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Heterogeneity of 5'-termini of nucleolar 45S, 32S and 28S RNA in mouse hepatoma

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ABSTRACT

The 5'-termini of nucleolar 45S, 32S and 28S RNA's were analyzed by means of thin layer chromatography and Dowex-1 column chromatography.

45S RNA did not bear a triphosphate at the 5'-terminus, but various monophosphates were found. 5'-termini of 32S and 28S RNA's were also heterogeneous.

These results indicate that 45S molecules as isolated with the conventional procedure may not contain the primary transcript of the ribosomal gene, but a collection of large precursors with different degrees of processing at the 5'-terminus. The processing of the primary transcript may thus involve some unknown trimming processes at the 5'-terminus before the first major cleavage takes place.

INTRODUCTION

Although many studies have been reported from this and other laboratories on the 5'-terminal structure of ribosomal 18S and 28S RNA (1-10), little information is available on those of ribosomal precursor RNA's.

One reason for this is that these precursor RNA molecules are of very large size and are present in much lower amounts within the cell as compared with mature ribosomal RNA molecules.

The 5'-terminal nucleotide sequence of 45S RNA is especially important on the view point of the initiation sequence of ribosomal RNA gene, since 45S RNA is thought to be the primary product of transcription (11), although precursor molecules larger than 45S RNA have been described *in vivo* (12) and *in vitro* (13).

If 45S RNA is the primary transcript, then we would expect it to bear a triphosphate group on its 5'-terminus.

Analysis of 5'-terminus of 40S precursor RNA from *Xenopus laevis* have indicated that this precursor has the 5'-terminal guanosine 5'-phosphate (10). We have reported previously 5'-terminal sequences of ribosomal 18S

and 28S RNA in various eukaryotic cells (9).

We have now examined the 5'-terminal nucleotides released by alkaline hydrolysis from nucleolar 45S, 32S and 28S RNA in mouse MH134 cells and have obtained evidence that the 5'-termini of these RNA's are heterogeneous. 45S RNA did not bear a terminal triphosphate suggesting that this molecule may not be the primary transcript.

We have also found that the 5'-terminal nucleotide of ribosomal 18S RNA was homogeneous, while that of ribosomal 28S RNA was heterogeneous (manuscript in preparation). Possible implications of these findings are discussed.

MATERIALS AND METHODS

Cells and labeling of RNA.

The MH134 cells were maintained in mice of C3H/He strain. Nine to twelve days after intraperitoneal transplantation, the ascites cells were harvested by abdominal drainage.

The cells were incubated with 10 to 20 mCi of carrier free [^{32}P] orthophosphate in the *in vitro* incubation system described earlier for five hours (7,9).

Isolation and purification of nucleolar RNA.

Highly purified nucleoli were prepared by a sonication procedure described previously (14). RNA was extracted by a modification of the hot sodium dodecyl sulfate-phenol method (15,16). Nucleolar 45S and 32S RNA were purified by repeated sucrose density gradient centrifugations as described in Fig. 1-a and b. Nucleolar 28S RNA was furthur purified by heat-quenching followed by recentrifugation (17). The specific activity of ^{32}P-labeled RNA was 5-10 x 10^6 cpm per A_{260} unit.

Hydrolysis of RNA and separation of oligonucleotides.

RNA was hydrolysed with 0.3 N NaOH at 37°C for 18 hr. The hydrolysate was neutralized with 1 N HCl, diluted and chromatographed on a DEAE-Sephadex column as described earlier (9).

Determination of 5'-terminal nucleotides.

Two methods have been utilized to identify nucleoside 3'(2'), 5'-diphosphates (pNp).

(1) The column fraction containing both alkali-stable trinucleotides and nucleoside diphosphates originated from 5'-terminus was chromatographed on a Dowex-1 column as described previously (9).

(2) The fraction was desalted on a DEAE-Sephadex column (0.5 x 2 cm),

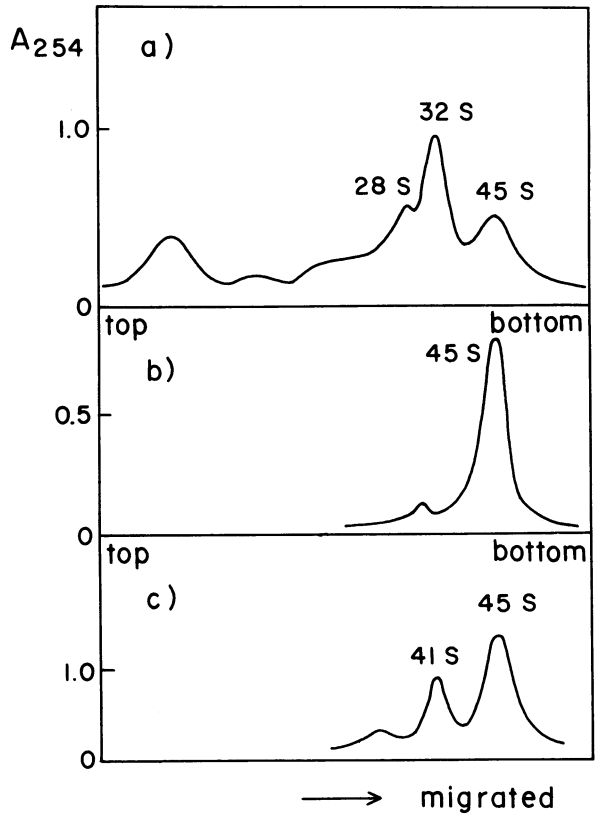

Fig. 1. Sedimentation profile of ^{32}P-labeled nucleolar RNA of MH134 cells. (a) Approximately 0.2 mg of nucleolar RNA was placed on a 10 ml of 10-40% (w/w) sucrose gradient and centrifuged in a Hitachi RPS-40T rotor at 28,000 rpm for 16 hr at 4°C. The gradient was fractionated with a ISCO Density Gradient Fractionator while recording the absorbance at 254.
(b) Re-sedimentation pattern of 45S RNA fraction.
(c) Agarose-acrylamide gel electrophoretic pattern of 45S RNA fraction. 45S RNA was electrophoresed on a 0.5% agarose-2.0% acrylamide composite gel according to Peacock and Dingman (21).

bicarbonate form, the nucleotides being eluted with 2 M triethylamine bicarbonate. The eluate was dried and dissolved in 10 µl of water and then was applied onto an Avicel-SF cellulose glass plate (Funakoshi Co. 10 x 10 cm) together with 0.05 A_{260} unit each of Ap, Cp, Gp, Up and Ψp as markers. The solvent systems used were isobutyric acid - 0.5 M NH_4OH (5:3, v/v) for the first dimension and 2-propanol-concentrated HCl-H_2O (70:15:15, v/v/v) for the second dimension (18). ^{32}P-labeled pAp, pCp, pUp and pGp markers were prepared by phosphorylation of ^{32}P-labeled AGp, CGp, UGp and GUp with ATP and polynucleotide kinase (a generous gift of Dr. M. Takanami, Kyoto

University) followed by alkaline hydrolysis (3). pNp was separated by two dimensional thin layer chromatography with Np markers. The 5'-terminal nucleotides were identified by Rf values after autoradiography.

RESULTS

Chromatography of alkaline hydrolysates of nucleolar 45S RNA and ribosomal 28S and 18S RNA.

When alkaline hydrolysates of 45S, 28S and 18S RNA were chromatographed on DEAE-Sephadex A-25 at pH 7.6, the elution profile shown in Fig. 2 was obtained; this system is designed to separate oligonucleotides on the basis of chain length (19). The contents of alkali-stable oligonucleotides in ^{32}P-labeled nucleolar and ribosomal RNA are shown in Table 1. Ribosomal alkali-stable oligonucleotides have been characterized previously in this laboratory (7). It is important to determine whether 5'-triphosphates are present in nucleolar 45S RNA isolated as such. After alkaline hydrolysis these should be liberated as molecules of the type pppNp which are eluted from the DEAE-Sephadex column between the fourth and the fifth isostich peaks (10,20). The DEAE-Sephadex columns did not show any significant peak at higher salt concentration than the fourth isostich peak which contained 2'-O-methylated tetranucleotide. No further radioactivity was eluted with 0.5 M NaCl. In the digest of 45S RNA, however, a shoulder was found in the trinucleotide peak as shown in Fig. 2. A similar shoulder was present in ribosomal 28S RNA, too. This shoulder fraction was previously shown to contain a nucleoside 3',5'-diphosphate originated from the 5'-terminus of 28S RNA. This suggested that nucleolar 45S RNA carried 5'-monophosphates rather than 5'-triphosphates at the 5'-terminus of the molecule. Almost identical results were obtained when RNase T_2 digestion was performed instead of alkaline hydrolysis.

Table 1, shows the distribution of radioactivity among mono-, di-, tri- and tetranucleotides. Characteristic distribution patterns are apparent for 45S, 32S and 28S RNA. Radioactivity ratio of tri/tetra fraction was 2, consistent with the theoretical value calculated from the assumption that the tri-fraction contained one mole of a 5'-terminal nucleotide and two moles of 2'-O-methylated trinucleotide.

The two dimensional thin layer chromatographic patterns of nucleoside 3',5'-diphosphates.

Nucleoside diphosphates (pNp) were identified in the following way.

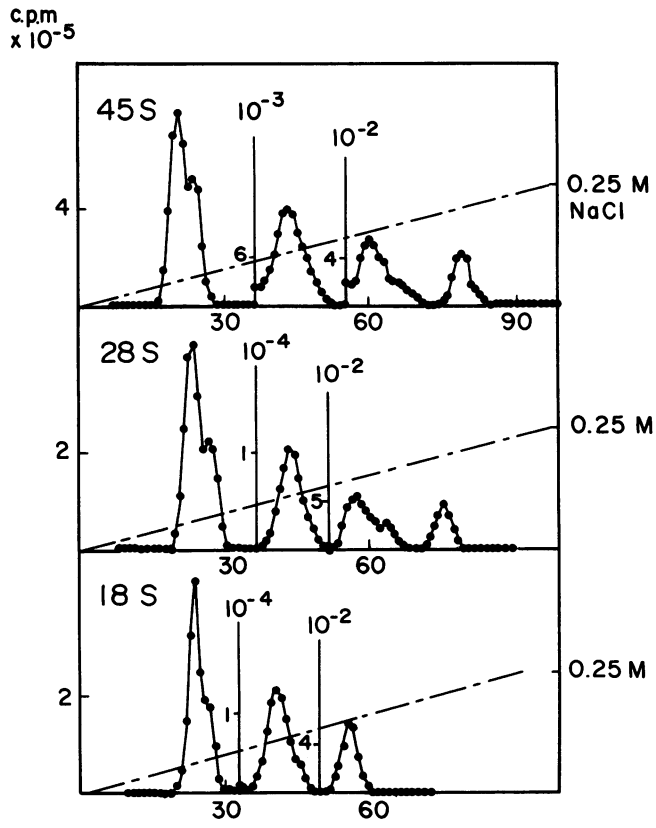

Fig. 2. DEAE-Sephadex chromatography of alkaline hydrolysate of ^{32}P-labeled 45S (a), 32S (b) and 28S (c) RNA.

RNA was hydrolyzed with 0.3 N NaOH for 18 hr at 37°C. Each hydrolysate was neutralized with 1 N HCl, diluted with 10 vol. of 7 M urea, 0.05 M Tris-HCl (pH 7.6) and applied to a column (0.5 x 20 cm) of DEAE-Sephadex and eluted with a 80 ml gradient of 0.05 M to 0.25 M NaCl in 7 M urea and 0.05 M Tris-HCl (pH 7.6). 1.0 ml fractions were collected every ten min. No significant radioactivity was eluted with 1 M NaCl.

Table 1 Distribution of radioactivity among mono-, di-, tri- and tetranucleotides (%)

Peak	Mono	Di	Tri	Tetra
Contents	Np	NmNp	UmGmUp UmGmψp	AmGmCmAp
RNA			pNp	
nucleolar 45S	98.1	1.79	0.090	0.045
nucleolar 32S	98.0	1.87	0.11	0.060
nucleolar 28S	96.8	3.01	0.15	0.072
ribosomal 28S	97.4	2.38	0.15	0.08
ribosomal 18S	96.4	3.52	0.10	

Dinucleotides (NpNp) were prepared from ^{32}P-labeled RNA by digestion with T_1 or pancreatic RNase, followed by separation with DEAE-Sephadex column chromatography. Each dinucleotide was separated by paper electrophoresis, and was 5'-terminally phosphorylated with polynucleotide kinase and ATP, and digested with T_2 RNase to yield 3'-phosphate labeled nucleoside 3',5'-diphosphates. These were separated by thin layer chromatography on the positions illustrated in the diagram (Fig. 3-a).

5'-terminal nucleoside diphosphates were separated by the two dimensional thin layer chromatography as shown in Fig. 3. The percentage distribution of radioactivity among nucleoside 3',5'-diphosphates in 45S RNA and other nucleolar RNA is presented in Table 2. Terminal nucleoside 3',5'-diphosphates were also chromatographed on a Dowex-1 column and characterized according to Takanami's method as shown in Fig. 4 (1). It is apparent from Table 2 that 5'-termini of 45S and other nucleolar RNA's are heterogeneous. Two possibilities of artifacts must be considered; (1) contamination of other kinds of RNA, (2) nicks which produce RNA fragments with a new 5'-phosphate and 3'hydroxyl group. In order to rule out these posibilities, we have calculated the recovery of 5'-terminus of RNA. Since 28S RNA have two 2'-O-methylated trinucleotides, UmGmUp and UmGmΨp, and the recovery of these trinucleotides was high and reproducible, it could be used as a reliable internal standard for the recovery of 5'-termini. As shown in Table 2 the sum of the recoveries of four different termini calculated from the ^{32}P radioactivity were approximately one mole. It is thus unlikely that the heterogeneity is the consequence of either the contamination of other RNA's or intramolecular nicks.

The 45S RNA purified by sucrose density gradient twice still contained 41S RNA, comprising up to 30% of the total 45S fraction. However, the 45S RNA purified by polyacrylamide-agarose composite gel electrophoresis (Fig. 1-c, 21) and almost devoid of 41S RNA also exibited a similar degree of 5'-terminal heterogeneity (Table 2).

Diversity of 5'-terminal structure of 45S RNA.

Although the above experiments have indicated that 45S RNA contained at least four kinds of 5'-termini, does it really mean that 45S RNA have many different 5'-terminal structures? To explore this possibility 45S RNA was digested with RNase T_1 and the digest was separated on DEAE-Sephadex column at neutral pH (Fig. 5). Fraction [A] designated in Fig. 5 was supposed to contain tetranucleotides and 5'-phosphodinucleotides (pNpGp) and fraction [B] was expected to have penta-to hexanucleotides and 5'-phospho-tri- or tetra-

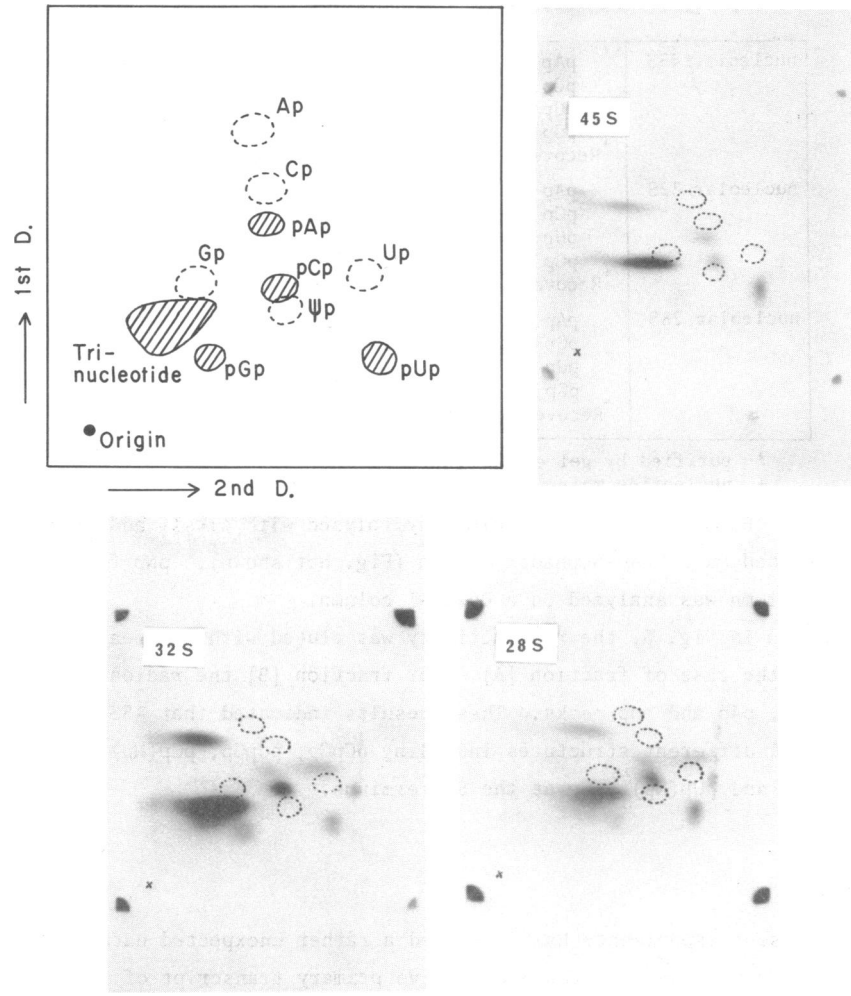

Fig. 3. (1) Autoradiographs of the two-dimensional thin layer chromatography of 5'-terminal nucleotides from nucleolar 45S (a,b), 32S (c) and 28S RNA (c).
^{32}P-labeled alkali-stable "Tri-fraction" eluted from the DEAE-Sephadex column was desalted on a column (0.5 x 1.5 cm) of DEAE-Sephadex, eluted with 2 M Tri-ethylamine bicarbonate, dried, dissolved in water, and applied onto an Avicel SF cellulose plate (10 x 10 cm) with 0.05 A_{260} unit each of Ap, Cp, Up, Ψp and Gp as markers. The solvent systems are described in Materials and Methods. Dotted circles show nucleotide markers detected by an ultraviolet lamp.
(2) Diagram of the autoradiograph.
Dotted circles on the diagram show nucleotide markers and shadowed circles indicate the positions of 5'-terminal nucleotides and alkali-stable tri-nucleotides.

Table 2 Distribution of radioactivity among nucleoside 3',5'-diphosphates derived from 5'-termini of rRNA precursors (%)

nucleolar 45S	pAp	22	34	45	22	37*
	pCp	30	8	20	39	23
	pUp	35	51	]36	]39	]40
	pGp	13	7			
	+Recovery	1.3	0.9	n.d.	n.d.	n.d.
nucleolar 32S	pAp	28	13	26		
	pCp	42	49	35		
	pUp	29	16	]39		
	pGp	0	22			
	+Recovery	1.5	0.7	n.d.		
nucleolar 28S	pAp	29	13	27		
	pCp	34	45	29		
	pUp	36	30	]44		
	pGp	2	12			
	+Recovery	0.9	1.2	n.d.		

* purified by gel electrophoresis
\+ nucleotide moles per RNA

nucleotides. Each fraction was pooled, hydrolysed with alkali and then chromatographed on a DEAE-Sephadex column (Fig. not shown). pNp fraction from each column was analyzed on a Dowex-1 column.

As shown in Fig. 5, the radioactivity was eluted with the peaks of pCp and pUp, in the case of fraction [A]. For fraction [B] the radioactivity was found in pCp, pAp and pUp peaks. These results indicated that 45S RNA had at least several different structures including pCpGp, pUpGp, $pCp(Np)_{1-2}Gp$, $pAp(Np)_{1-2}Gp$ and $pUp(Np)_{1-2}Gp$ at the 5'-terminus.

DISCUSSION

The present experiments has disclosed a rather unexpected nature of the 5'-terminus of the 45S RNA, the presumptive primary transcript of ribosomal RNA gene in mammalian cells. The fact that no triphosphates were found at the 5'-termini of 45S RNA strongly suggests that this RNA is probably not the primary transcript from the ribosomal RNA gene, although a limited dephosphorylation of the 5'-triphosphates to produce a 5'-monophosphate cannot be ruled out completely. The presence of pyrimidine nucleotides also argues for this contention, since only purine nucleotides have been found at the 5'-terminus of the primary transcript in both prokaryotic and eukaryotic cells (20,22,23,24). The heterogeneity of 5'-terminus of 45S RNA thus seems to indicate that this RNA is in fact a collection of large precursors with approximate molecular weight of 4.5×10^6, with different degree of processing at the 5'-terminus. The real primary transcript must then be

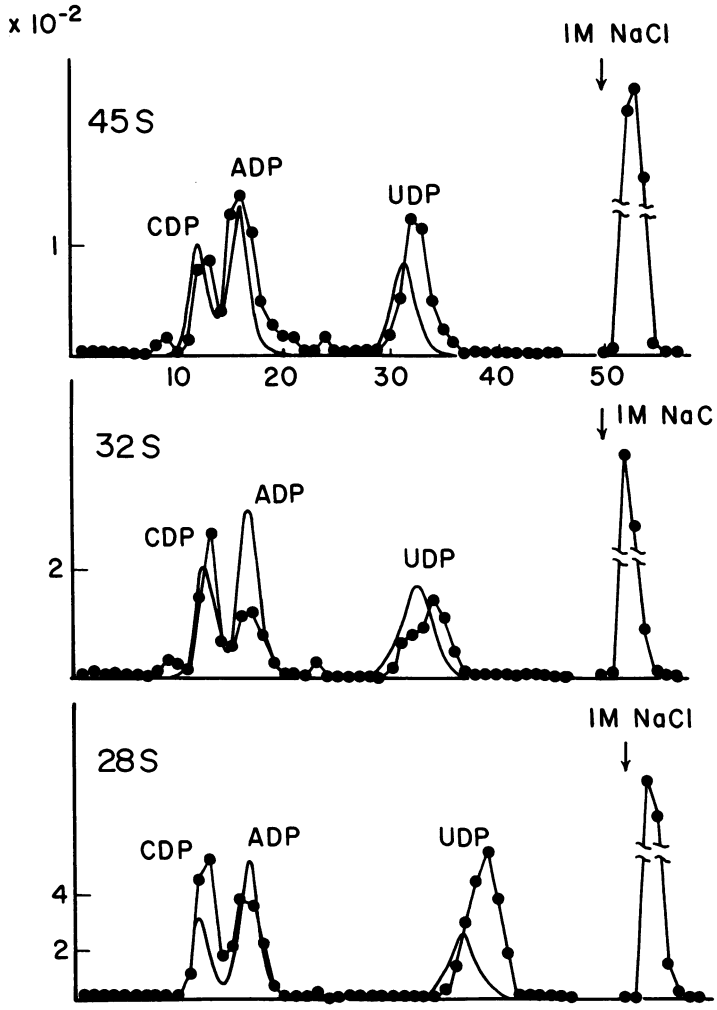

Fig. 4. Dowex-1 chromatography of 5'-terminal nucleotides. 45S (a), 32S (b) and 28S RNA (c).

The "Tri-fraction" on the DEAE-Sephadex column which contained 5'-terminal nucleoside diphosphates and alkali-stable trinucleotides was chromatographed on a Dowex-1 column with about 0.5 A_{260} unit each of the three nucleoside 5' diphosphates (ppC, ppA and ppU) as markers.

extremely short-lived and subjected to immediate processing at the 5'-terminal region. Thus the possibility of the presence of various 45S RNA's with different 5'-terminal sequences is not very likely.

If the 45S RNA is subjected to some trimming processes before it is cleaved into 41S and 20S RNA (25,11), the next question is the mechanism of

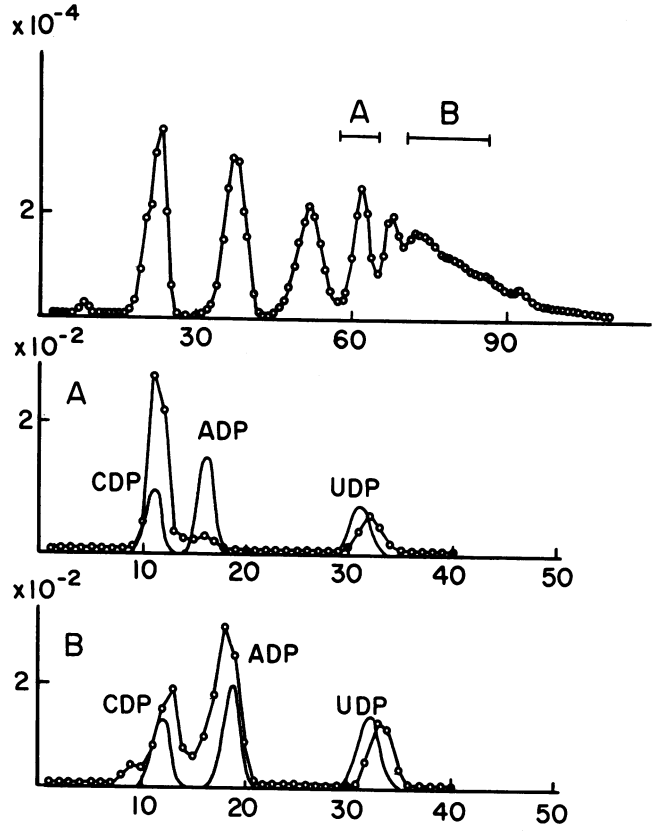

Fig. 5. (1) DEAE-Sephadex A-25 column chromatography of RNase T_1 digest of nucleolar 45S RNA.

Nucleolar 45S RNA containing 2.8 x 10^7 cpm was digested by RNase T_1 with an enzyme to substrate ratio of 1 : 20 (w/w) in 1 ml of a buffer containing 0.02 M Tris-HCl (pH 7.5) and 0.001 M EDTA at 37°C for 2 hr. Then 0.1 ml of 1 N HCl was added to the incubation mixture, and the incubation was continued for 1 hr at 37°C to break down any cyclic phosphates. The digest was loaded on a column (0.5 x 25 cm) of DEAE-Sephadex and eluted with a 140 ml gradient of 0.05 to 0.4 M NaCl in 7 M urea and 0.05 M Tris-HCl (pH 7.6). 1.2 ml fractions were collected every 10 min.

(2) Separation of chromatographic fractions [A] and [B] by Dowex-1 column. Fractions [A] and [B] were chosen to include pNpGp and $p(Np)_{2-3}Gp$ leaving 3 tubes between them to avoid cross-contamination. Each fraction was desalted, dissolved in water and hydrolyzed with 0.3 N NaOH for 18 hr at 37°C. Each hydrolysate was separated by DEAE-Sephadex chromatography as shown in Fig. 2 (Fig. not shown). The "Tri-fraction" was chromatographed on a Dowex-1 column as indicated in Fig. 4.

this trimming. An enzyme resembling RNase III, which is specific for double stranded region of RNA, may be a possible candidate for this trimming since this enzyme is already implicated in certain stages of RNA processing (26,27, 28) and also known to yield 5'-phosphate termini (29). Ribonuclease V, once

implicated as the messenger ribonuclease in E. coli (30,31), was assumed to degrade RNA exonucleolytically from 5'-terminus leaving 5'-phosphate end. However, this point has not been confirmed since then. Another ribonuclease which produces nucleoside 5'-phosphates is the nuclease P (32), although this enzyme does not show a strict specificity for bases (33). The enzyme involved in this process as well as the mechanism of trimming awaits further investigation.

Our arguement that the heterogeneity of 5'-termini of 45S and other RNA's is not an artifact is mainly based on the fact that the total recovery of pNp's was always nearly one. Both the contamination with other RNA's and the intramolecular nicks that produce pNp type 5'-termini could reasonably be excluded by this recovery. In a work which will be published elsewhere, we have found that the 5'-terminus of newly-labeled 18S RNA is exclusively pUp, the same as that of mature 18S RNA determined by a long labeling, while that of newly-labeled 28S RNA shows varying degrees of heterogeneity depending upon the labeling period (manuscript in preparation). The complete homogeneity of the 5'-terminus of 18S RNA prepared from both nuclei and ribosomes also argues against artifactitious nature of the present findings.

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