RNase III Cleavage of *Escherichia coli* rRNA Precursors: Fragment Release and Dependence on Salt Concentration†

Mark Birenbaum, David Schlessinger,* and Shuichi Hashimoto

ABSTRACT: Complete digestion of 30S pre-rRNA by RNase III yielded p16S and p23S pre-rRNAs, and at least six smaller species that varied in length from 100 to 525 nucleotides. The products of the reaction were dependent on the monovalent and divalent cation concentration. In the presence of 10 mM $MgCl_2$ at least one 400 nucleotide species was cleaved further by RNase III. This cleavage was inhibited in the presence of 0.5 mM $MgCl_2$. If the NH_4Cl concentration was raised from 100 to 400 mM, a species larger than p23S, termed 25S pre-rRNA, was accumulated. The six small species observed after cleavage in 0.5 mM $MgCl_2$ were 70 to 80% pure as determined by RNase T1 fingerprint analysis. One 200 nucleotide species contained pppNp; it and a second species with a very similar fingerprint were most likely cleaved from the 5′-terminal end of 30S pre-rRNA. One 300 nucleotide species contained 5S rRNA oligonucleotides; it and a species with a similar T1 fingerprint were inferred to originate from the 3′-terminal end of 30S pre-rRNA. The other two species were 400–525 nucleotides long. Each contained a UΨCG sequence and at least 8 modified nucleotides. They were therefore probably derived from the tRNA-containing spacer region of the 30S transcripts. The two tRNA-containing species were distinct in their content of modified nucleotides, and each small RNA was present in 0.5–0.6 mol per mol of 30S pre-rRNA cleaved. Therefore, the 30S transcripts include at least two sequence classes in the spacer regions, although a cleavage map consistent for all the transcripts has been constructed.

In *E. coli* mutant strain AB301/105 (Kindler et al., 1973), 16S and 23S rRNA sequences can be found initially transcribed in a single unit, 30S pre-rRNA, about 6250 bases long (Dunn and Studier, 1973; Nikolaev et al., 1973a, 1974). In normal cells, this large transcription product is cleaved or "processed" during its synthesis to yield p16S and p23S precursors of the large rRNAs (Pace, 1973; Nikolaev et al., 1975). The mutant AB301/105 is selectively deficient in RNase III (Robertson et al., 1968; Kindler et al., 1973; Nikolaev et al., 1973b; Studier, 1975), and the inference has been repeatedly verified that RNase III can cleave the purified 30S pre-rRNA to reproduce its initial cleavages in vivo (Nikolaev et al., 1973b; Dunn and Studier, 1973; Ginsberg and Steitz, 1975; Hayes et al., 1975).

In cleavage trials, several small fragments are also released from pre-rRNA by RNase III (Nikolaev et al., 1973b; Ginsberg and Steitz, 1975; Nikolaev et al., 1975; Hayes et al., 1975). However, the kinetics of the reaction and its products have not been completely clear. At least four problems were repeatedly encountered: (1) even in whole cells, some variation in the pattern of cleavage of 30S pre-rRNA was observed, for example, in cells incubated in media containing low phosphate levels (Nikolaev et al., 1975); (2) the first studies showed that there were at least three small fragments observed (Ginsberg and Steitz, 1975; Hayes et al., 1975), but their stoichiometry varied from less than one to several per 30S RNA chain processed; (3) the preparations of RNase III used were not completely pure, and the intrinsic capacity of RNase III to catalyze irrelevant cleavages was unclear (Robertson et al., 1968; Schweitz and Ebel, 1971; Westphal and Crouch, 1975; Dunn, 1976); and (4) experimentation with a single preparation of ^3H-labeled 30S pre-rRNA was limited by the tendency of the high molecular weight chains to accumulate breaks on storage over a period of more than 5 days, leading to some random fragmentation that obscured cleavage patterns.

Here we have remedied some of the technical problems and analyzed the in vitro cleavage of pre-rRNA in greater detail. More stable preparations of RNA and homogeneous RNase III were used. The data are discussed in relation to previous findings and to the questions: (1) is the in vitro reaction ordered, e.g., do some small fragments appear before others?; (2) are the small fragments stable to further attack by RNase III?; (3) does a change in the temperature or salt concentration of the reaction mixture alter the course of the cleavage reaction?; and (4) what is the arrangement of fragments in the original 30S pre-rRNA transcript?

Experimental Procedures

Materials. [^3H]Poly A (20 mCi/mmol) and [^3H]poly(C) (6 mCi/mmol) were from Schwarz/Mann. [^3H]Uridine (29 Ci/mmol) and $^{32}PO_4$ (carrier free, 126 Ci/mg) were purchased from Amersham. Unlabeled poly(I) and poly (dT) were products of Miles. Agarose–poly(I):poly(C) (type 6, 11.9 mg of pI:pC/mL of wet gel) was from P-L Biochemicals. Nucleases T_1 and T_2 were products of the Sankyo Co. Cellulose thin-layer plates were from Brinkman Instruments. DEAE[1]-cellulose paper, DEAE-cellulose (DE-52), and phosphocellulose (PC-11) were from Whatman. Cellulose acetate membrane strips were purchased from Schleicher and Schuell and x-ray film for autoradiography was Kodak XR-5, 35 × 43 cm. Lauroylsarcosine was purchased from K & K Laboratories. Acrylamide was the Eastman product, recrystallized before use. Dimethyl sulfoxide was from Fisher and was redistilled before use. Formamide was from Matheson Coleman and Bell and ethylene diacrylate from the Monomer-Polymer Labo-

† From the Department of Microbiology and Immunology, Division of Biology and Biomedical Sciences, Washington University School of Medicine, St. Louis, Missouri 63110 (M.B. and D.S.), and the Institute for Molecular Virology, St. Louis University School of Medicine, St. Louis, Missouri 63104 (S.H.). *Received December 7, 1976; revised manuscript received September 9, 1977.* Supported by National Science Foundation Grant BMS74-11779.

[1] Abbreviations used are: DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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ratories of Haven Chemical Co. Protosol solubilizer was purchased from New England Nuclear.

[^{3}H]Poly(C):poly(I) and [^{3}H]poly(A):poly(dT) were prepared by mixing equimolar amounts of the polymers in a buffer containing 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 0.1 mM EDTA, pH 7.4, and incubating the mixture for 2 h at room temperature. Before they were combined the homopolymers were heated for 15 min at 37 °C to denature any double-stranded forms.

Preparation of RNase III. RNase III was first prepared by methods previously described (Robertson et al., 1968; Crouch, 1974; Gotoh et al., 1974). The fraction from a 0.2 M NH_4Cl ribosome wash was applied to a Sephadex G-100 column (1.5 × 90 cm) after $(NH_4)_2SO_4$ precipitation. The low salt effluent was passed through a DEAE-cellulose column (1.5 × 43 cm) and the fraction that did not bind was applied to a phosphocellulose column (0.9 × 9.8 cm) preequilibrated with 50 mM NH_4Cl and 20 mM Tris-HCl, pH 7.5, at 4 °C. A salt gradient from 0.1 to 0.6 M NH_4Cl eluted the enzyme at a salt concentration of 0.3 M NH_4Cl. The enzyme obtained after phosphocellulose purification is referred to below as "PC-RNase III". RNase H also eluted at about this salt concentration. Peak fractions were pooled, diluted to approximately 0.1 M NH_4Cl, and applied to an agarose-poly(I):poly(C) column (0.9 × 5 cm). The column was washed with 0.6 M NH_4Cl followed by a salt gradient from 0.6 M to 2 M NH_4Cl. RNase III eluted at 1.3 M NH_4Cl (Figure 1), and is called "IC-RNase III" below. At this stage of purification the enzyme showed one band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Weber and Osborn, 1969). Peak fractions were pooled; a portion was stored at 4 °C, and another at −70 °C. At 4 °C the enzyme maintained about 80 to 90% of its activity after 4 months. Freezing to −70 °C resulted in a 40–50% loss of activity upon thawing, but the remaining activity has been stable at −70 °C for at least 15 months.

The standard assay for RNase III contained 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NH_4Cl, and 3 nmol (6000 cpm) of [^{3}H]poly(C):poly(I) in a final volume of 50 μL at 35 °C. Reactions were stopped after 30 min by the addition of 100 μL of 9% Cl_3CCOOH. Twenty microliters of 20 mg/mL bovine serum albumin was added as carrier and the solution mixed on a Vortex mixer. The mixture was then centrifuged 5 min at 2000×*g* and 100 μL of the supernatant was dissolved in toluene scintillation fluid containing 10% Protosol. Counting was done in a Packard Tri-Carb spectrometer, series 3000. For detection of RNase H, 1 nmol of [^{3}H]poly(A):poly(dT) (5000 cpm) was added per reaction and the assay performed as for RNase III. The assay for single-stranded activity utilized 1–2 nmole [^{3}H]poly(C) (4000 cpm) and a buffer containing 10 mM Tris-HCl, pH 7.5, and 1 mM $MgCl_2$.

Growth of Strains and Preparation of RNA. Strain AB301/105 was grown at 30 °C with aeration, both for ^{3}H and ^{32}P labeling as previously described (Nikolaev et al., 1975). The extraction of 30S and 17.5S pre-rRNA was based on the phenol method previously described (Nikolaev et al., 1973b). Three milliliters of a buffer containing 0.2 M Tris-acetate, pH 5.5, 10 mM EDTA, and 0.5% sodium dodecyl sulfate was added to the cells collected from a 50-mL culture. Two volumes of redistilled phenol saturated with the same buffer was then added.

The mixture was shaken for 15 min at 60 °C and centrifuged for 30 min at 12 000*g* to pack the protein pellet very firmly at the phenol–water interface. The 60 °C phenol extraction was repeated once, followed by precipitation of the RNA with 2.5 volumes of pure ethanol overnight at −20 °C. Two more phenol extractions were done at room temperature, followed by ethanol precipitation, usually for 5 h. The RNA was dissolved in 0.1 M Tris–acetate, pH 7.5, containing 0.5% sodium dodecyl sulfate and 10 mM EDTA, and layered on a 10–30% sucrose gradient. [^{32}P]RNA for fingerprint and thin-layer chromatographic analyses was dissolved in the same buffer containing 80% dimethyl sulfoxide, heated to 60 °C for 2 min, and immediately cooled on ice and applied to a 10–30% sucrose gradient. The heating in dimethyl sulfoxide was done to reduce the amount of 4S material bound to 30S pre-rRNA, which was otherwise observed in some preparations (see Discussion).

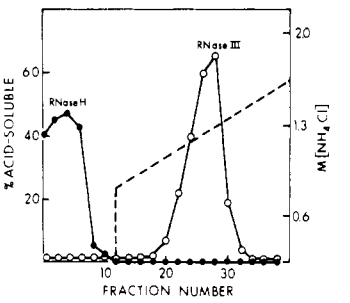

FIGURE 1: Elution of RNase III from an agarose poly(I):poly(C) column. Peak fractions from a P-11 phosphocellulose column were pooled, diluted to 0.1 M NH_4Cl, applied to an agarose-poly(I):poly(C) column, and eluted with a linear-salt gradient (as in Experimental Procedures). Details of the RNase III and RNase H assays are given in Experimental Procedures. (O- - -O) Cl_3CCOOH-soluble [^{3}H]poly(C) cts/min released by RNase III; (●—●) Cl_3CCOOH-soluble [^{3}H]poly(A) cts/min released by RNase H; (- - - -) NH_4Cl concentration.

One sucrose gradient was usually sufficient to separate the various rRNA precursor species. Peak fractions were pooled, washed at least three times by ethanol precipitation after resuspension in 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 300 mM NH_4Cl and then stored in 70% ethanol at −20 °C. Following this extraction procedure, the 30S pre-rRNA appeared in standard gel electrophoresis as a single species after storage for up to 3 weeks, the longest period tested.

The 25S pre-rRNA was recovered from pulse-labeled growing cells. To a logarithmically growing 50-mL culture of AB301/105 cells, rifampicin was added to a final concentration of 200 μg/mL. Thirty seconds later [^{3}H]uridine (3 mCi) was added to the 50 mL culture. Three minutes after the addition of label, the culture was poured over crushed ice and the cells were collected by centrifugation. Cells were then phenol extracted and their RNA was purified on sucrose gradients as above.

RNase III Cleavage of Pre-Ribosomal RNAs. In the experiments with ^{3}H-labeled 30S pre-rRNA, approximately 0.1 μg (10^5 cpm) was incubated with 3–5 μL of purified RNase III in a final volume of 50 μL. Three microliters of the RNase III preparation degraded 5% of [^{3}H]poly(C):poly(I) to acid solubility under identical reaction conditions. The reaction buffer usually contained 20 mM Tris-HCl, pH 7.6, at 35 °C, 10 mM $MgCl_2$, and 100 mM NH_4Cl. However, in some cases the $MgCl_2$ and NH_4Cl concentrations were varied (see Results). The temperature for all the reactions was 35 °C. Reactions were stopped by the addition of sodium dodecyl sulfate and EDTA, pH 7.2, to a final concentration of 1.5% and 15 mM, respectively. For the ^{32}P-labeled 30S pre-rRNA, approximately 20 × 10^6 cpm (10 μg) were dissolved in 2 mL of a buffer containing 20 mM Tris-HCl, pH 7.6, at 35 °C, and 0.6 mM $MgCl_2$. Purified RNase III (160 μL) was added (the enzyme was stored in 1.3 M NH_4Cl), and the reaction mixture was incubated for 30 minutes at 35 °C. One hundred and sixty microliters more of RNase III was then added and the incubation continued for 30 min. A 2:1 mixture (0.4 mL) of 10%

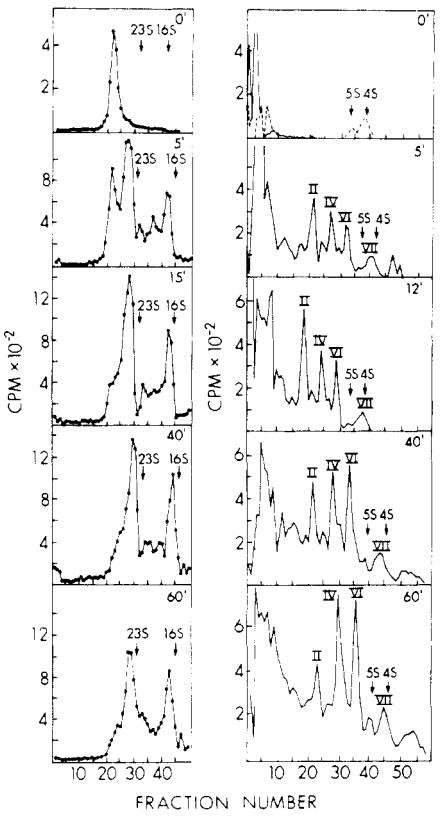

FIGURE 2: Kinetics of cleavage of 30S pre-rRNA by RNase III; production of small and large fragments. ^{3}H-labeled 30S pre-rRNA was incubated with PC-RNase III for the times shown. Products were analyzed on 0.5% agarose/2.2% acrylamide gels (left panels) to display fragments larger than 16 S. In addition, samples were also fractionated in 6.5% acrylamide gels to display smaller fragments (right panels; see Experimental Procedures). The positions of 4S, 5S, 16S, and 23S marker RNAs were detected by their content of ^{14}C; the dashed line in the upper right (time zero) panel shows a sample pattern of ^{14}C cpm.

sodium dodecyl sulfate and 0.2 M EDTA was added to stop the reaction, followed by 6 mL of 100% ethanol and 50 μL of 5 M NH_4Cl. The mixture was stored at −20 °C.

Gel Electrophoresis. For the analysis of rRNA precursors, 0.5% agarose/2.2% acrylamide gels were used (Nikolaev et al., 1975). Small fragments from 30S pre-rRNA were fractionated in 6.5% acrylamide gels containing 0.6% ethylene diacrylate cross-linker. Electrophoresis of 6.5% acrylamide gels was for 9–10 h with 80 V at 4 °C. In trials for the analysis of ^{32}P-labeled RNA, and for the determination of approximate molecular weights of the small fragments, the gels also contained 6 M urea, and the samples were dissolved in 90% formamide, heated for 5 min at 55 °C, and then applied to the gel. The gels were sliced and solubilized as described (Nikolaev et al., 1975).

Estimation of Label Content in [^{3}H]RNA Species Observed in Gels. The ^{3}H-label content in the RNA species was estimated as described (Nikolaev et al., 1975). In some figures (2 and 5B–D), the cpm in each gel slice are indicated; in other figures (4 and 5A), the cpm are plotted from paper tapes as a continuous line by a PDP-12 computer.

Elution of ^{32}P-Labeled RNA from Polyacrylamide Gels. Gel electrophoresis was done using a buffer containing 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, and 0.2% lauroylsarcosine, adjusted to pH 7.5 with acetic acid. Following autoradiography of a gel the position of each band was determined by comparison with the autoradiograph. The bands were sliced apart, and each section of gel containing a predominant band was reinserted into a siliconized gel tube. A small, open-ended siliconized glass rod was inserted to support the gel slice and the gel tube was filled with buffer containing 50 μg/mL carrier tRNA. A piece of dialysis tubing was then placed across the bottom of the gel tube. After electrophoresis for 2 h at 80 V in a jacketed tank maintained at 4 °C, the buffer between the gel slice and the dialysis tubing was removed, and its content of RNA was precipitated by addition of 2.5 volumes of absolute ethanol. One more ethanol wash after overnight storage was adequate to remove traces of lauroylsarcosine. Recoveries of RNA were greater than 90% (98% for the smallest bands).

Fractionation of Oligonucleotides. Two-dimensional electrophoresis for the separation of oligonucleotides was done on cellulose acetate membrane strips in the first dimension and DEAE-cellulose paper in the second dimension as described (Sanger et al., 1965; Hashimoto and Muramatsu, 1973). Nucleotide sequences or compositions of the spots detected by autoradiography were inferred by comparison with published fingerprints (Sanger et al., 1965) as well as fingerprints of RNase T1 digests of ^{32}P-labeled KB cell 28S rRNA and *E. coli* 5S rRNA.

To elute an oligonucleotide from the DEAE-cellulose paper the section of paper was cut out and saturated with a 2 M solution of triethylamine bicarbonate as described (Sanger et al., 1965). The eluent was dried in an evacuated desiccator and the resultant precipitate was washed twice with distilled water and redried. The precipitate was resuspended in 10 μL of 20 mM sodium acetate, pH 4.5, containing 4 μg of carrier tRNA. One unit of RNase T2 was added and the solution incubated for 2 h at 37 °C. The solution was spotted on a cellulose thin-layer plate and chromatographed.

Thin-Layer Chromatography. Two-dimensional thin-layer chromatography was performed as described (Nishimura, 1972). Nucleotides were scraped from the cellulose glass plates and eluted with 0.6 mL of 2 M triethylamine bicarbonate buffer in a 20-mL plastic scintillation vial. The ^{32}P cpm in each fraction were estimated by Cerenkov radiation.

DEAE-Sephadex Chromatography. DEAE-Sephadex chromatography of RNase T2 digests was done as described (Hashimoto and Green, 1976).

Results

Kinetics of RNase III Cleavage of 30S Pre-rRNA. The kinetics and products of cleavage were observed using PC-RNase III and the RNA species purified as in Experimental Procedures, in buffer containing 0.1 M NH_4Cl, 10 mM $MgCl_2$, and 20 mM Tris-HCl, pH 7.5. At the end of the incubation period part of the reaction mixture was applied to a 2.2% acrylamide/0.5% agarose gel (Figure 2, left panels) and the remainder, to a 6.5% acrylamide gel (Figure 2, right panels).

Precursors to the final "p23S" and "p16S" (Nikolaev et al., 1975) could always be observed at early times. In some experiments, for example, nearly all the RNA destined for the p16S species could be seen first at 17.5 S (data not shown). In other experiments, (Figure 2, for example) some p16S had already appeared when much more 17.5S RNA was still being formed. Thus, the relative rates of some cleavages varied from trial to trial.

Figure 2 shows one set of electrophoretic analyses after 0, 5, 15, 40, and 60 min of incubation. The progressive accumulation of "p23S" and "p16S" species (left panels) is clear up to 40 or 60 min. A fraction of 30S RNA, varying from 10 to 20% in various trials, remained uncleaved (cf. Ginsberg and Steitz, 1975). In experiments like that of Figure 2, where PC-RNase III was used, the final ratio of p23S/p16S tended

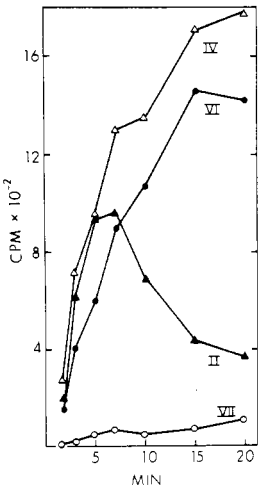

FIGURE 3: Amounts of small fragments produced during cleavage of 30S pre-rRNA by RNase III. Portions of reaction mixtures were fractionated on 6.5% acrylamide gels after the indicated times of incubation with RNase III. The amount of label in each fraction (II, IV, VI, and VII) was determined as described in Experimental Procedures. In these trials, PC enzyme was used, and the 30S pre-rRNA was not heated in dimethyl sulfoxide to remove bound 4S RNA.

to be less than the expected mass ratio of 1.8 (Nikolaev et al., 1974). With the same amount of IC-RNase III (Experimental Procedures), the results were the same except that the final ratio was consistently closer to the theoretical mass ratio of the two species (1.8 to 2.0), even after 60 min of incubation (Figure 4B).

RNase III cleavage of 30S pre-rRNA released several small fragments which were analyzed on 6.5% polyacrylamide gels (Figure 2, right panels). In all of six kinetic trials, four major bands appeared, labeled II, IV, VI, and VII (see also Figure 6). Several minor bands were better resolved in later experiments and studied further as bands I, III, and V (see below, Figure 6).

Even at the earliest time point (Figure 2b) all of the RNA bands were detectable. Thus, there did not appear to be a specific order to the appearance of the small RNA species, although some cleavage sites showed an apparently higher affinity for RNase III (below and Figure 9).

Early in the reaction (after 2 to 15 min of incubation) band II had more radioactivity than bands IV and VI. At later times, however, band II decreased while bands IV and VI increased further (Figure 3).

Effect of Salt Concentration on the Cleavage of 30S Pre-rRNA by RNase III. When the reaction conditions were varied, the course of the cleavage reaction changed. Three variables were tested to some extent: temperature of the reaction, and monovalent and divalent cation concentrations. Over a temperature range of 20 to 40 °C, the cleavage patterns were comparable, though the reaction rate varied as expected (data not shown).

A considerable change was, however, observed when the monovalent cation concentration was raised. In 0.4 M NH_4Cl, stoichiometric amounts of p16S were still produced, but a large intermediate en route to p23S RNA was detected even after 30 min of reaction (Figure 4D). Concomitant with the incomplete cleavage of he precursor to p23S RNA, the smaller RNA bands II and IV were observed at much lower levels than normal. In contrast, band VI was still produced to the same extent (Figure 4d).

Lowering the divalent cation concentration from 10 mM to 0.5 mM $MgCl_2$ produced no detectable change in the final pattern of large fragments (Figure 4C). However, band II was

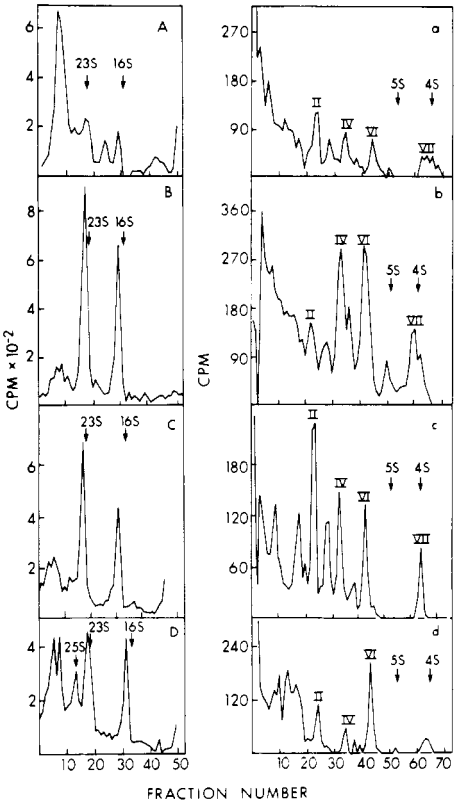

FIGURE 4: RNase III cleavage of 30S pre-rRNA using different concentrations of NH_4Cl and $MgCl_2$. The 30S pre-rRNA was purified and cleaved with IC-RNase III as described in Experimental Procedures. Reaction mixtures were divided and 40 μL was applied to 0.5% agarose/2.2% acrylamide gels (left panels A–D), and 80 μL was applied to 6.5% acrylamide gels (right panels a–d). ^{14}C marker RNAs were run with each sample and the positions of the peak fractions are indicated by arrows. All reactions contained 20 mM Tris-HCl, pH 7.5, and were stopped after 30-min incubation except for A, a which was stopped after 11 min. (A,a and B,b). In 10 mM $MgCl_2$, 100 mM NH_4Cl; (C, c) 0.5 mM $MgCl_2$, 100 mM NH_4Cl; (D, d) 10 mM $MgCl_2$, 400 mM NH_4Cl.

then present at markedly higher levels throughout the reaction, and two shoulders of band II were also more prominent (Figure 4c).

Cleavage of 25S and 17.5S Pre-rRNAs by RNase III. As expected for intermediate products from 30S pre-rRNA, the 25S and 17.5 pre-rRNAs each yielded characteristic fragments when treated with RNase III. ^3H-labeled 17.5S and ^3H-labeled 25S pre-rRNA were isolated from strain AB301/105 (as in Experimental Procedures). After an incubation of the 17.5S RNA with IC-RNase III, the small cleavage products were analyzed in 6.5% polyacrylamide gels (Figure 5B). The mobility of the predominant band observed corresponded closely to that of band VI from the reactions with 30S pre-rRNA (Figure 4c).

A comparable experiment was carried out with the 25S RNA species. Because p23S and partially cleaved 25S pre-rRNA predominated in cells labeled for 90 min in the presence of chloramphenicol, the 25S RNA for these trials was recovered from growing cells (see Experimental Procedures). RNase III cleavage of the 25S to p23S was observed (Figure 5C), in confirmation of earlier work (Nikolaev et al., 1975). When the release of small RNAs was assessed in 6.5% gels, the background was high, but the release of band IV was clear (Figure 5D). Larger fragments, including ones with the mobility of bands I and II (see below, Figure 6), were also observed.

RNase III Cleavage of ^{32}P-Labeled 30S Pre-rRNA for Structural Analyses. ^{32}P-labeled 30S pre-rRNA was prepared as described in Experimental Procedures. Care was taken to

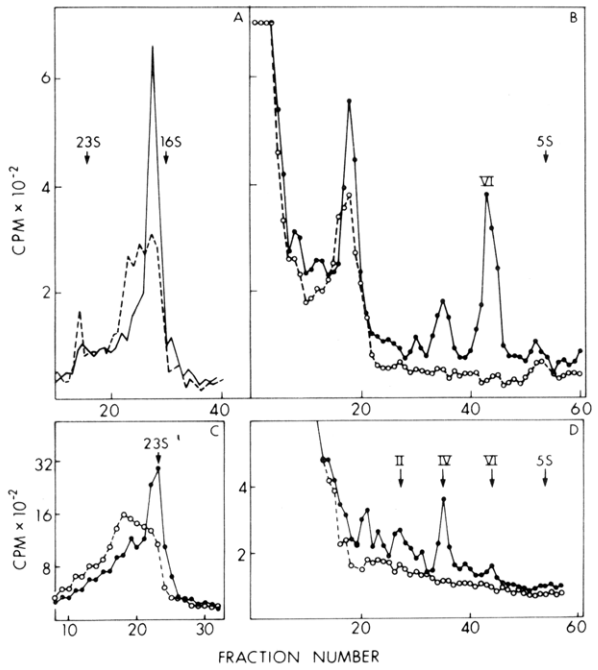

FIGURE 5: RNase III cleavage of 17.5S and 25S pre-rRNA. The "17.5S" pre-rRNA was isolated from in vivo after chloramphenicol addition as for 30S pre-rRNA. "25S" pre-rRNA was extracted 3.5 min after the addition of 0.2 mg/mL rifampicin to a logarithmically growing culture of AB301/105. The 17.5S and 25S precursors were incubated with IC-RNase III and the reaction products were applied to 0.5% agarose/2.2% acrylamide gels (A, C), and 6.5% acrylamide gels (B, D). ^{14}C marker RNA positions are indicated by the arrows. (O- - -O and - - -), RNA fractionated without RNase III treatment; (●—● and —) RNA after RNase III cleavage. (A, B) Cleavage products of 17.5S pre-rRNA; (C, D) cleavage products of 25S pre-rRNA.

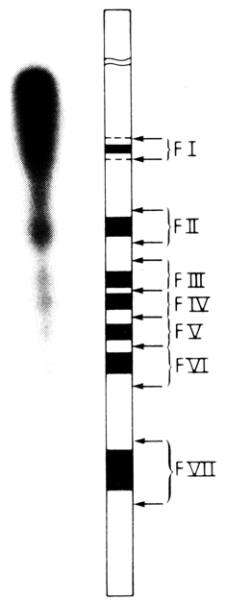

FIGURE 6: Fractionation of small ^{32}P RNA cleavage products after digestion of 30S pre-rRNA by RNase III. ^{32}P 30S pre-rRNA was cleaved with IC-RNase III and the reaction products were fractionated on a 6.5% polyacrylamide gel. The gel was removed from the gel tube and wrapped in Saran wrap to prevent its drying. Autoradiography was done by laying the gel on Kodak XR-5 x-ray film for 15 min. The gel was sliced at the positions marked by arrows. Fractions I–VII are labeled as FI–FVII.

TABLE I: Quantitation of Radioactivity in Small RNA Species Cleaved from 30S Pre-rRNA by RNase III.[a]

RNA fraction	^{32}P (cpm × 10^6)	% of 30S pre-rRNA	Nucleotide length	Moles per mole of 30S pre-rRNA
30S pre-rRNA	16.4	100	6250	1.00
I	0.76	4.6	525	0.54
II	0.70	4.3	420	0.64
III	0.46	2.8	360	0.48
IV	0.38	2.3	300	0.48
V	0.34	2.1	240	0.55
VI	0.34	2.1	200	0.66
VII	0.24	1.5	90	1.10
I–VII	3.22	19.7		

[a] The cpm in each RNA fraction was determined by counting the Cerenkov radiation emitted by the section of gel containing the RNA. The total length of spacer nucleotides was estimated to be 1050, assuming that 1800 nucleotides are contained in p16S and 3400 in p23S rRNAs. Thus, the spacer nucleotides comprise approximately 17% of the total 30S pre-rRNA transcript. The nucleotide length of each of RNA fractions I–VII was estimated by its mobility in 6.5% polyacrylamide–6 M urea gels, relative to 4S and 5S RNA.

minimize hydrogen bonding of tRNA to the 30S pre-rRNA (Lund and Dahlberg, 1977) by heat denaturing the RNA in dimethyl sulfoxide before purification on sucrose gradients. Also, ^{32}P-labeled 30S pre-rRNA was cleaved with IC-RNase III in the presence of 0.5 mM $MgCl_2$ (Figure 4c) in order to prevent further degradation of the material in band II.

The small RNAs were separated on a 6.5% polyacrylamide gel (Figure 6). Each band was cut out and its RNA content eluted from the gel. The quantitation of the fractions released by RNase III is calculated in Table I. The nucleotide length of each fraction was estimated by its mobility compared with marker 4S and 5S RNA on a 6.5% acrylamide gel containing 6 M urea. The radioactivity in each sample was determined by counting Cerenkov radiation from the segment of gel in 2 mL of water.

The total [^{32}P]RNA cpm in small fragments released by RNase III cleavage (19.7%; Table I) was close to the expected content of "extra" RNA sequences in 30S pre-rRNA as compared with the sum in p23S and p16S rRNAs (Nikolaev et al., 1974). Each predominant small RNA species released was present in only 0.5–0.6 mol per mol of 30S pre-rRNA (except for fraction VII). Thus, the spacer regions of the total 30S pre-rRNA transcripts are heterogeneous in their primary structure and/or in the number of cleavage sites for RNase III.

Two-Dimensional Fingerprints of the Bands from the 6.5% Polyacrylamide Gels. After it was eluted from the gel, the RNA was washed free of lauroylsarcosine and digested with ribonuclease T1. The resulting oligonucleotides were resolved with a standard two-dimensional electrophoretic technique (Sanger et al., 1965). Autoradiographs of the fingerprints are shown in Figure 7. All of the spots were cut out of the DEAE-cellulose paper and counted. Next to each autoradiograph is a sketch of all the spots that contained at least 0.4 mol of oligonucleotide.

Calculations of the molar amounts of some of the oligonucleotides produced by RNase T1 digestion are presented in Table II. If the RNA in any given band were 100% pure, all of the spots would occur in a molar ratio of 1.0 or multiples of that number. If the RNA were only 80% pure, then any spot that occurs only once in the sequence would yield 0.8 mol (and if its sequence occurs twice, 1.6 mol). Using this rationale, an estimate of the purity of each peak was made and is given at

the bottom of Table II. Most of the peaks were about 70% pure, with the exception of peak VII, which was only 50% pure.

All of the oligonucleotides present in 5S rRNA (excepting the mature 5′ and 3′ termini) were observed in fraction IV (Figure 7, shaded spots on sketch). Fraction III had a fingerprint very similar to that of fraction IV, but a few of the 5S oligonucleotides were present in low amounts (see Discussion). The fingerprints of fractions V and VI were nearly identical and were distinct from those of the other fractions (Figure 7). Fractions I and II also yielded fingerprints similar to one another, though a number of differences were found among the larger oligonucleotides (Figure 7).

Fraction VII, which was a diffuse band in the acrylamide gel (Figure 6), was heterogeneous in its oligonucleotide composition.

Two-Dimensional Thin-Layer Chromatography of Fractions I–VII. To determine its nucleotide composition, a portion of each RNA fraction was digested with ribonuclease T2, and the resulting mononucleotides were separated by two-dimensional thin-layer chromatography (Nishimura, 1972; Hashimoto et al., 1975). The thin-layer plates were autoradiographed for 1–2 days and the spots containing the four unmodified nucleotides, Ap, Cp, Gp and Up, were scraped from the plates. Autoradiography was repeated for 10 days and the autoradiograms are shown in Figure 8.

The longer exposure of the plates allowed the presence of modified nucleotides to be clearly seen. The position of each spot was compared with those of modified bases from total *E. coli* tRNA fractionated under the same conditions (Nishimura, 1972). By comparison, some of the modified nucleotides were tentatively identified (see legend to Figure 8).

The radioactivity in fraction VII was too low to permit observation of any modified nucleotides (Figure 8). A few were observed in fractions IV–VI, but the bulk of the modified nucleotides were contained in fractions I and II (Figure 8).

The content of modified bases suggested that fractions I and II contained tRNA sequences. Consistent with this possibility, a tetranucleotide at the position expected for TΨCG was observed in T1 fingerprints of fractions I and II (arrow near spot 20, Figure 7). This oligonucleotide is characteristic of tRNA. To verify its composition the oligonucleotide was eluted and digested with RNase T2, and the mononucleotides were separated by thin-layer chromatography. The autoradiogram revealed the presence of Ψ, U, C, and G (data not shown). T was not observed, but it was also not present in the total T2 hydrolysate (Figure 8). Thus, the oligonucleotide was probably UΨCG.

DEAE-Sephadex Chromatography of an RNase T2 Digest of 30S Pre-rRNA and Band VI. In order to determine the fate of the 5′-terminal triphosphate group of 30S pre-rRNA after RNase III treatment, a T2 digest of 30S pre-rRNA or fraction VI was applied to a DEAE-Sephadex column with oligonucleotide markers. pNp, ppNp, and pppNp are eluted in the tri-, tetra-, and pentanucleotide fractions, respectively (Takai et al., 1975). Small but distinct RNase stable peaks were observed in these fractions. The molar ratio of 5′-terminal nucleotides was estimated from the radioactivity in each peak and the nucleotide length of the corresponding RNA fraction (Table III). The 30S pre-rRNA contained very little pppNp, but a significant amount of ppNp (Table III). There were also two peaks eluting with pNp, possibly arising from tRNA that had been hydrogen bonded to the 30S pre-rRNA (Lund and Dahlberg, 1977).

A similar column analysis of a digest of fraction VI revealed the presence of a small amount of pppNp (Table III). The amount of triphosphate present in peak VI could account for at least 50% of the triphosphate in the 30S pre-rRNA, though the amount of ppNp was considerably less than that contained in 30S pre-rRNA.

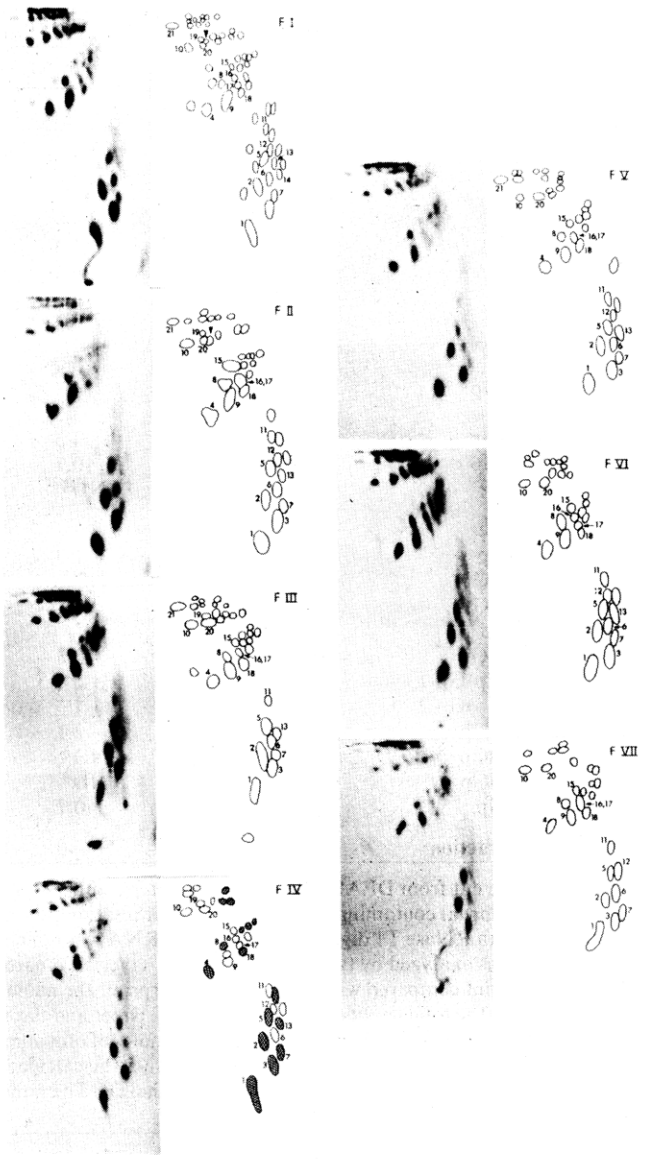

FIGURE 7: Two-dimensional electrophoresis of oligonucleotides (fingerprints) following an RNase T1 digest. Each RNA fraction (FI–VI) eluted from a 6.5% acrylamide gel was digested with RNase T1, followed by two-dimensional electrophoresis (Sanger et al., 1965). A sketch of each fingerprint was made showing the spots which contained at least 0.4 mol of oligonucleotide. The quantitation and nucleotide composition of the numbered spots are given in Table II. The fingerprint of an RNase T1 digest of 5S rRNA was done under the same conditions. Oligonucleotides present in fraction IV and 5S rRNA are indicated by cross-hatching. The unlabeled arrows in fractions I and II indicate the spots inferred to contain UΨCG (see text).

Discussion

When purified 30S pre-rRNA was incubated with pure RNase III, the production of p16S, p23S, and at least six smaller RNA fragments was completely reproducible. The cleavage patterns observed here are more complex than initial published trials had indicated (cf. Figures 2–6 with Ginsberg and Steitz, 1975; Hayes et al., 1975). Also, there was no apparent fixed order of cleavages (Figure 2), and the 30S pre-rRNA has proven to be heterogeneous (see below). However, from the fingerprint and thin-layer chromatographic analyses,

TABLE II: Moles of Oligonucleotides Produced by RNase T1 Cleavage of Small RNA Fractions.[a]

	RNA fraction						
	I	II	III	IV	V	VI	VII
Mononucleotide							
1 Gp	70	62	45	29.3	25.8	18.6	9.8
Dinucleotides							
2 A-Gp	17.3	15.3	11.8	8.4	7.3	6.4	2.4
3 C-Gp	22.7	20.8	11.3	11.3	9.0	6.5	2.0
4 U-Gp	14.5	14.5	9.6	5.9	6.5	4.9	2.0
Trinucleotides							
5 A-A-Gp	4.8	5.0	4.2	3.3	2.5	2.1	1.3
6 (Ap,Cp)Gp	4.6	4.3	3.5	2.7	2.0	1.7	0.8
7 C-C-GP	2.8	3.3	1.7	1.9	1.3	0.9	0.5
8 (Ap,Up)Gp	4.2	4.5	3.4	1.8	2.6	1.8	0.6
9 (Cp,Up)Gp	12.5	10.7	7.7	5.8	4.3	3.8	1.5
10 U-U-Gp	3.2	ND[b]	2.4	1.6	1.7	1.1	0.4
Tetranucleotides							
11 A-A-A-Gp	0.7	1.3	1.1	1.4	1.0	0.7	0.4
12 (2Ap,Cp)Gp	1.3	ND[b]		0.7	0.7	0.8	0.4
13 (Ap,2Cp)Gp	1.4	1.2	1.3	0.5	0.9	0.9	
14 C-C-C-Gp	0.6						
15 (2Ap,Up)Gp	0.7	0.8	0.9	0.6	0.6	0.4	<0.2
16 (Ap,Cp,Up)Gp-1	1.1	} 2.2	} 1.2	0.6	} 0.9	0.5	} 0.5
17 (Ap,Cp,Up)Gp-2	1.0			0.6		0.7	
18 (2Cp,Up)Gp	2.0	2.1	1.9	0.8	1.1	<0.3(−)	<0.3
19 (Ap,2Up)Gp	0.7	ND[b]	0.5	0.4	<0.3(−)	<0.2(−)	<0.1
20 (Cp,2Up)Gp	3.1	ND[b]	2.9	2.0	1.4	1.1	0.4
21 U-U-U-Gp	0.7	0.7	0.8	<0.3(−)	0.6	<0.2(−)	<0.3
Purity of the fraction	70%	70–80	80	60–70	70	70	50

[a] Spots were cut from DEAE-cellulose paper after comparison with the autoradiograph. Each piece of DEAE-cellulose paper was placed in a scintillation vial containing 5 mL of toluene scintillation fluid and the ^{32}P radiation counted in a Tri-Carb scintillation spectrometer. The fingerprint of an RNase T1 digest of KB cell 28S rRNA was done using the same conditions, and the base composition of an RNase T2 digest of each spot was analyzed by two-dimensional thin-layer chromatography (Hashimoto and Muramatsu, 1973). From the position of the spot on the fingerprint compared with the control fingerprint, the nucleotide chain length could be estimated and frequently the base composition assessed. From the total counts on the entire DEAE paper and the estimated nucleotide length of the RNA fraction a specific activity for each phosphate group was determined. The number of moles of oligonucleotide in each spot was calculated by dividing the total counts in each spot by the number of phosphate groups in that spot, times the calculated specific activity per phosphate; i.e., moles of oligonucleotide = total cts per spot/[(phosphate groups/spot) × (cpm/phosphate)]. The numbers 1–21 refer to the corresponding spots as numbered in Figure 7. [b] Not determined.

TABLE III: Detection of pppNp by DEAE-Sephadex Chromatography after Digestion of 30S Pre-rRNA and Fraction VI by RNase T2.[a]

	30S pre-rRNA		Fraction VI	
	Cts/5 min	Moles/mole 30S	Cts/5 min	Moles/mole FVI
Total cts	1.70×10^6		2.8×10^5	
pppNp	190	0.17	200	0.18
ppNp	530	0.64	99	0.12
pNp	980	1.78	485	0.87

[a] The ^{32}P radioactivity in each peak was summed after subtracting the background. The positions of the presumptive RNA termini were determined by comparison with the pattern of marker oligonucleotides derived from an RNase A digest of unlabeled tRNA, which was co-chromatographed with the radioactive sample. The excess pNp observed in the 30S pre-rRNA may be due to hydrogen-bonded tRNA (Lund and Dahlberg, 1977).

one can define better the number and position of cleavage points within the primary structure of 30S pre-rRNA. Furthermore, by varying the concentration of monovalent and divalent cations we have demonstrated that changes occur in the cleavage reaction, probably due to conformational differences in the substrate molecule.

The initial cleavage products of 30S pre-rRNA include two fragments, "17.5S" and "25S" (Figure 2). Fragments of comparable size are observed in pulse-labeled cells (Dunn and Studier, 1973; Nikolaev et al., 1973a, 1975; Kano et al., 1976). Judging by their positions in acrylamide gels relative to markers, and by the RNA species they produce during further RNase III treatment (Figure 5), these two fragments could add up to the total length of the 30S RNA transcript (Figure 9).

Prolonged incubation of 30S pre-rRNA (or 17.5S and 25S pre-rRNAs) with enzyme produces species of the size (Figures 2 and 5 and Nikolaev et al., 1974) and very likely the end groups (Ginsberg and Steitz, 1975) of the p16S and p23S RNAs that accumulate in normal bacteria treated with chloramphenicol (Pace, 1973).

Using PC-enzyme, the ratio of cpm in p23S/16S fell below their weight ratio of 1.8 in prolonged incubations. With the purer IC-enzyme, a final ratio of 1.8 to 2.0 was consistently seen. Thus, the physiological end point of RNase III processing seems to be the p16S and p23S species, and these are reproduced in the cleavage reactions.

Cleavage Fragments Smaller than 16S rRNA. As mentioned above (Results, Figure 3), the amount of RNA in fraction II decreased with time if the reaction buffer contained 10 mM $MgCl_2$. For this reason, the cleavage of ^{32}P-labeled

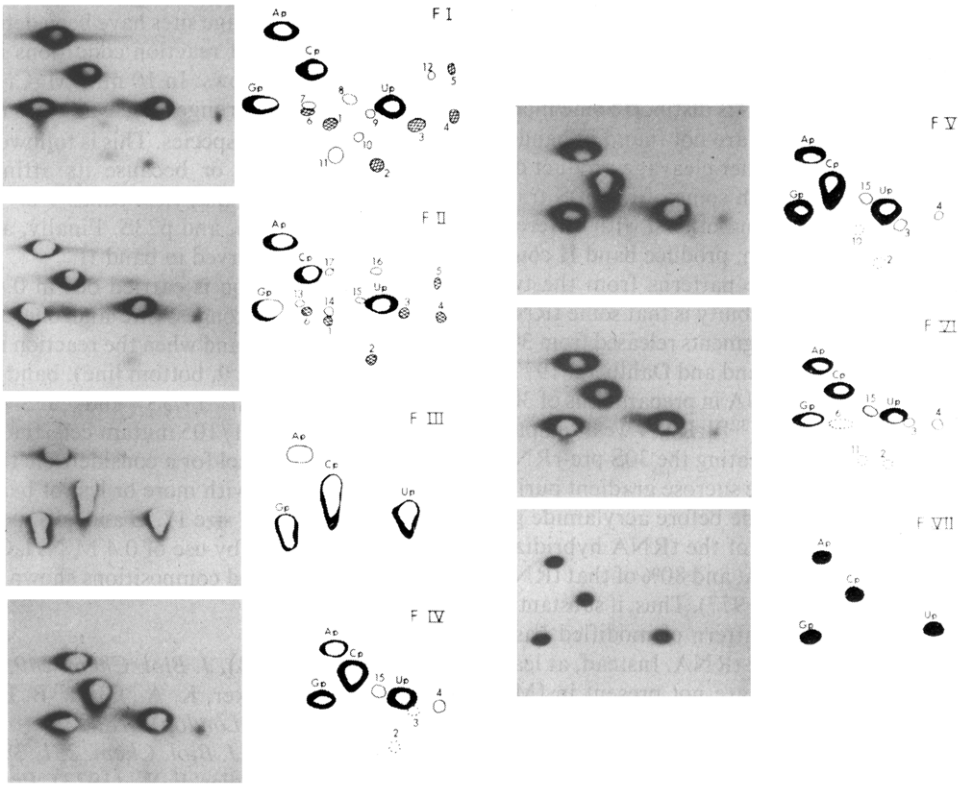

FIGURE 8: Two-dimensional thin-layer chromatography of a RNase T2 digest of RNA fractions I–VII. A portion of each RNA fraction eluted from a 6.5% polyacrylamide gel (Figure 6) was digested with RNase T2. The products were fractionated on thin-layer cellulose plates. The accompanying sketch outlines the modified nucleotides observed in each autoradiograph. Dashed lines indicate that the modified nucleotide was barely detectable. Cross-hatch spots indicate modified nucleotides present in both fractions I and II. Tentative identities of the modified nucleotides in the separated nucleotides are: (1) pseudouridine (Ψ); (2) uridine-5-oxyacetic acid (V); (3) pUp; (4) P_i; (5) 5-methylthiouridine (m^5S^2U); (6) 1-methylguanosine (m^1G); (7) 3-(3-amino-3-carboxypropyl)uridine (acp^3U) or unknown; (8) 2-thiocytidine (S^2C); (9–12) unknown; (13) 3-(3-amino-3-carboxypropyl)uridine (acp^3U); (14) unknown; (15) dihydrouridine (D); (16) methyl-*N*-[9-(β-D-ribofuranosyl)purine-6-carbamoyl]threonine (mt^6Ap); (17) 5-methylcytidine (m^5C).

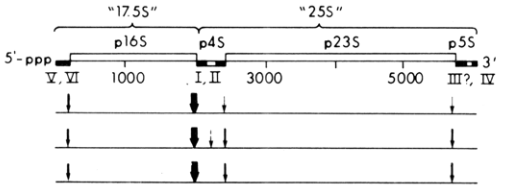

FIGURE 9: Cleavage sites in 30S pre-rRNA transcripts. The complete 30S transcript is depicted at the top, illustrating the suggested location of each RNase III cleavage product, including the 17.5S and 25S species. The entire length of the cleavage fragments adds up to 6.1 kb, which is close to the estimated length of 30S pre-rRNA, 6.25 ± 0.15 kb (Nikolaev et al., 1974). In the lower three lines, the relative affinity of cleavage sites for RNase III is indicated by the width of the arrows for three sets of reaction conditions. Below the representation of the 30S transcript are arrows appropriate for the reaction carried out in 10 mM $MgCl_2$, 400 mM NH_4Cl; then in 10 mM $MgCl_2$, 100 mM NH_4Cl; and finally in 0.5 mM $MgCl_2$, 100 mM NH_4Cl.

pre-rRNA to produce small fragments for structural studies was carried out in buffer containing 0.5 mM $MgCl_2$. Even under these conditions, in which fraction II is stable (Figure 4), it appeared that the RNA fragments released were heterogeneous (e.g., Figure 6). The quantitation in Table II suggests that each band is 70–80% pure. However, each band was present only at a 0.5 M level, and no 30S pre-rRNA chain could accommodate more than three of the fragments in addition to p16S and p23S sequences.

Two factors could account for the observed heterogeneity: (1) size and sequence heterogeneity in the "extra" sequences of 30S pre-rRNA; and (2) multiple cleavage sites within the 30S pre-rRNA for RNase III. At least the first alternative certainly occurs, as discussed below.

The 400- to 500-nucleotide chain lengths of bands I and II seem to exclude their origin from either the 5′-terminal or 3′-terminal end of 30S pre-rRNA. Electron microscopic mapping of a single 30S transcript has estimated the length of the extra sequence at the 5′-terminal end at only 200 nucleotides, and at the 3′-terminal end, at 300 nucleotides (Wu and Davidson, 1975; see Figure 9 below). Thus, fractions I and II presumptively arise from the spacer region between the 16S and 23S RNA sequences in the 30S pre-rRNA.

Important corroboration for this suggestion comes from the content of 8–10 modified nucleotides in each of fractions I and II (Figure 8). Of these, only one or two (e.g., m^5C) are also found in mature rRNA. Both bands also contained a UΨCG oligonucleotide that is diagnostic of tRNA. These almost certainly represent the tRNA sequences which are present in the middle spacer region of 30S pre-rRNA (Lund et al., 1976; Wu and Davidson, 1975; Lund and Dahlberg, 1977). Apparently many tRNA modifications can occur while the tRNA species are part of the large 30S transcript.

An interesting aspect of this interpretation is that the 6–10 30S transcription units may contain a number of different tRNAs in the middle spacer region (Lund and Dahlberg, 1977). As a result, after RNase III cleavage of 30S pre-rRNA, one would *expect* a heterogeneous collection of spacer regions from the middle of 30S pre-rRNA. Also, the middle spacer regions from different 30S transcription units may differ in

size, either because of a different primary structure or different cleavage sites for RNase III.

In fact, although the fingerprints of bands I and II are similar, the pattern of modified bases is distinct. Some modifications that are present in band II are not found in band I, suggesting that band II is not a smaller cleavage product derived from band I. A model in which some of the 30S transcripts produce the longer band I, and others, with different tRNAs in their middle spacer region, produce band II could explain the different modified base patterns from the two peaks. [An alternate, less likely possibility is that some tRNA was hydrogen bonded to the small fragments released from 30S pre-rRNA. We, as well as others (Lund and Dahlberg, 1977), have observed hydrogen-bonded tRNA in preparations of 30S pre-rRNA. In our experiments with [^{32}P]rRNA we attempted to minimize the tRNA binding by heating the 30S pre-rRNA in 90% dimethyl sulfoxide before the sucrose gradient purification and heating in 80% formamide before acrylamide gel electrophoresis. Furthermore, most of the tRNA hybridizes to m23S sequences in 30S pre-rRNA, and 80% of that tRNA is fMet-tRNA (Lund and Dahlberg, 1977). Thus, if substantial tRNA had remained bound, the pattern of modified bases should closely resemble that of fMet tRNA. Instead, at least six of the modified bases observed are not present in fMet tRNA (Figure 8 and Dube et al., 1968).]

Fraction IV was the only one which contained all of the 5S rRNA oligonucleotides (except for the mature 5′- and 3′-terminal oligonucleotides). 5S rRNA has been mapped at the 3′-end of 30S pre-rRNA (Wu and Davidson, 1975), and fraction IV must therefore be derived from the 3′-terminal end of 30S pre-rRNA. Since peak IV is present in less than a molar amount, however, some other RNA fraction(s) should contain 5S rRNA. Fraction III is the strongest candidate, since its fingerprint is very similar to that of fraction IV. However, a few of the 5S oligonucleotides, e.g., CCG and A_3CG, are present in relatively low amounts in fraction III. An alternative explanation is that a fraction of the initial 30S transcripts may not contain 5S rRNA, due to premature termination of transcription or to a nuclease activity which specifically releases the 5S rRNA from 30S transcripts. A previous report (Ginsberg and Steitz, 1975) stated that only one band on a 9% acrylamide gel contained 5S rRNA. However, that RNase III reaction was carried out in 10 mM Mg^{2+}, and the molar amount of the fragment containing 5S rRNA released was not clear.

Fraction VI was shown to contain pppNp (Table III), and a band with similar electrophoretic mobility was previously reported to contain pppNp (Ginsberg and Steitz, 1975). The fingerprints of bands V and VI were very similar, and we therefore infer that bands V and VI are both derived from the 5′-terminal end of 30S pre-rRNA chains. The different mobilities of these two fractions may be due to different promoter sequences for different 30S pre-rRNA transcription units, or to the existence of several different RNase III cleavage sites near the 5′-end of 30S pre-rRNA species.

Fraction VII was very heterogeneous and was eluted from a broad band in the 6.5% acrylamide gel. Its heterogeneity made further analysis inconclusive. It may contain breakdown products of larger RNAs and perhaps some residual tRNA that had been hydrogen bonded to the 30S pre-rRNA.

Tentative Map of RNase III Cleavage Sites in 30S pre-rRNA. Figure 9 summarizes the locations inferred for major cleavage sites and fragments of 30S pre-rRNA. Taken in pairs, fractions V and VI, I and II, and III and IV can account for molar yields from the 5′ end, middle, and 3′ end of 30S pre-rRNA.

At least five cleavage sites have been detected; their relative strength in different reaction conditions is indicated by the thickness of the arrows. In 10 mM $MgCl_2$, 100 mM NH_4Cl (middle line), the strongest cleavage site is that between the "17.5S" and "25S" species. This is followed—either because it is exposed later, or because its affinity for enzyme is lower—by cleavages that yield bands V or VI and p16S, bands II (I) and IV (III?), and p23S. Finally, at least one weaker cleavage site is observed in band II.

When the reaction is carried out in 0.4 M NH_4Cl, only cleavages at the strongest site and one other are observed (Figure 9, top line); and when the reaction is carried out in 0.5 mM $MgCl_2$ (Figure 9, bottom line), band II is preserved.

Very likely, when "17.5S" and "25S" RNA species are isolated from AB301/105 mutant cells that have been treated with chloramphenicol for a considerable time, they include a mixture of species with more or less of bands I to VI still attached. Only the full size 17.5S and 25S species, detected after limited cleavage or by use of 0.4 M NH_4Cl (Figure 4, panel D), have the inferred compositions shown in Figure 9.

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Nucleotide Sequences of Accessible Regions of 23S RNA in 50S Ribosomal Subunits†

Winship Herr‡ and Harry F. Noller*

ABSTRACT: Nucleotide sequences around kethoxal-reactive guanine residues of 23S RNA in 50S ribosomal subunits have been determined. By use of the diagonal paper electrophoresis method (Noller, H. F. (1974), *Biochemistry 13,* 4694–4703), 41 ribonuclease T_1 oligonucleotides, originating from about 25 sites, were identified and sequenced. These sites are single stranded and accessible in free 50S subunits, and are thus potential sites for interaction with functional ligands during protein synthesis. Examination of these sequences for potential intermolecular base-pairing reveals the following: (1) There are 19 possible complementary combinations between exposed sequences in 16S and 23S RNA containing more than 4 base pairs: 15 containing 5 base pairs and 4 containing 6 base pairs. Nine of these complementary combinations contain 16S RNA sequences which we have previously shown to be protected from kethoxal by 50S subunits (Chapman, N. M., and Noller, H. F. (1977), *J. Mol. Biol. 109,* 131–149). (2) One of the exposed sites in 23S RNA has a sequence which is complementary to the invariant GTψCR sequence in tRNA.

Elucidation of the functional role of ribosomal RNA in protein synthesis should benefit from a knowledge of the exposed, unpaired nucleotide sequences in ribosomes and ribosomal subunits. Such information is also valuable in the critical assessment of secondary or tertiary structural models. We have used the guanine-specific reagent kethoxal (2-keto-3-ethoxybutyraldehyde) as a probe of the accessibility of sequences in 16S and 5S RNA in 30S, 50S, and 70S ribosomes (Noller, 1974; Noller and Herr, 1974; Chapman and Noller, 1977). Here we report the use of this method to identify accessible 23S RNA sequences in 50S ribosomal subunits. Some of these sequences contain regions of complementarity with accessible sequences in 16S RNA and with the GTψCR sequences of transfer RNA.

Materials and Methods

Buffers are as follows: (buffer A) 0.05 M NH_4Cl, 0.5 mM $MgCl_2$, 0.006 M β-mercaptoethanol, 0.05 M Tris-HCl, pH 7.6; (buffer B) 0.02 M $MgCl_2$, 0.1 M potassium cacodylate, pH 7.0; (buffer C) 0.15 M NaCl, 0.01 M EDTA, 0.015 M sodium citrate, 0.025 M sodium borate, pH 7.0.

Ribonucleases T_1 and U_2 (Sankyo) were obtained from Calbiochem, ribonuclease A and snake venom phosphodiesterase were from Worthington, and bacterial alkaline phosphatase was from Sigma. Silkworm nuclease was a gift from Dr. John Abelson, and ε-carboxymethyllysine-41-ribonuclease A was a gift from Dr. Gary Paddock.

Preparation of ^{32}P-Labeled 50S Subunits. Cells (*Escherichia coli,* strain MRE 600) were labeled with 20 mCi of [^{32}P]orthophosphate in a volume of 50 mL as previously described (Chapman and Noller, 1977). The cells were lysed and the 70S ribosomes pelleted as described by Noller (1974). The 70S ribosome pellet was resuspended in 1 mL of buffer A by gentle shaking for 1 h at 0 °C, layered on a 35-mL 5–20% sucrose gradient in buffer A, and centrifuged for 13.5 h at 20 000 rpm (Beckman L3-40 centrifuge, SW-27 rotor) at 6 °C. Ten-drop fractions were collected and ribosomal subunits peaks were located by measurement of the radioactivity of each fraction. Fractions containing 50S subunits were pooled, made 10 mM in $MgCl_2$, and precipitated by addition of 0.65 volume of ethanol. The mixture was immediately centrifuged at 15 000 rpm (Sorvall RC-2B, SS-34 rotor) at 0 °C for 45 min. The pellet of ^{32}P-labeled 50S subunits was resuspended in 0.5 mL of buffer B, quick-frozen in dry ice–acetone, and stored overnight at −70 °C. The yield was typically $1–2 \times 10^9$ cpm of 50S subunits (specific activity $4–5 \times 10^6$ cpm/μg).

Kethoxal Reaction and Isolation of Modified RNA. Labeled 50S subunits ($1–2 \times 10^9$ cpm in 0.5 mL of buffer B) were incubated for 10 min at 37 °C prior to addition of kethoxal. Fifty microliters of a saturated solution of kethoxal in 20% (v/v) ethanol was then added, and incubation was continued for 1 h at 37 °C. Unreacted kethoxal was removed by precipitation of the ribosomes with 0.65 vol of ethanol. The precipitate was resuspended in 0.5 mL of buffer C and extracted three times with an equal volume of buffer C saturated phenol. RNA was recovered from the final aqueous phase by precipitation six times with 2 vol of ethanol. The final precipitate was dissolved in 50 μL of water and lyophilized.

Nuclease Digestion and Diagonal Electrophoresis. The lyophilized RNA sample was dissolved in 25 μL of a solution containing 1 mg/mL RNase T_1 and 5 mg/mL alkaline phosphatase in 10 mM Tris-HCl, pH 8.0, 20 mM potassium borate, pH 8.0, 0.01 mM $ZnCl_2$, and digested for 1 h at 37 °C. The digest was spotted 10 cm from the end of a 12×110 cm strip

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‡ Present address: The Biological Laboratories, Harvard University, Cambridge, Mass. 02138.

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