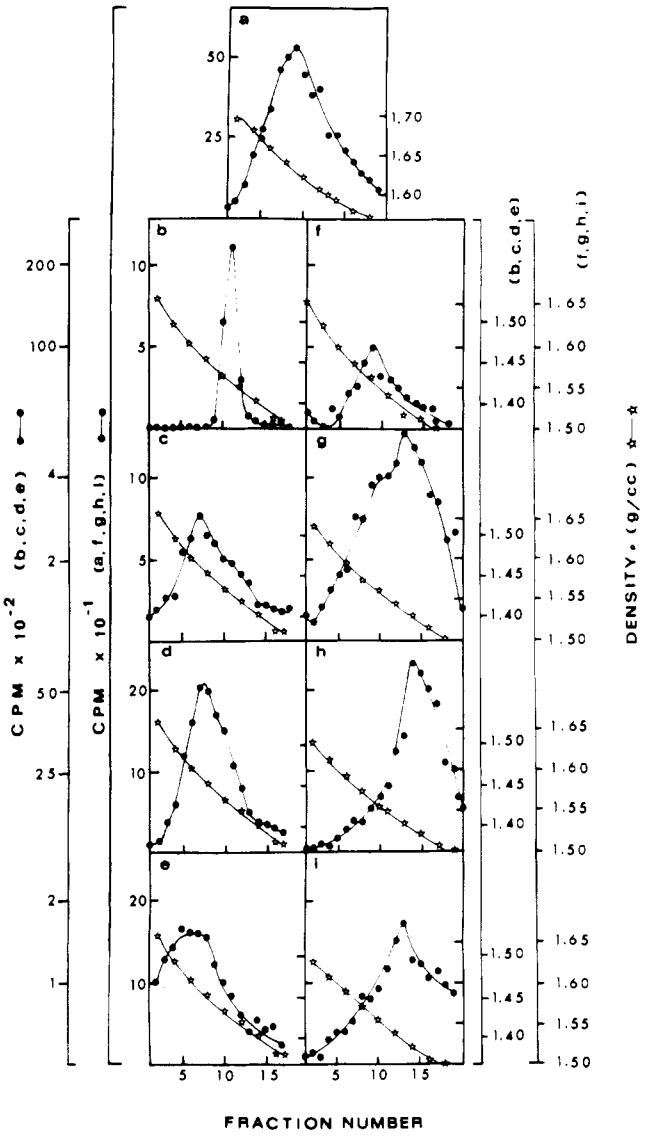

FIGURE 9: Buoyant density analysis of 5S cDNA:5S RNA in Cs_2SO_4. Experimental details as described under Materials and Methods. Panels: (a) 5S RNA; (b) KB DNA; (c) cDNA (1×10^7 counts min^{-1} μg^{-1}); (d) cDNA (3.35×10^5 counts min^{-1} μg^{-1}); (e) 5S RNA:cDNA hybrid incubated 16 h at 37 °C in 0.5 N NaOH; (f) 5S RNA:cDNA hybrid; (g) 5S RNA:cDNA hybrid treated with nuclease S_1; (h) 5S RNA:cDNA hybrid treated with RNase A and RNase T_1; (i) 5S RNA:cDNA hybrid treated with RNases A and T_1 and then nuclease S_1 as in g.

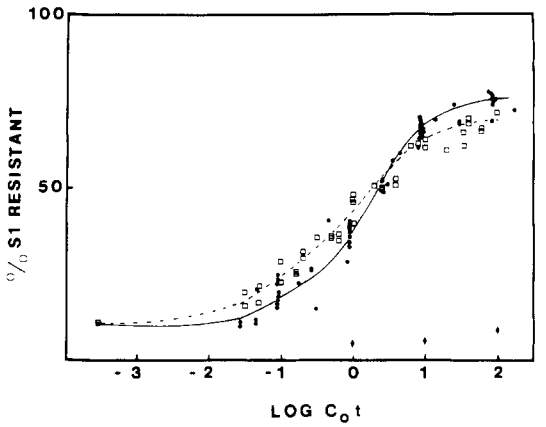

FIGURE 10: Annealing of 5S cDNA to calf thymus DNA and salmon sperm DNA. Experimental details as described under Materials and Methods. (●) Calf thymus DNA; (□) salmon sperm DNA; (♦) *E. coli* DNA.

Triton X-100 to wash nuclei, omission of high density sucrose in centrifugation of ribosomes, and the RNA extraction buffer also contribute to the increased yield. The use of a large Sephadex G-100 column permits the resolution of 5S RNA from 200 mg of unfractionated ribosomal RNA (Figure 1). A long, thin Sephadex column (1.5 × 210 cm) then makes it possible to obtain clean preparations of 5S RNA. We have also found that tRNA (pool III) is purified upon chromatography with this long column.

The 5S RNA serves as primer for calf thymus poly(A) polymerase and is not degraded by it. Most previous attempts to polyadenylate a defined RNA species for a specific purpose have used *E. coli* or plant poly(A) polymerase preparations (Thrall et al., 1974; Hell et al., 1976) which nicked or degraded the RNA (Hell et al., 1976). In contrast, calf thymus poly(A) polymerase prepared by the procedure of Keshgegian et al. (1975c) is free of RNase. Large quantities of calf thymus poly(A) polymerase can be obtained and remain stable when stored at −90 °C for 1–2 years.

Poly(A) tracts are initiated on 5S RNA molecules throughout the incubation period, since the amount of residual 5S RNA continuously decreases (Table I). The poly(A) tracts attached to 5S RNA increase in size with time. The apparently slow utilization of 5S RNA as primer, the presence of an initial lag period (Figure 2), and the fact that 5S RNA is not polyadenylated in vivo suggest that 5S RNA may be in an unfavorable conformational state for binding to the enzyme. The dependence of the initial states of the reaction on the concentration of enzyme, but not of the ATP substrate (Figure 4), is consistent with this hypothesis. Nevertheless, 5S RNA can be substantially polyadenylated in vitro.

There are other cellular RNA species that are not polyadenylated in vivo. By using the methodology of this study, these RNA species could also be polyadenylated in vitro. A complementary DNA could then be synthesized by using RNA-dependent DNA polymerase and an oligo(dT) primer that hybridizes to the poly(A) tract.

The polyadenylation reaction is a prerequisite for cDNA synthesis because 5S RNA does not serve as a template for AMV reverse transcriptase (Table II). The product of the reaction when polyadenylated 5S RNA is used as template appears to be a faithful copy of the template by base composition analysis (Table III) and nearest-neighbor analysis (Table IV). The 5S RNA is deemed to be pure by the following criterion: (i) ribosomes were prepared from a detergent treated postmitochondrial extract (eliminating mRNA contamination); (ii) purified ribosomes were extracted and the RNAs purified by several cycles of Sephadex chromatography to yield homogeneous species; and (iii) on denaturing (urea) and nondenaturing polyacrylamide gel electrophoresis, the 5S RNA migrates as a single, sharp band. This establishes that the cDNA is a copy of only one template, the 5S RNA.

The cDNA is full size (120 nucleotides plus 15 nucleotides of primer) as demonstrated in alkaline–sucrose gradients (Figure 6), but it is smaller than the polyadenylated 5S RNA (Figure 5) due to the extra poly(A) on the RNA template.

Hybridization analysis and T_m analysis demonstrate that the cDNA is an accurate (and complete) copy of 5S RNA. The observed T_m value for the 5S cDNA:RNA hybrid is identical with that reported by Kimmel & Gorovsky (1976) for a 5S RNA:DNA hybrid. While this could represent 4–5 base pairs mismatched (Laird et al., 1969), it is perhaps more likely that a lower T_m is observed because RNA:DNA hybrids usually melt at slightly lower temperatures than DNA:DNA duplexes (Kallenbach, 1968). An additional possibility is that

the 5S RNA:DNA molecules are smaller than that required for optimal thermal stability of duplexes (Crothers et al., 1965; Kallenbach, 1968). On the other hand, the sharpness of the thermal denaturation profile (melting occurs over a temperature range of 10 °C) indicates that there is no "fraying" at the ends of the molecule.

The hybrid does not appear to contain single-stranded regions since treatment with nuclease S_1, RNases A and T_1, and RNases A and T_1 followed by nuclease S_1 treatment does not significantly change the buoyant density in Cs_2SO_4 (1.560 g/cm^3 in controls as compared with 1.530 g/cm^3 in treated hybrids). If significant portions of the RNA were single stranded, the density would shift toward 1.630 g/cm^3; if the DNA were single-stranded, the density would shift toward 1.455 g/cm^3 (Figures 9a–e). The slight change in density after treatment (0.03 g/cm^3) may be due to the removal of the oligo(dT_{15}) primer.

Cavalieri & Carroll (1970) reported the synthesis of a cDNA to 5S RNA by using *E. coli* DNA polymerase I. However, in their experiments the amount of dAMP incorporated was twice the amount of dTMP, although the complement of 5S RNA should contain roughly equal amounts of dAMP and dTMP (Table III). The 5S cDNA prepared here is a complete and precise copy of 5S RNA and has a variety of applications. It is preferable, in many instances, to an RNA probe as DNA is more stable than RNA, and 5S cDNA of high specific activity can be obtained, higher than that which can be obtained when 5S RNA is isolated from labeled cells.

With the 5S cDNA probe, the frequency of 5S RNA genes in calf thymus has been determined. This value, 1500–3000 per genome (haploid), can be compared with 800 rat liver 5S RNA genes (Quincey & Wilson, 1969) and 2000 5S RNA genes in the HeLa cell genome (Hatlen & Attardi, 1971). These values are significantly less than the number of 5S RNA genes in *Xenopus* (10 000 to 25 000) (Birnsteil et al., 1972; Brown & Weber, 1968).

The sequence of 5S RNA is very similar in higher organisms. It appears to be identical in all mammalian cells (Walker et al., 1975) and *Xenopus* 5S RNA differs from mammalian 5S RNA by only seven base changes, mainly purine–purine and pyrimidine–pyrimidine substitutions (Brownlee et al., 1972). Annealing of the 5S cDNA to salmon sperm DNA would, therefore, be expected. Since the DNA complexity of fish is the same or slightly less than that of mammals (Sober, 1970), there appears to be at least the same number of 5S RNA genes (3000) in salmon sperm.

In addition to determining gene frequency, 5S ribosomal cDNA can be used in studying specificity of transcription, analogous to studies using a 5S DNA obtained from plasmids (Hershey et al., 1977; Carroll & Brown, 1976a,b; Parker & Roeder, 1977; Yamamoto & Seifart, 1977). Preparation of a 5S cDNA probe directly from 5S RNA is an alternative approach which has the advantage of eliminating hybridization to spacer regions of the isolated 5S gene (cf. Parker & Roeder, 1977; Yamamoto & Seifart, 1977). In addition, with 5S cDNA, hybridization to the antisense portion of the 5S ribosomal RNA gene does not occur. By utilizing this probe, preferential transcription of the 5S RNA gene by RNA polymerase III in vitro from both native and denatured DNA templates has been demonstrated (Ackerman et al., 1978; Ackerman & Furth, 1979).

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