# Accurate Processing of Human Pre-rRNA In Vitro

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We report here that the mature 5' terminus of human 18S rRNA is generated in vitro by a two-step processing reaction. In the first step, SP6 transcripts were specifically cleaved in HeLa cell nucleolar extract at three positions near the external transcribed spacer (ETS)-18S boundary. Of these cleavage sites, two were major and the other was minor. RNase $T_1$ fingerprint and secondary nuclease analyses placed the two major cleavage sites 3 and 8 bases upstream from the mature 5' end of 18S rRNA and the minor cleavage site 1 base into the 18S sequence. All three cleavages yielded 5'-hydroxyl, 2'-3'-cyclic phosphate termini and were 5' of adenosine residues in the sequence UACCU, which was repeated three times near the ETS-18S boundary. In the second step, the initial cleavage product containing 3 bases of ETS was converted to an RNA with a 5' terminus identical to that of mature 18S RNA by an activity found in HeLa cell cytoplasmic extracts.

It has been established for 20 years that mature rRNAs are derived from a large precursor which is cleaved by an efficient machinery capable of processing this abundant transcript. In procaryotes but not eucaryotes, the mechanism of rRNA processing has been worked out in detail. RNase III, an endonuclease discovered in *Escherichia coli*, generates intermediates which are later trimmed at their 5' and 3' ends to give mature species (reviewed in reference 14). It is important to learn whether enzymes facilitating similar processes can be identified in eucaryotic cells as well.

Early studies in mammalian cells localized the site of synthesis and processing of rRNAs to the nucleolus and defined a complex set of rRNA processing intermediates. This information has provided a basis for seeking cell extracts able to produce both these intermediates and molecules with the termini of mature rRNAs (7, 22). In this regard, Sollner-Webb and colleagues have characterized an endonucleolytic processing site which lies about 3.6 kilobases (kb) upstream of the 5' end of the mouse and human 18S rRNAs. Processing at this site has been efficiently reproduced in vitro with an S-100 extract of mouse cells (6, 13, 16). Similar extracts have been shown to catalyze the endonucleolytic cleavage of mouse pre-rRNA 105 nucleotides upstream from the 5' end of 18S rRNA (17). However, generation of mature rRNA termini has not been achieved previously with these or any other cell-free systems.

Using sequential incubations in nucleolar and cytoplasmic extracts of HeLa cells, we have found that the mature 5' terminus of human 18S rRNA is produced in vitro by processing of SP6 transcripts spanning the boundary between the external transcribed spacer (ETS) and 18S rRNA. In nucleolar extract, the SP6 transcripts were cleaved at three positions near the 5' terminus of 18S rRNA. In a second reaction, one of these cleavage products was converted to an RNA with the 5' terminus of mature 18S rRNA by an activity present in cytoplasmic extracts. Our results suggest that the maturation of 18S rRNA involves an initial endonucleolytic cleavage followed by "trimming" and thus

may be formally analogous to the processing of 16S and 23S rRNAs in *E. coli*.

## MATERIALS AND METHODS

**Construction of transcription templates and RNase protection probes.** Portions of two cloned fragments of human rRNA (*Eco*RI-A and *Eco*RI-B [11]) (kindly provided by R. Schmickel and J. Sylvester) were subcloned into the transcription vector pSP64. The resultant plasmid contained most of the human rDNA transcription unit from a *Sal*I site ~750 base pairs (bp) upstream of 18S coding sequence to an *Eco*RI site near the 3' end of 28S RNA (Fig. 1A). To provide a termination site for in vitro transcription, this *Eco*RI site was destroyed and replaced with an *Hpa*I site. Transcription of this plasmid yielded a 9.7-kb RNA. For most of the experiments reported, an *Nci*I-*Xba*I restriction fragment spanning the ETS-18S boundary was subcloned into pSP65 (Fig. 1B). RNase protection probes were constructed by the insertion of the *Sal*I-*Xba*I fragment into the M13 phage vector mp19, yielding the phage M13SX.

**Preparation of RNA substrates.** Pre-rRNA substrates were synthesized by transcription of the templates detailed above with SP6 polymerase (New England Nuclear Corp.) under conditions specified by the manufacturer. Transcription reactions were carried out in the presence of none, one, or all four [α-$^{32}$P]rNTPs (Amersham Corp.) as described in the legends accompanying individual figures.

5'-Terminally labeled substrates were prepared by dephosphorylating substrate RNAs by treatment with calf intestinal alkaline phosphatase (CIAP) (Boehringer) for 20 min at 37°C. Following phenol-chloroform extraction and ethanol precipitation, RNAs were suspended and treated with polynucleotide kinase in the presence of [γ-$^{32}$P]ATP (5,000 Ci/mmol; Amersham) for 20 min at 37°C. 5'-Terminal phosphorylation of purified nucleolar extract products was performed under identical conditions except that 1 mM ATP was substituted for [γ-$^{32}$P]ATP.

3'-Terminally labeled substrates were prepared by the ligation of [$^{32}$P]pCp to nonradioactive pre-rRNAs by using T4 RNA ligase as described before (9). Alternatively, for the preparation of 3'-terminally blocked transcripts for preparative trimming reactions, internally $^{32}$P-labeled 260-nucleotide (nt) transcript was ligated to an excess of unlabeled pCp

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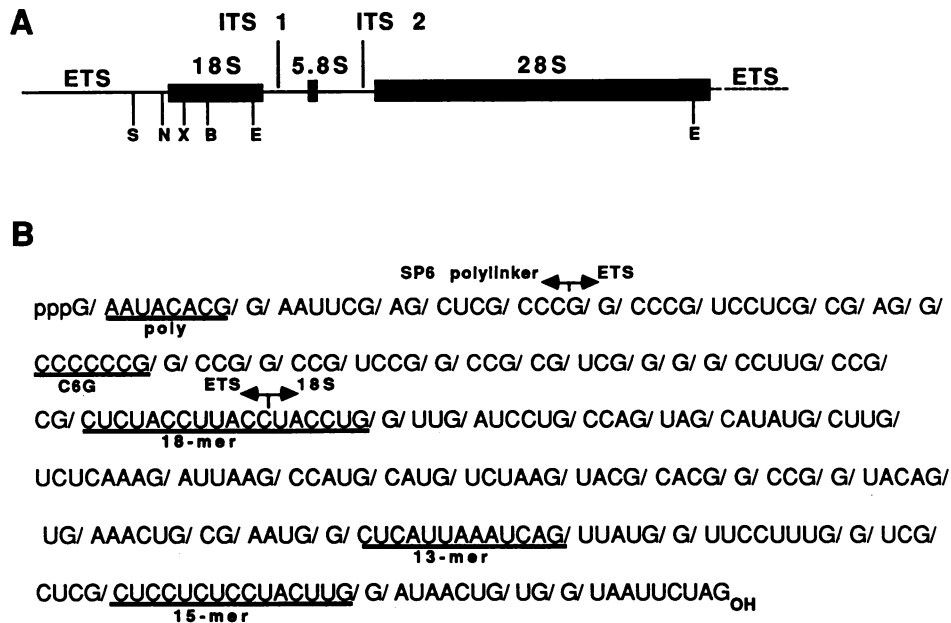† Present address: Department of Biochemistry, Cornell University Medical College, New York, NY.

**A**



**B**

SP6 polylinker ←→ ETS

pppG/ AAUACACG/ G/ AAUUCG/ AG/ CUCG/ CCCG/ G/ CCCG/ UCCUCG/ CG/ AG/ G/
　　　　poly

CCCCCCG/ G/ CCG/ G/ CCG/ UCCG/ G/ CCG/ CG/ UCG/ G/ G/ G/ CCUUG/ CCG/
　C6G　　　　　ETS ←→ 18S

CG/ CUCUACCUUACCUACCUG/ G/ UUG/ AUCCUG/ CCAG/ UAG/ CAUAUG/ CUUG/
　　　18-mer

UCUCAAAG/ AUUAAG/ CCAUG/ CAUG/ UCUAAG/ UACG/ CACG/ G/ CCG/ G/ UACAG/

UG/ AAACUG/ CG/ AAUG/ G/ CUCAUUAAAUCAG/ UUAUG/ G/ UUCCUUUG/ G/ UCG/
　　　　　　　　　　　　13-mer

CUCG/ CUCCUCUCCUACUUG/ G/ AUAACUG/ UG/ G/ UAAUUCUAG$_{OH}$
　　　15-mer

FIG. 1. Pre-rRNA processing substrate. (A) Map of the human rRNA transcription unit. An NciI (N)-XbaI (X) restriction fragment spanning the ETS-18S boundary was subcloned into the SP6 transcription vector pSP65 to give the plasmid pNX. Following digestion with XbaI, transcription of this plasmid yields a run-off RNA containing 25 bases of polylinker sequence, 70 bases of 5' ETS, and the first 165 bases of 18S sequence. (B) Sequence of this run-off transcript, with slashes between oligonucleotides predicted to result from digestion with RNase T$_1$. RNase T$_1$ oligonucleotides referred to in the text are underlined. S, SalI; N, NciI; X, XbaI; B, BamHI; E, EcoRI; ETS, external transcribed spacer; ITS, internal transcribed spacer.

which was prepared by the method of England and Uhlenbeck (10) by the phosphorylation of Cp (Sigma Chemical Co.) with polynucleotide kinase in the presence of ATP. Substrates used in both nucleolar extract and trimming reactions were electrophoresed on polyacrylamide gels, excised from the gels, and eluted in 50 mM Tris hydrochloride (Tris-HCl), pH 7.5–0.2 M ammonium acetate–1 mM EDTA–50 mM NaCl–0.2% sodium dodecyl sulfate (SDS) as described before (1).

In vivo-labeled cytoplasmic RNA was prepared by incubating 2 × 10$^7$ HeLa cells in 50 ml of phosphate-free medium supplemented with 5% dialyzed fetal bovine serum and 0.5 mCi of carrier-free $^{32}$P$_i$ (ICN) per ml for 16 h. Labeled RNA prepared as described before (20) was then hybridized to 20 μg of unlabeled M13SX DNA for 1 h at 65°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) in a volume of 200 μl. Following hybridization, the sample was chilled on ice, diluted with an equal volume of 50 mM sodium acetate (pH 4.5)–2 mM EDTA–200 mM NaCl and digested for 30 min at 23°C with 10 U of RNase T$_2$ (Sankyo). The reaction was terminated by the addition of 2 volumes of 50 mM Tris-HCl (pH 7.5)–200 mM NaCl–30 μg of yeast tRNA (Bethesda Research Laboratories)–SDS to a final concentration of 0.2%. The sample was then digested for 1 h at 37°C with 100 μg of proteinase K (Boehringer) per ml, phenol-chloroform extracted, ethanol precipitated, and applied to a nitrocellulose filter (Millipore, type GS) which had been equilibrated in 50 mM Tris-HCl (pH 7.5)–200 mM NaCl. The filter was then washed extensively in the same buffer. The protected RNA was eluted from the filter by treatment with 100 μg of RNase-free DNase I (Bethesda) per ml for 30 min at 37°C in 10 mM Tris-HCl (pH 7.5)–5 mM MgCl$_2$. RNA samples were phenol-chloroform extracted and denatured before use.

**Preparation of cellular extracts.** The following solutions were used. Solution A: 10 mM Tris-HCl, pH 7.5, 1.5 mM

MgCl$_2$, 10 mM NaCl, and 50 μg of dextran sulfate per ml. Solution B: 0.25 M sucrose and 10 mM MgCl$_2$. Solution C: 0.88 M sucrose and 0.05 mM MgCl$_2$. Solution D: 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 0.05 mM MgCl$_2$, and 50 μg of dextran sulfate per ml. Solution E: 20 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 20% (vol/vol) glycerol.

HeLa cells were maintained in suspension culture at a density of 4 × 10$^5$ to 6 × 10$^5$ cells per ml in SMEM (M.A. Bioproducts) supplemented with 7.5% horse serum (GIBCO Laboratories).

For nucleolar extracts, 2 × 10$^9$ to 4 × 10$^9$ cells were collected by centrifugation at 1,000 × g for 5 min and washed with 100 volumes of phosphate-buffered saline. Nuclei were then isolated as described before (21). Briefly, cells were suspended in 5 packed-cell volumes of solution A, incubated for 10 min on ice, and homogenized with a Dounce homogenizer (type B pestle) until lysis was greater than 95%. An equal volume of solution A containing 0.5% Triton X-100 was added, and nuclei were collected by centrifugation at 1,500 × g for 5 min. Nucleoli were then prepared by a modification of the procedure described before (18). Nuclei were suspended in 10 packed-cell volumes of solution B, layered over a cushion of an equal volume of solution C, and centrifuged at 2,500 × g for 10 min. The purified nuclei were then suspended in solution D (2 × 10$^7$ nuclei per ml) and broken by shearing for 11 min at 5,000 rpm in a Virtis 60 homogenizer with the macroblade. Microscopic examination indicated >95% nuclear breakage. This suspension was layered over an equal volume of solution C and centrifuged at 3,500 × g for 15 min. The resulting pellet of purified nucleoli was suspended in 1 packed nucleolar volume of solution E. This preparation was then made 0.5 M KCl and extracted on ice for 10 min with gentle agitation. Following dilution with solution E to a final KCl concentration of 120

mM, the extract was cleared by centrifugation for 10 min at 225,000 × g in a Ti50 rotor (Beckman). Samples of this extract were stored at −80°C. Nucleolar extract contained approximately 0.5 mg of protein per ml. Dot blot hybridization with an rDNA probe indicated that DNA prepared from nucleolar extract was approximately fivefold enriched for rDNA sequences compared with identical hybridizations to total HeLa cell DNA (data not shown).

The P100 fraction of HeLa cell cytoplasmic extract was prepared from HeLa cells lysed by homogenization as described before (8). The supernatant from the initial nuclear isolation was cleared by centrifugation at 20,000 × g for 10 min. The resulting cleared cytoplasm was then centrifuged at 100,000 × g for 60 min in a Ti50 rotor to yield a P100 pellet. This pellet was rinsed in solution E and then suspended in 0.2 initial packed-cell volume of solution E by gentle homogenization in a 1-ml Dounce homogenizer with a Teflon pestle. The extract was stored at −80°C and diluted 10-fold for use. P100 extract contained approximately 12 mg of protein per ml.

Nuclear extracts of HeLa cells were prepared as described before (8) and contained 3 mg of protein per ml. Cytoplasmic extracts, produced as described above for the preparation of P100 extract, contained 10 mg of protein per ml.

**In vitro processing reactions.** Incubation mixes for nucleolar extracts contained 1 ng of a pre-rRNA processing substrate, 2 μl of nucleolar extract in 10 mM Tris-HCl (pH 7.5)–75 mM KCl–2 mM MgCl$_2$, and 0.2 mM spermidine in a final volume of 20 μl at 30°C for the amount of time specified in each figure legend. Following the reaction, RNAs were recovered by phenol-chloroform extraction followed by ethanol precipitation with 15 μg of yeast tRNA. Electrophoretic analysis of products was carried out on 40-cm, 6% acrylamide–7 M urea gels containing 50 mM Tris-HCl, pH 8.3, 50 mM boric acid, and 1 mM EDTA. Preparative processing reactions were performed identically except that they contained up to 500 ng of pre-rRNA substrate so that individual products could be isolated from gels for further analysis.

These products served as substrates for trimming in the cytoplasmic P100. Trimming reaction mixes contained 5 μl of diluted P100 extract, and the reaction was carried out under ionic conditions identical to those used for nucleolar extract reactions at 37°C for the amount of time specified in the individual figure legends. Analysis of products was carried out by polyacrylamide gel electrophoresis as described above.

**RNase T$_1$ fingerprint and secondary nuclease analysis.** RNase T$_1$ fingerprint analysis was performed essentially as described before (19). Substrates and reaction products were eluted from gels as described above and ethanol precipitated twice with 10 μg of yeast tRNA. Samples were then suspended in 2μl of 10 mM Tris-HCl (pH 7.4)–1 mM EDTA containing 2 μg of RNase T$_1$ (Sankyo) (RNase T$_1$ cleaves 3′ to guanosine residues) and digested for 45 min at 37°C. RNase T$_1$ oligonucleotides were separated in the first dimension (right to left) by electrophoresis in a 10% polyacrylamide gel containing 6 M urea and 25 mM sodium citrate (pH 3.5) until the blue and yellow dyes were separated by 10.5 cm. RNase T$_1$ fragments in each gel lane were then transferred to DEAE-cellulose plates (Polygram Cell 300; Brinkmann). Separation in the second dimension (bottom to top) was by homochromatography (5). Fingerprint spots were eluted and prepared for secondary enzymatic digestions essentially as described before (2).

**Secondary analysis of oligonucleotides eluted from fingerprints.** For digestion with pancreatic RNase, samples were suspended in a 3-μl volume containing 1 mg of pancreatic RNase A (Worthington Diagnostics) (RNase A cleaves 3′ to pyrimidines) per ml in 0.001 M EDTA–0.01 M Tris-HCl (pH 7.4) and incubated for 1 h at 37°C. For digestion with RNase U2, samples were suspended in a 3-μl volume containing 10 U of RNase U2 (Sankyo, Calbiochem) (RNase U2 cleaves 3′ to adenosine residues) per ml in 0.002 M EDTA–0.05 M ammonium acetate (pH 4.5) and incubated for 2 h at 37°C. For nuclease P1 digestion, samples were suspended in 3 μl containing 0.33 mg of nuclease P1 (Calbiochem) (nuclease P1 cleaves 5′ of any base) per ml in 0.01 M sodium acetate (pH 6.0) and incubated for 1 h at 37°C. For digestion with CIAP, samples were suspended in 3 μl containing 50 U of CIAP (Boehringer Mannheim) per ml in 17% glycerol–0.042 M NaCl–0.0007 M EDTA–0.015 M Tris-HCl (pH 7.6) and incubated for 1 h at 37°C, allowed to heat up to 65°C in a water bath, and then incubated for 15 min at 65°C. To prevent evaporation, digestions were carried out in sealed glass capillary tubes. For removal of cyclic phosphate groups, samples were first suspended in 5 μl containing 0.035 U of 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (Sigma) in 0.02 M sodium acetate, pH 6.6, and then incubated for 30 min at 25°C. Five microliters containing 50 U of CIAP per ml in 17% glycerol–0.042 M NaCl–0.0007 M EDTA–0.015 M Tris-HCl (pH 7.6) was added, and then the sample was incubated for 1 h at 37°C, allowed to heat up to 65°C in a water bath, and then incubated for 15 min at 65°C. To identify 5′-terminal phosphate groups, oligonucleotides were first treated with RNase T$_2$ (RNase T$_2$ cleaves 3′ to any base) at 37°C for 1 h, releasing 3′-nucleoside monophosphates from most positions and 3′,5′-nucleoside diphosphates (pXps) from any 5′-phosphorylated termini. RNase T$_2$ digestion mixes contained 3 μl of RNase T$_2$ (2 U/ml) in a solution containing 0.05 mg of RNase T$_1$ per ml, 0.05 mg of RNase A per ml, and 0.05 M ammonium acetate, pH 4.5. Following this incubation, 3 μl of nuclease P1 was added, and samples were incubated for 1.3 h at 37°C. The 3′-phosphatase activity of nuclease P1 removed all labeled phosphates from the 3′-nucleoside monophosphates produced by RNase T$_2$ and released 5′-nucleoside monophosphates from the pXps. To provide markers for pUp, pCp, and pAp, RNase T$_1$-resistant oligonucleotides of potato spindle tuber viroid were labeled at their 5′ ends by [γ-$^{32}$P]ATP and polynucleotide kinase, eluted from characteristic positions in gels, and then digested with RNase T$_2$.

(Throughout this text, terminal phosphate groups are indicated at the appropriate position on all species; cyclic phosphate groups generated during incubation in nucleolar extract are designated >p; hydroxyl groups occur on all unmarked termini of RNA oligonucleotides.)

## RESULTS

**Pre-rRNA is specifically cleaved in nucleolar extract.** The human rRNA transcription unit is depicted schematically in Fig. 1A. To prepare substrates for in vitro processing reactions, a small restriction fragment (NciI-XbaI) spanning the 5′ end of 18S rRNA was subcloned into a vector containing the SP6 promoter. Transcription of this plasmid generated a 260-nt RNA containing 25 bases of polylinker sequence, 70 bases of 5′ ETS, and the first 165 bases of 18S rRNA (Fig. 1B). We chose to focus on the processing events which generated the 5′ end of 18S rRNA because previous experiments (3, 4) suggested that this is the first mature rRNA terminus produced in vivo.

To assay for potential processing activities, the 260-nt transcript (Fig. 1B) was either 3′- or 5′-end labeled and

FIG. 2. Cleavage of pre-18S rRNA substrates in nucleolar extract. The pre-18S rRNA substrate transcribed from pNX (described in Fig. 1) was either 3'-end labeled (A) or 5'-end labeled (B) as described in the text and incubated in an extract of purified HeLa cell nucleoli prepared as described in Materials and Methods. Following phenol-chloroform extraction and ethanol precipitation, reaction products were electrophoresed on a 6% acrylamide–7 M urea gel. (A and B) Lanes 1 to 5 were incubated for 0, 30, 60, 120, or 180 min, respectively. (A) Lanes 6 and 7 are identical reactions carried out for 180 min in the presence of either nuclear (lane 6) or cytoplasmic (lane 7) extract.

incubated in an extract prepared from HeLa cell nucleoli (see Materials and Methods). We reasoned that such an extract might be enriched for rRNA processing activities, since rRNA processing takes place within this organelle. A time course of incubation is shown in Fig. 2. With both 3'- and 5'-end-labeled molecules, a limited number of discrete cleavage products accumulated as a consequence of incubation. The sizes of cleavage products observed individually with 3'-end-labeled molecules (Fig. 2A, RNAs a, b, and c) and 5'-end-labeled molecules (Fig. 2B, RNAs a', b', and c') suggested that the substrate was cleaved endonucleolytically at three positions near the 5' end of 18S rRNA. A detailed analysis of these cleavage products confirmed the interpretation that incubation produced three pairs of 5' and 3' half-molecules (RNAs a and a', b and b', and c and c') (see below). In incubations with 3'-end-labeled RNAs, an additional smaller triplet of cleavage products was produced whose size (~110 nt) indicated that these cleavages occurred within the 18S rRNA sequence. It seems possible that these internal cleavages occurred following or in addition to cleavage near the 5' end of 18S rRNA, since 5'-end-labeled half-molecules expected from internal cleavage did not accumulate to an appreciable level (Fig. 2B). The extent of these internal cleavages was markedly reduced when substrates containing additional 18S rRNA sequence were analyzed following similar incubations (data not shown).

Cleavages near the ETS-18S boundary of the 260-base-

long transcript were found only following incubation with nucleolar extract; similar cleavages were not observed when the same substrate was incubated in nuclear or cytoplasmic extracts (Fig. 2). The extent of cleavage was proportional to the amount of nucleolar extract included in incubations and was not affected by the addition of ATP; no attempt was made to deplete endogenous ATP. The extent of cleavage was not sensitive to the inclusion of nonspecific competitor RNA at concentrations of up to 1 mg/ml [yeast tRNA, poly(A), poly(A · U), HeLa cell nonpolyadenylated RNA] (data not shown). The activity of the extract was not affected by preincubation with micrococcal nuclease but was abolished by treatment with proteinase K. Furthermore, the cleavage activity was specific. Other SP6 transcripts (beta-globin, adenovirus) of similar or larger size than the 260-base-long ribosomal transcript remained intact even upon prolonged exposure to nucleolar extract (data not shown).

To define the positions of cleavages observed with nucleolar extract, internally labeled, gel-purified reaction products (the pairs of 5' and 3' half-molecules described above) were studied by two-dimensional RNase $T_1$ fingerprinting and secondary nuclease digestion. The analysis of cleavage sites was facilitated by the fact that the ETS-18S boundary lies within the longest RNase $T_1$-resistant oligonucleotide, an 18-mer, present within the sequence of the substrate molecule (Fig. 1). As would be expected if cleavage occurred near the in vivo terminus of 18S rRNA, the fingerprints of individual cleavage products lacked this 18-mer (Fig. 3). Concomitant with the disappearance of the 18-mer was the appearance of novel RNase $T_1$ oligonucleotides characteristic of each cleavage reaction (Fig. 3). To determine the exact sites and phosphate polarity of the cleavages, these newly arising oligonucleotides were recovered from fingerprints and analyzed in detail by further enzymatic treatments.

For these analyses, the 18-mer (CUCUACCUUACCUAC CUGp) was recovered from fingerprints of substrate RNA so that its secondary nuclease digestion products could be used as markers in the identification of the novel fingerprint spots generated by the cleavage reactions. As detailed above, both fragment sizes and gel band intensities indicated that cleavage occurred at three sites and produced three pairs of bands (Fig. 2). Therefore, the novel oligonucleotides were eluted from fingerprints and analyzed in sets of two. They are designated spots a, a', b, b', and c' according to the cleavage product from which they are derived. Spot c, the partner of spot c', did not consistently resolve well in fingerprints and could not be analyzed. The first pair of oligonucleotides analyzed were derived from the shortest 5' (ETS) cleavage product (RNA a', Fig. 2) and its partner, the longest 3' (18S) cleavage product (RNA a, Fig. 2). Detailed analysis revealed that spot a' was resistant to phosphatase treatment, but could be made sensitive to this enzyme by treatment with 2',3'-cyclic-nucleotide 3'-phosphodiesterase. This activity converts the phosphatase-resistant cyclic phosphate to a sensitive form. This result indicated that spot a' terminated in a 2',3'-cyclic phosphate. Furthermore, it was resistant to RNase U2, but produced pU>p, pC, and pU upon nuclease P1 treatment. Together, these data show that while spot a' had no A or G residues, it contained an internal C residue and ended with a U residue attached to a cyclic phosphate. Assuming that this oligonucleotide was generated by cleavage within the 18-mer, it follows that spot a' has the sequence CUCU and terminates with a 2',3'-cyclic phosphate group. Spot a contained RNase U2 products which comigrated with CCUUAp, CCUGp, CCUAp, and Ap, suggesting, based upon the known sequence of the 18-mer,
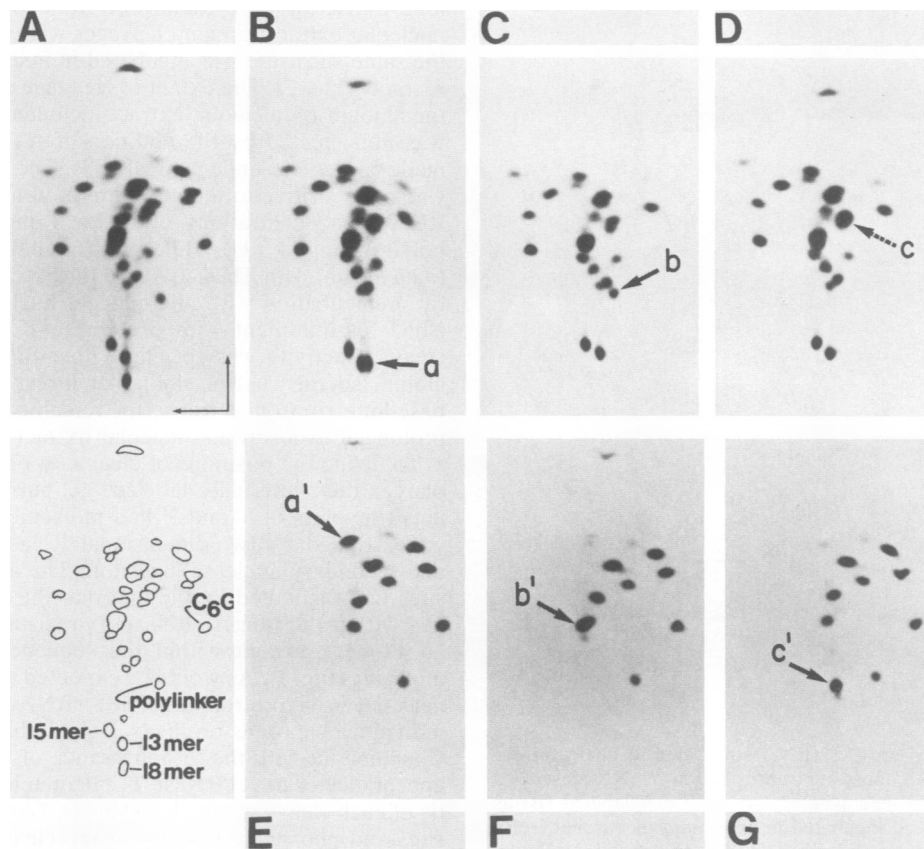
FIG. 3. RNase $T_1$ fingerprint analysis of nucleolar cleavage products. The pre-rRNA substrate generated by transcription of pNX in the presence of all four [$\alpha$-$^{32}$P]rNTPs was incubated in nucleolar extract as described in Materials and Methods, and individual reaction products as well as the unreacted substrate were recovered from a 5% acrylamide–7 M urea gel. These RNAs were further purified on a 7% acrylamide–7 M urea gel and then subjected to RNase $T_1$ fingerprint analysis. (A) Fingerprint of the intact RNA substrate, with a schematic diagram below. Diagnostic spots labeled $C_6G$ and polylinker are from the 5' (ETS) side of the molecule, and those labeled 13-mer and 15-mer are from the 3' (18S) side. The 18-mer is the oligonucleotide containing the cleavage sites (Fig. 1 and Table 1). (B–D) Fingerprints of 3' half-molecules. Below each is the fingerprint of the corresponding 5' half-molecules (E–G), as proposed in the text. In each of these fingerprints, the arrows denote the location of the novel oligonucleotide resulting from endonucleolytic cleavage within the 18-mer. These spots are designated a, a', b, b', c, and c' according to the cleavage products from which they arose (see Fig. 2). In panel D, the dotted arrow indicates a novel oligonucleotide which could not be clearly resolved from other spots.

that spot a has the sequence ACCUUACCUACCUGp (Table 1). Thus, RNAs a and a' (Fig. 2) could result from a single cleavage event within the 18-mer. A similar analysis of the other novel fingerprint spots was carried out, and the results are summarized in Table 1.

We conclude from these analyses that the three cleavages produced by incubation in nucleolar extract occur on the 5' side of adenosine residues in the sequence UACCU, which is repeated three times near the 5' end of human 18S rRNA, i.e., CUCU/ACCUU/ACCU/ACCUG. The cleavages produce 5' half-molecules which have 2',3'-cyclic phosphate groups at their 3' ends and 3' half-molecules which have 5'-hydroxyl termini (Table 1). The cleavages occurred at positions –8, –3, and +1 with respect to the mature 5' terminus of 18S rRNA, which begins with the sequence pUACCUG (15, 24). Thus, for the remainder of this report, the 3' half-molecules initially designated RNAs a, b, and c will be referred to as the –8, –3, and +1 RNAs.
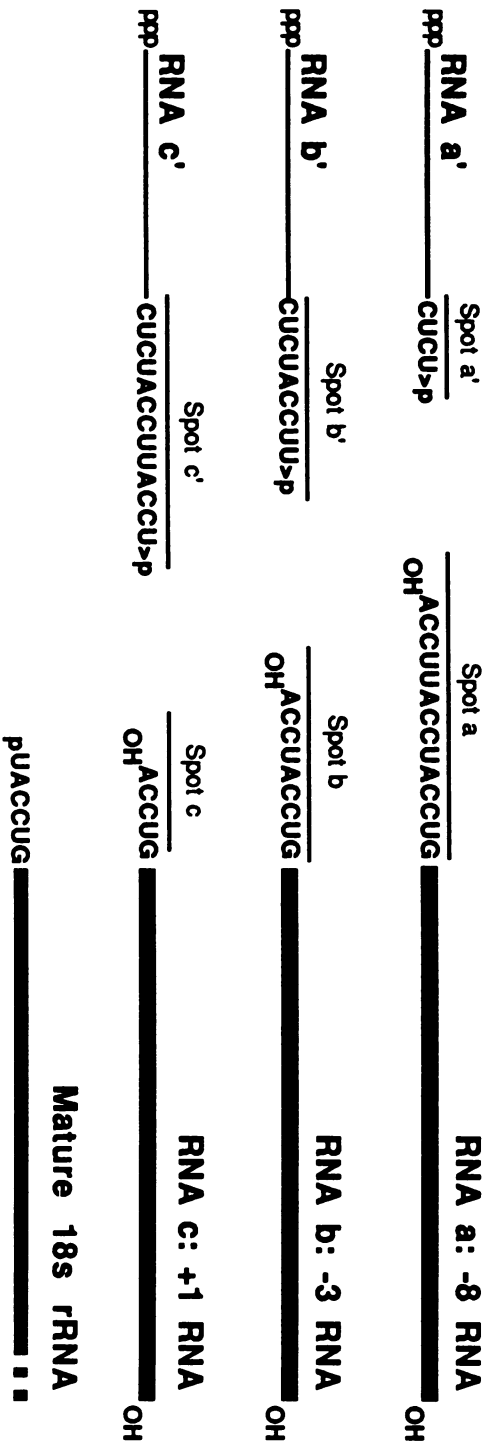
**–8 RNA and –3 RNA are specifically "trimmed" in a fractionated HeLa cell cytoplasmic extract.** We speculated that the cleavages observed in nucleolar extract might be an initial processing event which could be followed by a trimming reaction to generate the mature 5' terminus. If this

were the case, maturation of 18S rRNA in vitro would require an additional activity absent from the nucleolar extract. To address this possibility, we purified the 3' half-molecules (–8 RNA, –3 RNA, and +1 RNA) generated in the nucleolar extract reactions and assessed the ability of a number of HeLa cell subcellular fractions to trim these species. For these experiments, we first assayed the processing of 3' half-molecules which had been labeled by the addition of [$^{32}$P]pCp to their 3' ends, since any trimming of these substrates would necessarily result from removal of nucleotides from the 5' end of the molecule. We observed a small increase in the electrophoretic mobility of two of the 3' half-molecules (–8 and –3 RNAs) as a consequence of incubation in a resuspended P100 fraction of HeLa cell cytoplasmic extract (the +1 RNA was not altered by these incubations). A time course of this reaction is shown in Fig. 4. This result suggested that the P100 fraction contained a "trimming" factor active on the 5' ends of –3 and –8 RNAs. These substrates were largely unchanged by incubation in either nuclear or nucleolar extracts (Fig. 4).

To accurately determine the extent of trimming which occurred in cytoplasmic P100, internally labeled 3' half-molecules were purified from nucleolar extract reaction

TABLE 1. Secondary nuclease digestion analysis of oligonucleotides recovered from fingerprints of nucleolar extract reaction products[a]

| Fingerprint Spot | RNase U2 | RNase A | Nuclease P1 | Phosphatase | 2',3' Cyclic Phosphodiesterase then Phosphatase | Proposed Sequence |
|---|---|---|---|---|---|---|
| a' | Insensitive | Cp,Up | pU>p,pC,pU | Insensitive | sensitive | CUCU>p |
| a | CCUUАp,CCUUGp, CCUАp,Ap | ACp,Cp,Up | pA,pC,pU,pG, phosphate | sensitive | sensitive | ACCUUACCUACCUGp |
| b' | CUCUАp,CCUUp | ACp,Cp,Up [AAUp,Gp]* | pU>p,pC,pU,pA | Insensitive | sensitive | CUCUACCUU>p [AAUUCGp] |
| b | CCUGp,CCUАp,Ap | ACp,Cp,Up | pA,pC,pU,pG, phosphate | sensitive | sensitive | ACCUACCUGp |
| c' | CUCUАp,CCUUАp, CCUp | ACp,Cp,Up | pU>p,pC,pU,pA | Insensitive | sensitive | CUCUACCUUACCU>p |
| c | n.d. | n.d. | n.d. | n.d. | n.d. | ACCUGp |

RNA a'  ppp ———[Spot a']——— CUCU>p

Spot a  OH-ACCUUACCUACCUG   RNA a: -8 RNA  -OH

RNA b'  ppp ———[Spot b']——— CUCUACCUU>p

Spot b  OH-ACCUACCUG   RNA b: -3 RNA  -OH

RNA c'  ppp ———[Spot c']——— CUCUACCUUACCU>p

Spot c  OH-ACCUG   RNA c: +1 RNA  -OH

pUACCUG ——— Mature 18s rRNA

[a] Oligonucleotide designations refer to the fingerprint spots marked in Fig. 3. Conditions for secondary enzymatic digestion are given in Materials and Methods. The term insensitive means that treatment with a particular enzyme failed to produce a detectable change in the electrophoretic mobility of an oligonucleotide. Products of RNase U2 digestion were analyzed by high-voltage electrophoresis on DEAE paper, pH 1.9. Electrophoresis on DEAE paper at pH 3.5 was used for analysis of all secondary products of pancreatic RNase A. Products of nuclease P1 digestion were analyzed on two different systems. Whatman 3MM paper, pH 3.5 (to permit resolution of all four nucleotides), and DEAE paper, pH 1.9 (to distinguish pUp from phosphate; these species nearly comigrate on 3MM paper). Products of CIAP treatment and products generated by sequential treatment with 2',3'-cyclic-nucleotide 3'-phosphatase and CIAP were analyzed on DEAE paper, pH 1.9. With respect to this sequential treatment, sensitive means that a readily detectable mobility change occurred; this was accompanied by the appearance of free phosphate in all but the faintest samples. *, Brackets identify a specific oligonucleotide present in control fingerprints.
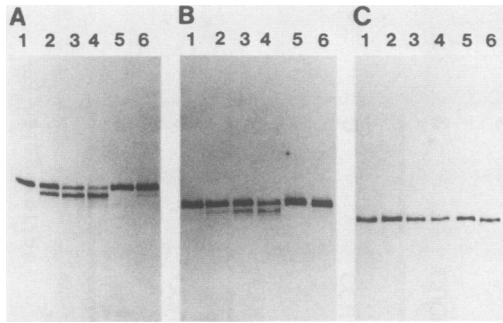
FIG. 4. Initial cleavage products of nucleolar extract are specifically trimmed in HeLa cell cytoplasmic extracts. 3′-End-labeled −8 RNA (A), −3 RNA (B), and +1 RNA (C) were gel purified following a preparative nucleolar extract reaction as described in Materials and Methods. Lanes 1 to 4 of each panel are incubations of these RNAs in the presence of a P100 fraction of HeLa cell cytoplasmic extract for 0, 15, 30, or 60 min, respectively, under conditions described in Materials and Methods. Lanes 5 and 6 of each panel are 60-min incubations of these same substrates in nucleolar or nuclear extract, respectively, under identical conditions. The products of these reactions were analyzed by electrophoresis on a 5% acrylamide–7 M urea gel.

mixes. However, upon incubation in P100, these RNAs appeared highly unstable, apparently due to a 3′ exonuclease present in the extract which could act upon these molecules because of their 3′-hydroxyl termini. As shown above, 3′-end-labeled molecules which contained a 3′-terminal phosphate as a consequence of the end-labeling procedure were largely resistant to this activity (Fig. 4). Accordingly, unlabeled pCp was ligated to internally labeled substrates prior to incubation in nucleolar extract. The stability of the −8 and −3 RNAs prepared in this way was similar to that of 3′-end-labeled molecules, and thus it was possible to isolate trimmed reaction products for fingerprint analysis (Fig. 5A and data not shown).

Although trimming of both the −8 and −3 RNAs was analyzed in parallel, only the results obtained with the −3 RNA are shown because of their relevance to accurate

processing. In initial experiments, unfractionated reaction products (mixtures of trimmed and untrimmed RNAs) resulting from incubation of the −3 or −8 RNA with P100 extract were analyzed by RNase $T_1$ fingerprinting. As a consequence of incubation, the relative intensity of some RNase $T_1$-resistant oligonucleotides was reduced and a number of novel oligonucleotides appeared (Fig. 5C and data not shown). With both RNAs, oligonucleotides derived from the 3′ end of the substrate molecules (Fig. 5C, note the spot horizontally to the right of the spot designated 1 and immediately adjacent to the spot designated 2) were reduced in relative intensity (Fig. 5C and data not shown). Additionally, one novel spot (Fig. 5C, note the spot above and to the right of the spot designated 1) appeared in the fingerprints of trimmed products of both the −3 and −8 RNAs (Fig. 5C and data not shown). It seems likely that the reduction in intensity of 3′-terminal oligonucleotides and the appearance of a common novel oligonucleotide following exposure of the −8 and −3 RNAs to P100 extract resulted from limited 3′ exonuclease activity on these substrates. This conjecture is supported by the fact that these features were not apparent in fingerprints of purified trimmed products (Fig. 6).

The relative intensity of the 5′-terminal oligonucleotides of both the −3 (Fig. 5B to D, spot marked by a light arrow) and −8 RNAs was also reduced in fingerprints of unfractionated trimmed products (Fig. 5 and data not shown). This reduction in intensity was accompanied by the appearance of a distinct pair of additional novel oligonucleotides specific for either the −3 (Fig. 5C, spots labeled 1 and 2) or −8 RNA (data not shown). Since these different pairs of oligonucleotides were derived from molecules which differed only at their 5′ ends it seemed likely that they could result from trimming at the 5′ end of the substrate. Furthermore, since two products of different size were evident, these fingerprints suggested that the trimmed band observed with end-labeled substrate (Fig. 4) was actually a mixture of species which differed in size by 1 or 2 bases.

If either of the oligonucleotides generated from the 5′ terminus of the −3 RNA corresponded to mature 18S rRNA terminus, it would be predicted to have a 5′-terminal phosphate. When the unfractionated trimmed products of the −3
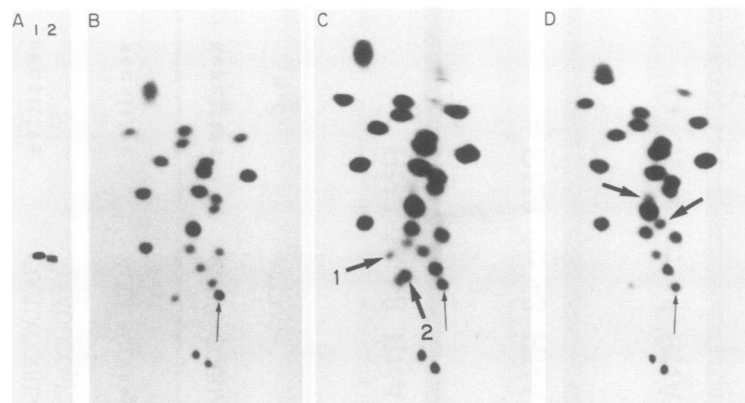


FIG. 5. RNase $T_1$ fingerprint analysis of trimmed products. [α-$^{32}$P]GTP-labeled −3 RNA containing pCp at its 3′ terminus (see text) was incubated in P100 extract. A sample was removed at 0 min for fingerprint and gel analysis. After 60 min, a small sample of the reaction mix was saved for gel analysis and the remainder was subjected to RNase $T_1$ fingerprint analysis either with or without prior treatment with CIAP. (A) A 6% acrylamide–7 M urea gel analysis of the trimming reaction. Lane 1 is the unincubated sample, and lane 2 had been incubated for 60 min. (B) Fingerprint of the unincubated −3 RNA. The oligonucleotide marked by the arrow is from the 5′ end of the RNA. (C) Fingerprint of the −3 RNA which had been incubated for 60 min; bold arrows identify oligonucleotides designated 1 and 2 which arise as a result of the trimming reaction. The bold arrows in panel D mark the positions of these oligonucleotides when the trimmed RNA was treated with CIAP prior to fingerprinting.
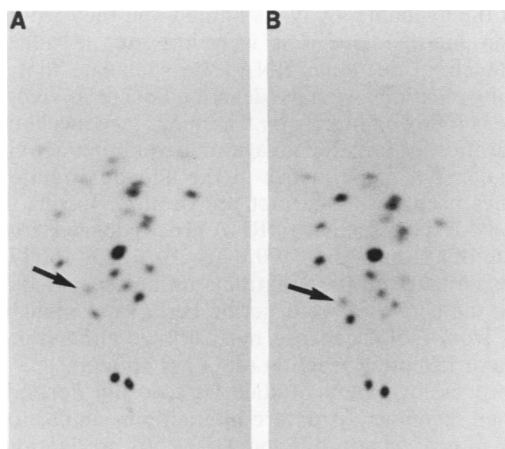
FIG. 6. Comparison of the 5' termini of mature 18S rRNA and trimmed −3 RNA. Uniformly ${}^{32}$P-labeled −3 RNA was gel purified from a preparative nucleolar extract reaction and trimmed in the cytoplasmic P100. This reaction was phenol-chloroform extracted, ethanol precipitated, and analyzed on a 5% polyacrylamide gel. The leading edge of the trimmed band was excised in order to purify one of the two reaction products (see text). The RNA eluted from this gel slice was subjected to RNase $T_1$ fingerprint analysis (A) for comparison to cytoplasmic 18S rRNA from HeLa cells. ${}^{32}$P-labeled cytoplasmic RNA was isolated from HeLa cells, and the first 166 nucleotides of 18S rRNA were protected from RNase $T_2$ digestion by hybridization to M13SX DNA (see Materials and Methods). The protected RNA was gel purified and subjected to RNase $T_1$ fingerprint analysis (B). Arrows indicate the 5'-terminal oligonucleotide of each RNA.

RNA were treated with CIAP prior to fingerprinting, the position of both oligonucleotides was shifted up and to the right (Fig. 5D), as expected if they terminated in 5' phosphates. Unexpectedly, the relative intensity of the 3'-terminal oligonucleotide (note the spot immediately adjacent to the spot labeled 2 in Fig. 5C) was also reduced by CIAP treatment. This reduction in intensity could have resulted if the terminal C residue (added by RNA ligase) was removed from the substrate during incubation, leaving a 3' phosphate on the G residue at position 165.

To explore the possibility that one of the novel spots generated form the 5' end of the −3 RNA by trimming

represented the authentic 5' terminus of 18S rRNA, we wished to separate the population of trimmed −3 RNAs into its two components. Upon prolonged electrophoresis, the reaction products formed a single wide band. Since two distinct bands could not be generated, RNA from the top and bottom halves of this band was eluted and analyzed. Furthermore, it was necessary to have an appropriate marker for accurate processing. Accordingly, HeLa cells were labeled with ${}^{32}$P$_i$, and cytoplasmic RNA was prepared. This RNA was annealed with a single-stranded DNA spanning the ETS-18S boundary and digested with RNase $T_2$ (see Materials and Methods). The resultant protected RNA, corresponding to the first 166 bases of 18S rRNA, was analyzed by RNase $T_1$ fingerprinting (Fig. 6B) and the 5'-terminal RNase $T_1$ oligonucleotide was identified by its sensitivity to phosphatase treatment. A comparison of fingerprints showed that the smaller trimmed product of the −3 RNA and the RNA prepared from HeLa cells were nearly identical (Fig. 6). While the 5'-terminal oligonucleotides of the two RNAs migrated identically, the migration of some oligonucleotides, including the U-rich oligonucleotides flanking the 5'-end spots, differed. These differences in migration result from the fact that these oligonucleotides are methylated in vivo (15).

To further substantiate the structure of the terminal oligonucleotide of the trimmed RNA, it was recovered from preparative-scale fingerprints such as the one shown in Fig. 5C and subjected to detailed RNA secondary nuclease digestion analysis. While the 5'-terminal oligonucleotide of the −3 RNA yielded the RNase U2 products Ap, CCUAp, and CCUGp, the new oligonucleotide yielded the RNase U2 products pUAp and CCUGp. The presence of a 5'-terminal phosphate was further confirmed by pancreatic RNase A digestion. Products of this digestion included pUp (in addition to ACp and nucleoside monophosphates). Moreover, when this spot was treated with RNase $T_2$ followed by nuclease P1, pU and phosphate were produced (Table 2). From this analysis, we conclude that trimming of the −3 RNA yields an RNA with a 5'-terminal RNase $T_1$ oligonucleotide (pUACCUGp) identical to that of mature 18S RNA (15, 24).

Similar analysis indicated that the other trimmed product from the −3 RNA was pCUACCUGp (Table 2), although pCCUACCUGp could not be completely ruled out. Prelim-

TABLE 2. Secondary nuclease digestion analysis of the novel 5' end spots generated by trimming of the −3 RNA[a]

| Trimmed fingerprint spot | Novel spot generated after trimming with: | | | Proposed sequence |
|---|---|---|---|---|
| | RNase U2[b] | RNase A | RNase $T_2$ followed by nuclease P1 | |
| 1 | CCUGp, pUAp | ACp, pUp, Gp, Cp, Up | pU, phosphate | pUACCUGp |
| 2 | CCUGp, pCUAp | ACp, pCp, Gp, Cp, Up | pC, phosphate | pCUACCUGp |

[a] Oligonucleotide designations refer to the fingerprint spots marked in Fig. 5. Conditions for secondary enzymatic digestions are given in Materials and Methods. The products of RNase U2 digestion of spot 1 separated well during high-voltage electrophoresis on DEAE paper at pH 1.9 or 3.5. However, under these conditions, the RNase U2 products of spot 2 comigrated; to separate these products, electrophoresis on Whatman 3 MM paper, pH 3.5, was used. Electrophoresis on DEAE paper at pH 3.5 was used for analysis of pancreatic RNase A products, while Whatman 3 MM paper, pH 3.5, was used to separate the products remaining after the sequential treatment of RNase $T_2$ followed by nuclease P1.

[b] The RNase U2 products of spot 1 were identified as follows. (i) Fingerprint analysis indicated that spot 1 arose from spot b, ACCUACCUGp; (ii) one product comigrated with a marker for CCUGp derived from the 18-mer; (iii) the other RNase U2 product was eluted from DEAE paper and directly shown to be pUAp by tertiary analysis (in one experiment, pancreatic RNase released pUp and Ap, and in another, RNase $T_2$ released pUp and one or more mononucleotides). The presence of pU at the 5' end was further confirmed by digestion of spot 1 with RNase $T_2$, which released a spot comigrating with a marker for pUp plus mononucleotides. The RNase U2 products of spot 2 both comigrated with a marker for CCUGp under the standard conditions for electrophoresis of RNase U2 digests. However, after electrophoresis on Whatman 3 MM paper, two products were resolved. One comigrated with a marker for CCUGp wand was thus identified. The other product was concluded to be pCUAp because (i) fingerprint analysis indicated that spot 2 arose from spot b; (ii) RNase $T_2$ followed by nuclease P1 digestion showed that spot 2 began with pC; and (iii) pancreatic RNase A digestion of spot 2 released pCp plus other products. Furthermore, preliminary studies done on the purified U2 product eluted from 3 MM paper indicated that it lacked Cp; however, the technical limitations of this final study leave open the possibility that the 5'-terminal RNase U2 product is pCCUAp.
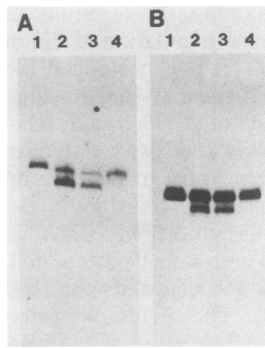
FIG. 7. Phosphorylation of −3 and −8 RNAs abolishes trimming. 3′-End-labeled −8 (A) and −3 (B) nucleolar products, containing 5′-hydroxyl termini, were either phosphorylated with polynucleotide kinase or mock phosphorylated prior to incubation in the P100 extract (Materials and Methods). For comparison, lanes 1 and 2 are unmodified RNAs incubated in the P100 extract for 0 or 60 min, respectively. Lanes 3 are 60-min incubations of mock-phosphorylated RNAs treated with polynucleotide kinase in the absence of ATP. Lanes 4 are 60-min incubations in the P100 extract of RNAs which were quantitatively phosphorylated (as shown by fingerprint analysis) prior to incubation. Analysis of reaction products was carried out by electrophoresis on a 5% acrylamide–7 M urea gel.

inary studies indicate that analogously trimmed derivatives (pCUUACCUACCUGp and pUUACCUACCUGp) were produced from the −8 RNA (data not shown).

Because the foregoing results indicated that the P100 trimming activity yielded the mature 5′ terminus of 18S RNA, it was of interest to study this reaction in more detail. While pretreatment of the P100 extract with micrococcal nuclease had no effect, the activity was abolished by proteinase K. To explore the specificity of termini required for trimming activity, the −8 RNA and −3 RNA were phosphorylated with polynucleotide kinase prior to trimming. When these RNAs were incubated with P100, no trimming was detected (Fig. 7).

## DISCUSSION

We have shown that a small synthetic transcript containing 70 bases of 5′ ETS and the first 165 bases of 18S rRNA is processed in vitro to yield the mature 18S rRNA 5′ terminus. Accurate processing results from sequential catalysis by two distinct activities found in separate subcellular extracts derived from HeLa cells.

In nucleolar extract, the substrate is cleaved at three positions near the mature 5′ terminus of 18S rRNA. The significance of multiple products is unclear, since only the cleavage which yields the −3 RNA creates a molecule which can be accurately trimmed by the extracts we have tested. While it is possible that cellular activities might be found which could salvage the −8 and +1 RNAs, cleavage at the −8 and +1 positions may reflect relaxed specificity due to the in vitro conditions and could indicate the potential for each of the repeats of the sequence UACCU to occupy equivalent positions in similar higher-order structures.

We used a number of techniques, including primer extension, S1 nuclease mapping, and direct two-dimensional fingerprint analysis of pulse-labeled RNA, in an attempt to detect a processing intermediate in HeLa cell RNA corresponding to the −3 RNA. These experiments did not reveal the presence of the predicted intermediate (data not shown). This negative result may not be surprising since the sensi-

tivity of the available assays is limited and they would only detect an intermediate if it were present at substantive (≥3%) levels in nucleolar RNA. We speculate that, if the processing reactions we have described occur in vivo, initial cleavage is followed rapidly by trimming. It seems likely that the separation of initial cleavage and trimming activities in vitro could be a consequence of the aqueous fractionation techniques used during extract preparation. In this regard, previously described in vitro rRNA processing systems have made use of cytoplasmic S100 extracts (6, 13, 16, 17). The activities detected in these extracts do not appear to correspond to the activities we describe here, since similar S100 extracts from HeLa cells do not catalyze either the initial cleavage or trimming reaction (data not shown).

The processing events studied here do not depend upon long-range secondary-structure interactions similar to those involved in procaryotic 16S and 23S rRNA production. This conclusion was also reached by Vance et al. (25), who observed accurate processing of truncated 18S rRNA precursors in a transfection system. It also appears unlikely that the initial cleavage reactions in nucleolar extract are catalyzed by a eucaryotic enzyme with a substrate specificity similar to that of RNase III, since cleavage reactions are insensitive to double-stranded RNA at concentrations which would effectively compete for an RNase III-like activity.

Extensive studies employing direct techniques as well as phylogenetic comparisons have established a secondary structure for mammalian 18S ribosomal RNA (12, 23). In this structure, the first 165 bases of 18S rRNA (the portion of 18S rRNA present in our processing substrate) form extensive base-paired contacts with regions downstream in the 18S sequence. These secondary structural features clearly could not form in our truncated substrates since the downstream elements are absent. To see whether the relative reactivity of the three initial cleavage sites was strongly affected by the inclusion of additional pre-rRNA sequence, we assayed the processing of a longer substrate molecule in nucleolar extract. This substrate (9.7 kb) extended from the 5′ SalI site to the 3′ EcoRI site of the rRNA transcription unit depicted in Fig. 1. However, RNase $T_1$ fingerprint analysis indicated that cleavage of this substrate occurred at all three sites near the ETS-18S boundary (data not shown).

We have not defined the signals which mediate the initial cleavage or trimming reactions. Sequence analysis of rRNA genes in higher eucaryotes has revealed that the 5′ coding sequence of 18S rRNA is highly conserved while there is little primary sequence conservation in the 5′ ETS (7). While it seems likely that processing signals could reside within the 18S rRNA sequence, it is unclear whether these signals involve primary sequence elements or higher-order structures. Appropriate mutational analysis will be required to distinguish between these possibilities.

As discussed above for the initial cleavage reaction, the specificity of the trimming reaction also suggests that each of the repeated UACCU elements of the ETS-18S boundary could occupy equivalent positions within similar structures. Both the −3 and −8 RNAs were trimmed to the same extent, yielding a mixture of products. In these reactions, the correctly trimmed product was produced inefficiently (Fig. 5). It seems possible that the inefficiency of the trimming reaction could result either from the fact that assays were carried out with crude extracts or as a consequence of the structure of the truncated substrate. Purification of the trimming activity should help to distinguish between these possibilities. The fact that phosphorylation of the −3 and −8 RNAs rendered them insensitive to the trimming activity

emphasizes the relationship between the products resulting from cleavage by the nucleolar extract and the substrate specificity of the trimming activity, which appears to have end recognition as an important element of its selectivity. However, the detailed mechanism of trimming as well as the significance of alternatively trimmed products remain to be determined.

Despite the apparent dissimilarities between procaryotic and eucaryotic rRNA processing, the pathway leading to formation of the 5' end of 18S rRNA may be analogous to the pathway of formation of 16S rRNA. In both cases, maturation proceeds through two steps, initial endonucleolytic cleavage and subsequent trimming. The availability of an in vitro system to analyze pre-rRNA processing in eucaryotic cells should allow the determination of the intrinsic features of the precursor which mediate processing as well as provide the opportunity to purify and characterize relevant processing activities.

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