5′-Terminal Processing of Ribosomal 28S RNA†

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ABSTRACT: The 5′-terminal nucleotides of ribosomal 28S, 18S, and 5.8S RNA of mouse hepatoma cells were analyzed by means of DEAE-Sephadex column chromatography followed by two-dimensional thin-layer chromatography at various times of in vivo labeling with [^{32}P]orthophosphate. The 5′-termini of 18S RNAs prepared from both nucleus and cytoplasm were pUp and homogeneous irrespective of labeling periods. The 5′ terminus of 5.8S RNA consisted of approximately equal moles of pCp and pGp, which, however, did not change with the labeling periods. On the contrary, the 5′ terminus of 28S RNA showed various degrees of heterogeneity depending upon the labeling periods. Not only the percentage of pCp, mature terminus, increased with the labeling period, but a "chase" with cold phosphate made the 5′ terminus of this RNA almost homogeneously pCp. These results indicate that a unique 5′-terminal processing is taking place on newly made 28S RNA in the cytoplasm. Neither size difference by agarose–acrylamide gel electrophoresis nor sequence difference in RNase T_1 fingerprints by homochromatography was detected between these molecules, suggesting that the processed sequence of the 5′ terminus was relatively short. Both nucleolar and ribosomal 28S RNAs had the same and homogeneous 3′ terminus, Uoh. 5′-Terminally immature 28S RNAs were also found in the cytoplasmic polyribosomes, indicating that they were functionally active in protein biosynthesis.

The processing of ribosomal RNA has been extensively studied in various eukaryotes and a common pathway of major cleavage established (see Perry, 1976; Hadjiolov & Nikolaev, 1977).

In mammalian cells, the presumptive primary transcript, 45S RNA, is cleaved at four sites, sequentially producing lower molecular weight precursors and transcribed spacers which are eventually degraded. Depending upon the temporal order of the second and the third cleavage site, the precursor may take either one of the following two pathways; i.e.,

$$\begin{array}{l} 45S \longrightarrow 41S \longrightarrow 32S \longrightarrow 28S \\ \qquad\qquad\quad \searrow 20S \longrightarrow 18S \end{array} \qquad (1)$$

or

$$\begin{array}{l} 45S \longrightarrow 41S \longrightarrow 36S \longrightarrow 32S \longrightarrow 28S \\ \qquad\qquad\quad \searrow 18S \end{array} \qquad (2)$$

It has been demonstrated that in Hela cells pathway 1 is the major one, whereas in L cells pathway 2 is more predominant (Weinberg and Penman, 1970; Wellauer et al., 1974).

Apart from these major cleavages, little is known about the possible trimming processes at both ends of the precursors. Considering the sequential occurrence of cleavage and trimming in the processing of transfer RNA precursors (Shimura and Sakano, 1977), the possibility may exist that some trimming processes are involved in the formation of ribosomal RNA. Mere size analysis on gels or under the electron microscope is not sufficient to answer this question, but structural analysis near both termini of these molecules is essential. We have previously found that the presumptive primary transcript, 45S RNA, is not a homogeneous molecular species but rather a collection of larger precursor molecules with heterogeneous 5′ termini, and we concluded that a certain trimming process was taking place on 45S RNA molecules before they are cleaved into 41S RNA (Kominami & Muramatsu, 1977). Nazar (1977) recently reported similar findings.

We have now examined the 5′ terminus of 28S RNA of mouse hepatoma cells under different labeling conditions and found that it is again heterogeneous. Kinetic analysis indicates that newly made 28S RNA must be subjected to a trimming process at the 5′-terminal region until the mature 5′ terminus, pCpGp, is exposed. Furthermore, this processing takes place in the cytoplasm as well as in the nucleus, probably even in the polysomes.

Materials and Methods

Cell and Labeling Conditions. Mouse ascites hepatoma cell lines, MH134/C and MH134/M, were maintained in C3H/He mice by transplantation into the abdominal cavity (Hashimoto et al., 1975). MH134/C and MH134/M cells both had different karyotypes and different ribosomal 28S RNA sequences, even though they are derived from one original strain, MH134 (manuscript in preparation). MH134/C-c and MH134/M-c were culture cell lines derived from MH134/C and MH134/M, respectively.

The labeling of the ascites cells with [^{32}P]orthophosphate was performed in a siliconized flask. The cells were incubated at 37 °C with gentle shaking at a concentration of 3×10^8 cells/80 mL in phosphate-free Eagle's minimal essential medium (MEM) containing 10% dialyzed calf serum.

The culture cells were labeled in Roux bottles with [^{32}P]-orthophosphate at a concentration of 1×10^8 cells/40 mL in phosphate-free Eagle's MEM containing 10% dialyzed calf serum. The labeling schemes are described in the legend to Figure 1.

Preparation of Ribosomal and Nuclear RNAs. The cells were collected by a low-speed centrifugation, washed with RSB (10 mM Tris-HCl,[1] pH 7.6, 0.01 M NaCl, 1.5 mM $MgCl_2$) and resuspended in the same buffer for 10 min at 0 °C. Then Nonidet P40 (Shell Chemical Co.) and deoxycholate were

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[1] Abbreviations used are: DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

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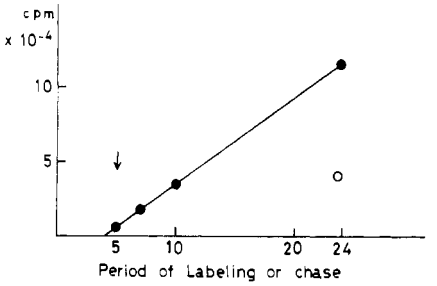

FIGURE 1: Labeling of stable RNA and the effect of "chase". MH-134/C-c cells were propagated in Eagle's medium supplemented with 10% calf serum in Roux bottles. The cells were collected by centrifugation and resuspended in phosphate-free Eagle's medium containing 10 μCi of [^{32}P]orthophosphate at a concentration of about 1 × 10^8 cells/40 mL. The culture was divided into two or four equal parts. Each subculture was then labeled for 5, 7, 10, or 24 h. The chase was performed with a final concentration of 2.5 mM phosphate after 5 h of labeling. The cells were harvested at specified periods and RNA was extracted by the NaDodSO$_4$–cold phenol method from the postmitochondrial supernatant. Aliquots were precipitated by 7% Cl_3AcOH and counted in toluene-based scintillator: (●) pulse labeling; (○) 5-h pulse and then 19-h chase.

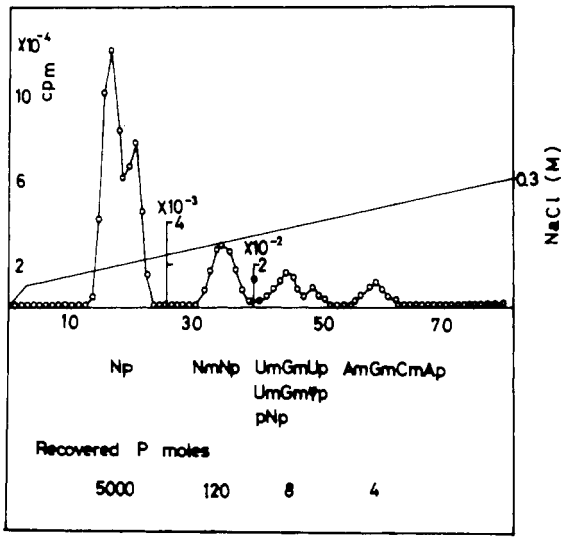

FIGURE 2: Chromatography of the alkaline hydrolysate of uniformly labeled 28S RNA. 28S RNA was hydrolyzed with 0.3 N NaOH for 18 h at 37 °C. The hydrolysate was neutralized with 1 N HCl, diluted with 10 volumes of 7 M urea, 0.05 M Tris-HCl, pH 7.6, and applied onto a column (0.5 × 20 cm) of DEAE-Sephadex and eluted with an 80-mL gradient of 0.05 to 0.30 M NaCl in 7 M urea and 0.05 M Tris-HCl, pH 7.6. One-milliliter fractions were collected every 10 min. No significant radioactivity was eluted with 1 M NaCl.

added at final concentrations of 0.3 and 0.2–0.3%, respectively, and homogenization was carried out in a Potter-Elvehjem homogenizer, giving seven to ten strokes. The homogenate was centrifuged at 12 000g for 15 min. Ribosomal RNA was prepared from the supernatant by the sodium dodecyl sulfate method. The pellet fraction containing nuclei and mitochondria was suspended in 0.25 M sucrose and 3.3 mM $CaCl_2$, layered on the top of an equal volume of 0.88 M sucrose, and centrifuged at 2500 rpm for 15 min to yield purified nuclei (Muramatsu et al., 1974). Nuclear RNA was extracted by the sodium dodecyl sulfate–hot phenol method as described previously (Muramatsu et al., 1970). The 18S and 28S RNAs were separated by sucrose density gradient centrifugation or, in some cases, by 1.5% agarose gel electrophoresis. Heat treatment of these RNAs was performed as described previously (King and Gould, 1970; Sakuma et al., 1976), and 5.8S RNA was recovered from low-molecular-weight fractions by 10% acrylamide gel electrophoresis.

Polysomes were prepared essentially as described by Taylor and Schimke (1973), and RNA was obtained as described above.

Determination of the 5′-Terminal Nucleotides. Each RNA was hydrolyzed with 0.3 M NaOH at 37 °C for 18 h. DEAE-Sephadex column chromatography was performed as described previously (Hashimoto et al., 1975; Sakuma et al., 1976).

The third peak containing nucleoside 5′–3′(2′)-diphosphates derived from the 5′-terminal nucleotide was pooled, desalted by a small column of DEAE-Sephadex, and analyzed by means of two-dimensional thin-layer chromatography on an Avicel SF cellulose plate (Nishimura, 1972; Hashimoto et al., 1975; Kominami and Muramatsu, 1977). In brief, desalted samples were spotted on a corner of a 10 × 10 cm Avicel SF cellulose plate and developed with isobutyric acid–6 N ammonia (5:3) for the first dimension and with a propanol–concentrated HCl–water (70:15:15) for the second dimension, respectively. After autoradiography, pNp spots were eluted and counted.

Determination of the 3′-Terminal Nucleosides. The method of RajBhandary (1968) for the labeling of the 2′,3′-terminal glycol group of an RNA chain was followed with minor modifications (Hamada et al., in preparation). The ^{3}H-labeled RNA was repurified by Sephadex G-50 gel filtration followed by 1.5% agarose gel electrophoresis. The RNase T_2 digest of the [^{3}H]RNA was chromatographed on an Avicel SF cellulose thin-layer plate together with four kinds of nucleoside trialcohols as markers (Furuichi & Miura, 1972).

Homochromatography. RNase T_1 digestion and the two-dimensional fractionation of oligonucleotides were carried out essentially according to Brownlee & Sanger (1969). 28S RNA was digested by RNase T_1 (Sankyo Pharmaceutical Co., Tokyo) with an enzyme to substrate ratio of 1:20 in 10 μL of a buffer containing 0.01 M Tris-HCl, pH 7.6, and 1 mM EDTA at 37 °C for 1 h. A strip (70 × 2.5 cm) of cellulose acetate was used for the first dimension. Electrophoresis in the first dimension was performed in 5% acetic acid in 7 M urea, pH 3.5, for 1 h at 3 kV. Oligonucleotides were transferred to a DEAE-cellulose plate (Polygram cell 300 DEAE, 20 × 40 cm) by the blotting procedure. Homomixture was prepared by a controlled alkali digestion of 3% yeast RNA (Miles Laboratories, Inc.). Homochromatography was carried out for 16 h at 60 °C. The plate was dried, covered with a sheet of Saran Wrap, and exposed onto a Fuji X-ray film Rx.

Results

Kinetics of Labeling of Cytoplasmic RNA with [^{32}P]Orthophosphate and the Effect of a "Chase" with Cold Phosphate. First, kinetics of labeling of cytoplasmic RNA were studied in a parallel culture. The MH134/C-c cells were labeled with [^{32}P]orthophosphate for various time periods. The RNA was extracted from the postmitochondrial supernatant. Figure 1 shows that the incorporation of [^{32}P]orthophosphate was almost linear from 4 h through 24 h, although it took approximately 4 h before a significant amount of labeled RNA appeared in the cytoplasm. This suggests that the cells and the labeling conditions did not change appreciably at least for these periods.

The effect of a chase with cold phosphate on the labeling of cytoplasmic RNA was studied in this system. Cold phosphate was added to the culture bottle at a concentration of 2.5 mM (the same concentration as the standard MEM) after labeling for 5 h, and the labeled RNA was chased for 19 h. Figure 1 shows that the labeling of cytoplasmic RNA decreased to approximately 35% of the control by a chase with cold phosphate, indicating that the chase was in fact effective, though not complete. It may be calculated that, if the chase was complete,

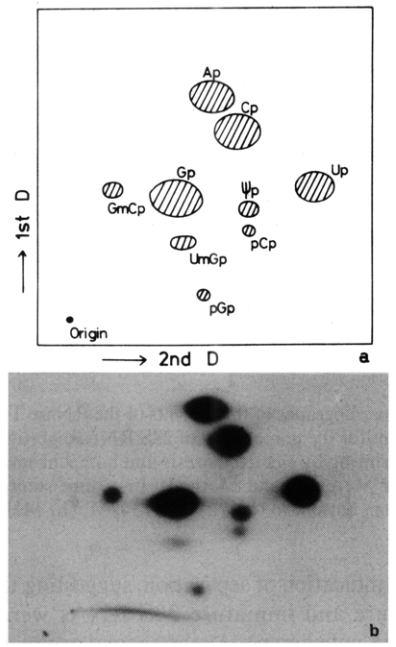

FIGURE 3: Autoradiograph of the two-dimensional thin-layer chromatography of RNase T_2 digest of 5.8S RNA. 5.8S RNA was isolated from heat-quenched 28S RNA of MH134/C by sucrose density gradient centrifugation followed by gel electrophoresis. The RNase T_2 digest was directly applied onto an Avicel SF thin-layer plate (20 × 20 cm) and developed as described under Materials and Methods. The position of Ψp and pCp was reversed in this experiment than other experiments with 10 × 10 plates shown in Figure 4 for some unknown reasons.

the count appearing in the cytoplasm at 24 h would have been 2.9×10^6 cpm.[2] Since the actual radioactivity which appeared in the cytoplasm was 4.1×10^6 cpm, the effect of the chase was calculated to be 70%, although this was only an approximation.

Determination of the 5′ Termini of Ribosomal RNAs. When the alkaline hydrolysate of ribosomal 28S RNA was chromatographed on a DEAE-Sephadex A-25 column at pH 7.6, the elution profile shown in Figure 2 was obtained; this system is designed to separate oligonucleotides on the basis of chain length (Bell et al., 1963). Nucleoside 5′,3′(2′)-diphosphates originated from the 5′ terminus of 28S RNA are eluted in the trinucleotide peak (Hashimoto et al., 1975). The trinucleotide fraction was previously described to contain 1 mol of nucleoside 5′,3′(2′)-diphosphate (pNp) and 2 mol of 2′-O-methylated trinucleotide (UmGmU and UmGmΨp) (Hashimoto et al., 1975; Kominami & Muramatsu, 1977).

The alkaline hydrolysate of 18S RNA was also chromatographed in the same manner. pNp derived from the 5′-terminus of 18S RNA was detected in the trinucleotide peak (data not shown) and determined in the same way as that of 28S RNA.

5′-Terminal nucleoside diphosphates were separated and identified by the two-dimensional thin-layer chromatography as described under Materials and Methods. As shown in the diagram, nucleoside 3′-phosphates, nucleoside diphosphates, and trinucleotides are well separated and identified.

Since 28S RNA has two 2′-O-methylated trinucleotides and the recovery of these trinucleotides was high and reproducible, it could be used as a reliable internal standard for the recovery of the 5′ terminus of 28S RNA.

Homogeneity of the 5′ Terminus of 18S RNA and the

[2] 3.5×10^6 (labeling at 10 h) − 0.6×10^6 cpm (labeling at 5 h) = 2.9×10^6 cpm.

TABLE I: 5′-Termini of 5.8S RNA of MH134 Cells.

	mol/5.8 S RNA		
cell type:	MH134/M	MH134/C-c	MH134/C- c
labeling time (h):	20	5	20
pCp	0.4	0.4	0.5
pGp	0.5	0.5	0.6
UmG	0.5	0.3	0.7
GmC	1.0	0.9	1.0
A	27	31	30
C	44	43	43
U	31	37	35
G	50	42	42
Ψ	1.7	1.7	1.9

Heterogeneity of 28S RNA. The 5′ terminus of 5.8S RNA was determined by directly applying the RNase T_2 digest onto a large Avicel SF thin-layer plate (20 × 20 cm), since the interference by mononucleotides was relatively small for this low-molecular-weight RNA (Figure 3). The results summarized in Table I indicate that, in agreement with the previous reports (Nazar et al., 1974), these molecules consist of two species of almost equal numbers with pCp and pGp at the 5′ termini, respectively. Incidentally, the presence of 2 mol of Ψ, 1 mol of GmC, and ~0.5 mol of UmG was also confirmed with this technique. It should be pointed out here that both the species (pCp and pGp) and their ratio (pCp/pGp) did not change with labeling periods (5 and 20 h) and with different cell types (ascites type M and culture type C).

The 5′-terminal nucleotide of 18S RNA prepared from either the nuclei or the cytoplasm was invariably pUp and homogeneous for both MH134/C and MH134/M cells (Figure 4). The 5′-terminal nucleotide of ribosomal 18S RNA from either MH134/C-c or MH134/M-c cells was also pUp and homogeneous, regardless of the labeling period (data not shown).

On the contrary, the 5′ termini of 28S RNA from both MH134/C and MH134/M showed varying degrees of heterogeneity depending upon the length of labeling (Figure 4c–e, Table II). As seen in Table II, the 5′ terminus of 28S RNA extracted from the nucleus of MH134/M cells after 5 h of labeling was very heterogeneous, and no pCp was detected, which was the main 5′ terminus of the mature 28S RNA (Hashimoto et al., 1975; Sakuma et al., 1976). This was also true for MH134/C cells (data not shown). It was striking that the 5′ terminus of 28S RNA prepared from cytoplasm was also heterogeneous at such a short labeling period as 5 h, although about 60% of the 5′ terminus was already converted to pCp (Figure 4c). At 20 h, the percentage of pCp in the 5′ terminus increased to 83%, indicating some maturation process was under way in the cytoplasm during this period (Figure 4d). In the case of culture cells, the rate of processing appeared apparently slower. The percentage of pCp did not increase by a continuous labeling with $^{32}PO_4$ until 20 h (Table II), probably because of the continuous influx of newly labeled 28S RNA. It is also possible that 5′-terminal processing of 28S RNA was impaired by the phosphate starvation, as will be discussed later. However, a chase with cold phosphate for 19 h after a 5-h labeling with [^{32}P]orthophosphate converted 70–90% of the 5′-terminus into pCp (Table II), indicating that maturation at the 5′ end of the 28S RNA was taking place also in these cultured cells.

Estimation of the Extent of 5′-Terminal Processing. As an attempt to estimate how many nucleotides are involved in this

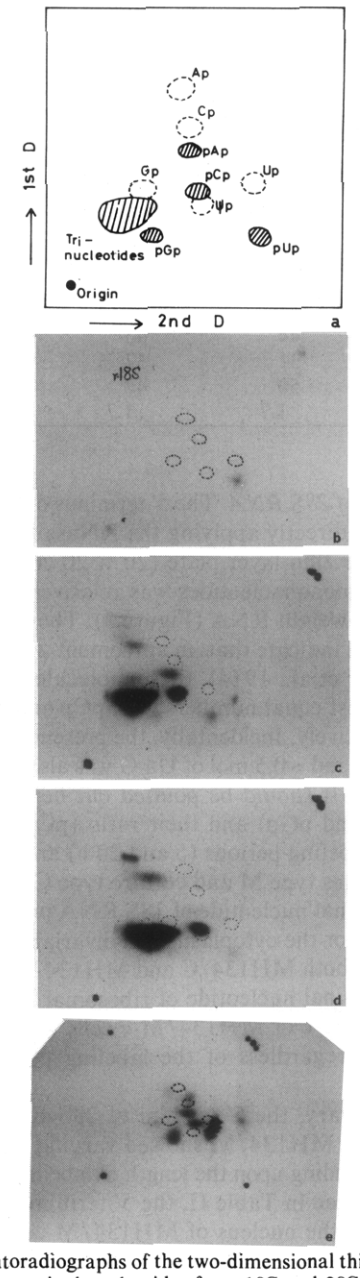

FIGURE 4: Autoradiographs of the two-dimensional thin-layer chromatography of 5′-terminal nucleotides from 18S and 28S RNA. The ^{32}P-labeled, alkali-stable "trinucleotide" fraction eluted from the DEAE-Sephadex column (Figure 2) was desalted on a column (0.5 × 1.5 cm) of DEAE-Sepahdex, eluted with 2 M triethylamine bicarbonate, dried, dissolved in water, and applied onto an Avicel SF cellulose plate (10 × 10 cm) with 0.05 A_{260} unit each of Ap, Cp, Up, Ψp, and Gp as the markers. The solvent systems are described under Materials and Methods. Dotted circles show nucleotide markers detected by an ultraviolet lamp: (a) diagram, (b) 18S RNA, (c) ribosomal 28S RNA of MH134/C, labeled for 5 h, (d) ribosomal 28S RNA of MH134/C, labeled for 20 h, (e) ribosomal 28S RNA from the polysomes of MH134/C-c, labeled for 10 h. For b through d, −4 charge fractions from DEAE-Sephadex column were all collected, including 2′-O-methylated trinucleotides, whereas for e only the latter peak containing pNp was collected (Figure 2). Therefore, no radioactive spots are seen on the area of trinucleotide in this chromatogram.

5′-terminal processing of 28S RNA, we have first compared the size of "mature" and "immature" 28S RNA on an agarose–acrylamide composite gel (Peacock and Dingman, 1968).

Two 28S RNAs, one labeled for 20 h with [^{32}P]orthophosphate and the other for 2 h with [^{3}H]uridine, were comigrated for a prolonged period (15 h), until they moved up to 4 cm.

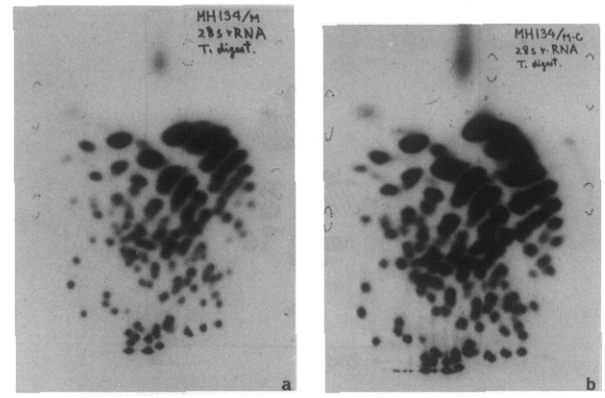

FIGURE 5: Autoradiographs of fingerprints of the RNase T_1 digest of 28S RNA. Conditions for the preparation of 28S RNA, digestion with RNase T_1, and fingerprinting by electrophoresis and homochromatography are described under Materials and Methods: first dimension, right to left; second dimension, bottom to top; (a) MH134/M; (b) MH134/M-c.

There was no indication of separation, suggesting that the sizes of these mature and immature 28S RNAs were not much different (data not shown). As the second approach, we have compared the RNase T_1 fingerprints of 28S RNA derived from MH134/M and MH134/M-c that were labeled for 20 h. Since there was a considerable difference in the 5′ termini between these cells under these labeling conditions, some extra spots might be detected if the 5′-terminal processing involved relatively long sequences. However, as shown in Figure 5, no extra spots were detected in the pattern of MH134/M-c as compared with MH134/M, supporting the above conclusion that the trimming at the 5′ terminus of 28S RNA was relatively short.

Homogeneity of the 3′ Terminus of 28S RNA. In order to examine the possible processing at the 3′ end of 28S RNA during the course of maturation at the 5′ end, we have determined the 3′-terminal nucleoside of 28S RNA with the method of periodate oxidation followed by [^{3}H]borohydride reduction. After digestion with RNase T_2, the products were identified by thin-layer chromatography as described under Materials and Methods. As shown in Figure 6, the 3′ terminus of 28S RNA isolated from either nucleoli or cytoplasmic ribosomes was always Uoh and almost homogeneous. These data appear to indicate that there is no concomitant 3′-terminal processing during the course of 5′-terminal processing on newly made 28S RNA.

The Presence of 5′-Terminally Heterogeneous 28S RNA in the Polysomes. It is interesting to know whether these presumptive immature 28S RNAs with different 5′ termini are functionally active. To test this, we have isolated polyribosomes from MH134/C-c cells after 10 h of labeling with [^{32}P]orthophosphate (Figure 7), and the 5′-terminus of the labeled 28S RNA was analyzed as described above. As shown in Figure 4e and Table II, the 5′ terminus was again heterogeneous, the highest percentage being found in pGp. In another experiment where the yield of polysomes was rather low, we also found heterogeneity in the 5′ terminus of 28S RNA, with the highest percentage in pGp. These findings clearly indicate that the immature 28S RNA with respect to the 5′ end is actively engaged in protein biosynthesis in vivo.

Discussion

The heterogeneity of the 5′ terminus of ribosomal 28S RNA is rather unexpected and deserves further consideration in the context of ribosomal RNA maturation.

The possibility of artifacts due to contamination of other

TABLE II: 5′-Termini of 28S RNA of MH134 Cells.

cell type	cell fract	labeling time (h)	% of total 5′ end				Yield (mol/28S RNA)
			pCp	pUp	pAp	pGp	
MH134/C	nuclei	5	0	47	24	29	0.6
	cytoplasm	5	62	6	17	15	0.8
	cytoplasm	5	63	12	18	6	0.9
	cytoplasm	5	57	19	15	9	0.4
	cytoplasm	20	83	6	6	5	0.6
MH134/C-c	cytoplasm	20	36	19	25	19	1.0
	cytoplasm	20	19	39	0	42	0.5
	polysomes	10	22	24	18	36	0.7
	cytoplasm	5 → 20	92	8	0	0	1.0
MH134/M	nuclei	5	0	24	6	70	0.8
	cytoplasm	20	79	8	8	5	0.5
MH134/M-c	cytoplasm	20	48	0	52	0	1.0
	cytoplasm	20	26	20	55	0	0.5
	cytoplasm	5 → 20	73	9	7	10	0.6

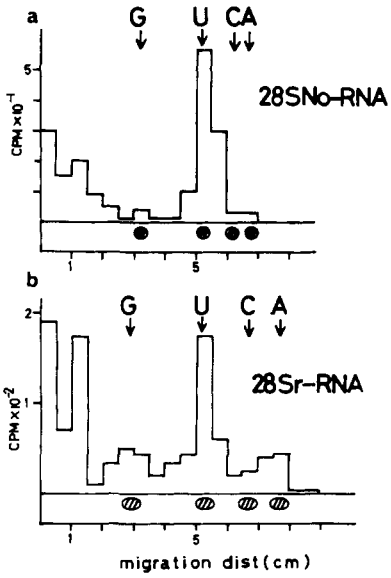

FIGURE 6: Thin-layer chromatography of the RNase T_2 digest of 3′-terminally labeled 28S RNA. The labeled trialcohols were separated on Avicel SF cellulose plates with a solvent system of *tert*-butyl alcohol/methyl ethyl ketone/water/concentrated ammonium hydroxide (8:6:3:2, v/v). Strips of adsorbent, 5-mm wide, were serially removed from the glass plate and nucleoside derivatives extracted in 0.2 mL of distilled water. The radioactivity was counted in 10 mL of Bray's scintillator: (a) nucleolar 28S RNA; (b) ribosomal 28S RNA. The relatively high radioactivity at the origin was due to a small amount of contaminating polysaccharide.

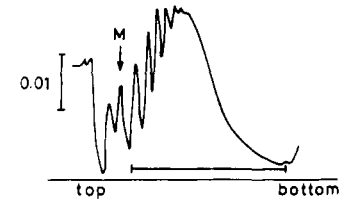

FIGURE 7: A sucrose density gradient profile of polysome from MH134/C-c cells. The cells were collected by centrifugation, washed once with ice-cold hypotonic 10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, containing heparin (500 μg/mL), resuspended in 10 volumes of the same buffer, and allowed to swell for 10 to 15 min. The cell suspension was then brought to room temperature, and 10% NP-40 was added to a final concentration of 1.5%. The suspension was rapidly mixed and gently swirled. The cells were examined by microscopy, and the suspension was chilled after 90 to 95% of the cells was lysed. The suspension was centrifuged at 10 000 rpm for 15 min, and the supernatant was centrifuged on a discontinuous sucrose density gradient of 0.5 (1 mL), 1.0 (5 mL) and 2.5 M (2.5 mL) in TKMH buffer (50 mM Tris-HCl, pH 7.6, 25 mM KCl, 5 mM $MgCl_2$, 500 μg/mL heparin) at 26 000 rpm for 5 h using a SW 27.1 rotor. The polysomes, which formed an opalescent band at the 2.5 M interface, were pooled and dialyzed overnight against TKMH buffer on ice. Subsequently, the polysome was applied on a 0.5 to 1.5 M linear sucrose density gradient in TKMH buffer and centrifuged at 26 000 rpm in a SW 27.1 rotor for 180 min. The optical density profile was monitored by an ISCO Model UA-4 fractionator and the polysome fraction, indicated by a bar, was pooled for the extraction of RNA. M designates the position of the monosomes.

RNA molecules or intramolecular nicks could be ruled out because of the following reasons.

In the first place, the 28S RNA used in these experiments has been heat quenched to remove 5.8S RNA hydrogen bonded to it. This treatment must have removed most of the possibly entangled low-molecular-weight RNAs and also the nicked 28S RNA molecules into small fragment regions lower than 28S.

Second, and most important, the recovery of the 5′-terminal nucleotides (pNp) added up close to one. Nicks or contamination of other low-molecular-weight RNAs would have increased the total recovery of pNp.

Third, although shorter or continuous labelings revealed heterogeneity of the 5′ terminus of 28S RNA, longer labelings or especially "chase" experiments almost abolished the heterogeneity, making pCp the most predominant 5′ terminus. These kinetics may only be explained by the gradual processing of 28S RNA at the 5′ terminus to form the final pCp terminus.

Fourth, the uniqueness of the 5′ terminus of 18S RNA and the invariability of those of 5.8S RNA studied under the same conditions and techniques may attest to the reality of the heterogeneity of the 5′ terminus of 28S RNA.

All considered, it may be concluded that the 28S RNA which is newly cut out from 32S precursor has an extra sequence at the 5′ terminus, which is trimmed during the course of maturation in the cytoplasmic ribosomes as well as in the nucleolus to form mature 28S RNA having a pCpGp structure at the 5′ end (Sakuma et al., 1976). It is interesting that immature 28S RNAs with different 5′ termini appear to be functional in protein synthesis, since they are in fact found in the cytoplasmic polyribosomes. A logical conclusion may be that short extra sequences at the 5′ terminus of 28S RNA would not affect the ribosome function. It should be mentioned in this connection that Mangiarotti et al. (1974) have shown

that immature 16S RNA is present in active 30S subunits in *Escherichia coli*.

Although the present experiments have been done with mouse hepatoma cell lines, we would suspect that this phenomenon of 5′-terminal processing would probably be ubiquitous in eukaryotic cells considering the fundamental nature of this sort of reaction. One possibility which cannot be ruled out is that, in these experiments, the persistence of immature 5′ termini may have been exaggerated by some metabolic disturbances caused by the phosphate starvation which must have occurred under the present labeling conditions, especially with a longer labeling period, although there is no evidence that phosphate starvation does inhibit the 5′-terminal processing of 28S RNA.

We have previously shown that mouse 45S RNA has heterogeneous 5′ termini and concluded that some trimming mechanisms might be operating before the first major cleavage took place (Kominami & Muramatsu, 1977). We also have evidence that the 3′ terminus of 45S RNA is altered as it is processed into 28S RNA (Hamada et al., in preparation). In view of these findings, we would like to point out the general presence of trimming processes, besides the major cleavages, during the course of ribosomal RNA formation. Their possible significance in the regulation of the processing itself or of the ribosome function awaits further studies.

References

Bell, D., Tomlinson, R. V., and Tener, G. M. (1963), *Biochem. Biophys. Res. Commun. 10,* 304.

Brownlee, G. G., and Sanger, F. (1969), *Eur. J. Biochem. 11,* 395.

Furuichi, Y., and Miura, K. (1972), *J. Mol. Biol. 64,* 619.

Hadjiolov, A. A., and Nikolaev, N. (1976), *Prog. Biophys. Mol. Biol. 31,* 95.

Hashimoto, S., Sakai, M., and Muramatsu, M. (1975), *Biochemistry 14,* 1956.

King, H. W., and Gould, H. (1970), *J. Mol. Biol. 51,* 687.

Kominami, R., and Muramatsu, M. (1977), *Nucleic Acids Res. 4,* 229.

Mangiarotti, G., Turco, E., Ponzetto, A., and Altruda, F. (1974), *Nature (London) 247,* 147.

Muramatsu, M., Hayashi, Y., Onishi, T., Sakai, M., Takai, K., and Kashiyama, T. (1974), *Exp. Cell Res. 88,* 345.

Muramatsu, M., Shimada, N., and Higashinakagawa, T. (1970), *J. Mol. Biol. 53,* 91.

Nazar, R. N. (1977), *Biochemistry 16,* 3215.

Nazar, R. N., Sitz, T. O, and Busch, H. (1974), *FEBS Lett. 45,* 206.

Nishimura, S. (1972), *Prog. Nucleic Acid Res. Mol. Biol. 12,* 49.

Peacock, A. C., and Dingman, C. W. (1968), *Biochemistry 7,* 668.

Perry, R. P. (1976), *Annu. Rev. Biochem. 45,* 605.

RajBhandary, U. L. (1968), *J. Biol. Chem. 243,* 556.

Sakuma, K., Kominami, R., Muramatsu, M., and Sugiura, M. (1976), *Eur. J. Biochem. 63,* 339.

Shimura, Y., and Sakano, H. (1977), Nucleic Acid-Protein Recognition, Vogel, H. J., Ed., Academic Press, New York, N.Y., p 293.

Taylor, J. M., and Schimke, R. T. (1973), *J. Biol. Chem. 248,* 7661.

Weinberg, R. A., and Penman, S. (1970), *J. Mol. Biol. 47,* 169.

Wellauer, P. K., Dawid, I. G., Kelly, D. E., and Perry, R. P. (1974), *J. Mol. Biol. 89,* 379.