# 2′-O-Methylated Oligonucleotides in Ribosomal 18S and 28S RNA of a Mouse Hepatoma, MH 134†

S. Hashimoto, M. Sakai, and M. Muramatsu*

ABSTRACT: Simple two-dimensional thin-layer chromatography was found to be useful for the separation of sugar methylated dinucleotides in RNA. Of the 16 possible sequences of the type Nm-Np, 15 were separated and all the sequences were determined. In a mouse hepatoma, MH 134, the levels of the sugar methylation in the 18S and 28S RNA molecules were 17–18 and 11–12 per 1000 nucleotides, respectively. Thus, 18S RNA contained approximately 35 2′-O-methylated dinucleotides and 28S RNA approximately 60 2′-O-methylated dinucleotides. The pattern of distribution was also distinct between these two molecules. Two 2′-O-methylated trinucleotides were identified in the 28S RNA with the sequences Um-Gm-Up and Um-Gm-Ψp. A unique 2′-O-methylated tetranucleotide was present also in the 28S RNA, the sequence of which was Am-Gm-Cm-Ap. The 5′-terminal nucleotides of both 18S and 28S RNA were obtained as nucleoside 3′,5′-diphosphates (pNp) in the trinucleotide fraction of the RNase $T_2$ digest. The 5′-termini of 18S and 28S RNA were pUp and pCp, respectively, and found to be almost homogeneous.

The methylation of ribosomal RNA has been a subject of considerable interest from the viewpoint of structure, function, and evolution of this RNA (for a review, see Attardi and Amaldi, 1970). It is known that ribosomal RNAs (rRNA) from eukaryotic cells contain a few percent of methylated nucleosides, some 80–90% of which represents the sugar methylation at the 2′-OH position of the ribose (Singh and Lane, 1964b; Brown and Attardi, 1965; Wagner et al., 1967; Vauhgan et al., 1967; Lane and Tamaoki, 1969). When the RNA is hydrolyzed with either alkali or RNase $T_2$ (EC 3.1.4.23), the phosphodiester linkages adjacent to 2′-O-methylated ribose remain uncleaved and a series of dinucleotides of the type Nm-Np is obtained. These dinucleotides have been analyzed with the aid of either two-dimensional paper chromatography (Singh and Lane, 1964a,b), electrophoresis (Klootwijk and Planta, 1973), or combined electrophoresis and chromatography (Wagner et al., 1967). However, in addition to the fact that two or three dinucleotides could not be separated by those procedures, they require relatively large amounts of the sample for the purpose of quantitation of each nucleotide.

In the course of the study of the primary structure of ribosomal RNA of animal cells, we have found that a two-dimensional thin-layer procedure which has been used for the analysis of various nucleotides (Nishimura, 1972) could separate all but one 2′-O-methylated dinucleotide in RNA. It requires only very small amounts of material and is especially useful for highly labeled RNA that is used for Sanger's fingerprinting. We have used this technique to establish the pattern of 2′-O-methylation in ribosomal RNA of a mouse hepatoma, MH 134. In this paper, we describe the details of the method and the kind and frequency of the 2′-O-methylated dinucleotides that are separated. In addition, the 5′-terminal nucleotide of each ribosomal RNA species, two 2′-O-methylated trinucleotides, and a unique 2′-O-methylated tetranucleotide found in the 28S RNA will be described.

## Materials and Methods

*Cells and Labeling of RNA.* The maintenance of the mouse hepatoma MH 134 cells and their labeling in vivo with $^{32}P$ were described in a previous paper (Hashimoto and Muramatsu, 1973).

For in vitro labeling with [$^{32}P$]orthophosphate, the cells were harvested on the 12th day after inoculation and incubated in vitro at a concentration of 1 g/80 ml in phosphate-free Eagle's minimal essential medium (MEM), with 10% calf serum, to which 5–10 mCi of carrier-free [$^{32}P$]orthophosphate was added.

For labeling with [*methyl*-$^3H$]methionine, MH 134 cells were harvested and cultured in methionine-free MEM medium containing 10% calf serum and 2.0 mCi of [*methyl*-$^3H$]methionine. To suppress the labeling of the purine ring (Tamaoki and Lane, 1968; Maden et al., 1972), $2 \times 10^{-5}$ *M* adenosine, $2 \times 10^{-5}$ *M* guanosine, and $1 \times 10^{-2}$ *M* sodium formate were added. The incorporation of radioactivity into the purine ring was almost completely suppressed with this procedure as indicated by the counts found in purine nucleotides after hydrolysis with RNase $T_2$.

For the double labeling with [$^{32}P$]orthophosphate and [*methyl*-$^3H$]methionine, total doses of 20 mCi/mouse of [$^{32}P$]orthophosphate and 1.6 mCi/mouse of [*methyl*-$^3H$]methionine (9 Ci/mmol) dissolved in 0.8 ml of saline were injected intraperitoneally into the mouse which had been transplanted with MH 134 cells, in two injections with an 18-hr interval. Eighteen hours after the second injection, 0.8 mCi/mouse of [*methyl*-$^3H$]methionine in 0.4 ml of saline was further injected. Twelve hours after the third injection, the animals were killed.

*Isolation and Purification of the Two Ribosomal RNA Components.* Ribosomal RNA was extracted and purified from ribosomes essentially as described previously (Hashimoto and Muramatsu, 1973).

For the purpose of determining the recovery of mono- and oligonucleotides as well as for 5′-terminal analysis both the 18S and 28S RNA were further purified by heat

†From the Department of Biochemistry, Tokushima University School of Medicine, Tokushima, Japan. *Received November 4, 1974.* This work was supported in part by grants from the Ministry of Education of Japan and from Chiyoda Life Insurance Fund for Health Development.

quenching followed by recentrifugation on a sucrose gradient (King and Gould, 1970; Hashimoto and Muramatsu, 1973). However, for the analysis of the dinucleotide sequences, these procedures were omitted. The specific activity of purified $^{32}$P-labeled RNA was 5–7 × $10^5$ and 2–5 × $10^6$ cpm per $A_{260}$ unit for in vivo and in vitro labeling, respectively. Specific activities of all four nucleoside 3′-monophosphates obtained by alkaline hydrolysis were almost identical indicating a sufficient equilibrium of $^{32}$P isotope in the RNA under these labeling conditions.

The specific activity of $^3$H-labeled RNA was 1–3 × $10^4$ cpm per $A_{260}$ unit. A sufficiently long labeling period was chosen for in vitro methyl labeling of ribosomal RNA with [*methyl*-$^3$H]methionine. Since all of the 2′-O-methylation of ribosomal RNA takes place at the level of 45S precursor RNA which turns over rapidly (Greenberg and Penman, 1966; Muramatsu and Fujisawa, 1968; Salim and Maden, 1973) the specific activity of each 2′-O-methylated nucleotide must be the same under these labeling conditions.

*Hydrolysis of RNA and Separation of Oligonucleotides.* Ribonuclease $T_2$ or alkaline hydrolysis of rRNA was performed as described previously (Hashimoto and Muramatsu, 1973). Separation of oligonucleotides according to the chain lengths was achieved by a column chromatography essentially as described previously except that DEAE-Sephadex was utilized instead of DEAE-cellulose. The former ion exchanger showed an apparently superior resolution capacity to the latter especially for the separation of 5′-terminal nucleoside diphosphate from the 2′-O-methylated trinucleotides in 28S RNA.

*Two-Dimensional Thin-Layer Chromatography.* Approximately 10,000 to 30,000 cpm of the $^{32}$P-labeled or the $^{32}$P- and methyl-$^3$H-labeled 2′-O-methylated dinucleotide was applied onto an Avicel-SF cellulose glass plate (Funakoshi Co., 20 × 20 cm) together with 0.05 $A_{260}$ unit each of Cp, Ap, Ψp, Up, and Gp as markers. Two or three plates were run in an identical manner. 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 (Nishimura, 1972). The possibility of depurination by the low pH of the second solvent could be ruled out since the base compositions determined by this solvent system were almost identical with those determined by a standard Dowex-1 chromatography in which much dilute acid was used (Hashimoto and Muramatsu, 1973). The spots from the plates identified with the autoradiograph were scraped out and counted by the Čerenkov scintillation or in the toluene scintillator.

*Characterization of Dinucleotides.* Two methods have been utilized to identify the compounds of the two-dimensional thin-layer chromatography. (1) The compounds were treated with snake venom phosphodiesterase (EC 3.1.4.1, Sigma) in order to convert dinucleotides to 2′-*O*-methyl ribonucleoside and mononucleoside diphosphate.

The dinucleotide dissolved in 0.5 ml of 0.05 *M* Tris buffer (pH 8.3) was mixed with about 2 $A_{260}$ units of dinucleotides from pancreatic ribonuclease (EC 3.1.4.22) digest or ribonuclease $U_2$ [ribonucleate purine nucleotide-2′-transferase (cyclizing), kindly supplied by Dr. T. Uchida, Mitsubishi Kasei Institute for Life Sciences] digest of yeast RNA. To the mixture was added 30–50 μg of snake venom phosphodiesterase and incubation was done at 37° for 15 hr. The digest was analyzed on a Dowex-1 column. The 3′ side of the dinucleotide was determined by the mononucleoside diphosphate identified in this way.

(2) The compounds were treated with spleen phosphodiesterase (EC 3.1.4.1, a generous gift from Professor S. Takemura, Nagoya University) to obtain the 2′-*O*-methyl nucleoside 3′-monophosphate that was present at the 5′ side of the dinucleotide as well as the nucleoside 3′-monophosphate that was present at the 3′ side of the dinucleotide.

The dinucleotide (less than 0.02 $A_{260}$ unit) dissolved in 0.5 ml of 0.01 *M* sodium acetate buffer (pH 4.5) was mixed with 0.2–0.5 $A_{260}$ unit each of Cp, Ap, Ψp, Up, and Gp and 1 unit of spleen phosphodiesterase. In the purified enzyme fraction, no activity of ribonuclease or phosphomonoesterase was detected as long as substrates detectable by ultraviolet (uv) absorbance were used (Hashimoto et al., 1972). The mixture was incubated at 37° for 15 hr. The digest was analyzed on a Dowex-1 column.

*Characterization of Tri- and Tetranucleotides.* (i) DIGESTION WITH *Escherichia coli* ALKALINE PHOSPHATASE AND SNAKE VENOM PHOSPHODIESTERASE. Labeled tri- or tetranucleotides (~0.2 μg) were completely digested with 5 μg of *E. coli* alkaline phosphatase (EC 3.1.3.1, Worthington) and 5 μg of snake venom phosphodiesterase in 50 μl of 0.05 *M* Tris-HCl (pH 8.3) at 37° for 16 hr. Resultant 2′-*O*-methyl nucleosides were separated by descending paper chromatography on Whatman No. 3MM paper strips with the following two solvent systems: solvent I, 1-butanol–0.8 *M* boric acid–concentrated HCl (2000:270:8, v/v/v; Al-Arif and Sporn, 1972); solvent II, 2-propanol–concentrated HCl–water (680:176:144, v/v/v; Hall, 1965).

(ii) DIGESTION WITH SNAKE VENOM PHOSPHODIESTERASE AND NUCLEASE $P_1$. $^{32}$P-Labeled 2′-O-methylated tri- or tetranucleotides (approximately 0.2 μg) were mixed with $^3$H-labeled homologous fractions, and completely digested with 5 μg of snake venom phosphodiesterase and 0.1 μg of nuclease $P_1$ (a generous gift of Dr. A. Kuninaka, Yamasa Shoyu Co.) in 50 μl of 0.05 *M* Tris-HCl (pH 8.3) at 37° for 16 hr. Nuclease $P_1$ hydrolyzed exclusively phosphodiester and 3′-phosphomonoester linkages, producing nucleoside 5′-monophosphates (Fujimoto et al., 1969). This enzyme does not cleave 2′-O-methylated phosphodiester linkage at low concentrations but does cleave 3′-phosphomonoester linkages completely. The digests were analyzed on a Dowex-1 column with a mixture of nucleoside 5′-monophosphates (pN) as a marker. The nonabsorbed $^3$H radioactivity peak was further analyzed by paper chromatography as described above. The digests of 2′-O-methylated trinucleotides were also analyzed by thin-layer chromatography as described above.

The tetranucleotide was also partially digested with snake venom phosphodiesterase and nuclease $P_1$ for the purpose of sequence determination. Snake venom phosphodiesterase (0.1–2.0 μg) and 0.1 μg of nuclease $P_1$ were added to $^{32}$P-labeled tetranucleotide (0.2 μg) and digested at 0 or 37° for 5–30 min in 20–50 μl of 0.05 *M* Tris-HCl (pH 8.3). The digests were separated by electrophoresis on DEAE paper at pH 1.9 (7% formic acid) at 30 V/cm for 4 hr. Separated fragments were analyzed by a complete digestion with snake venom phosphodiesterase or spleen phosphodiesterase followed by Dowex-1 column chromatography.

*Determination of 5′-Terminal Nucleotides.* The fractions corresponding to mononucleoside diphosphates on the DEAE-Sephadex column chromatograph were pooled, diluted tenfold with water, and loaded on a Dowex-1 column with marker mononucleoside diphosphates, which had been prepared from yeast RNA in the following manner. Ap-

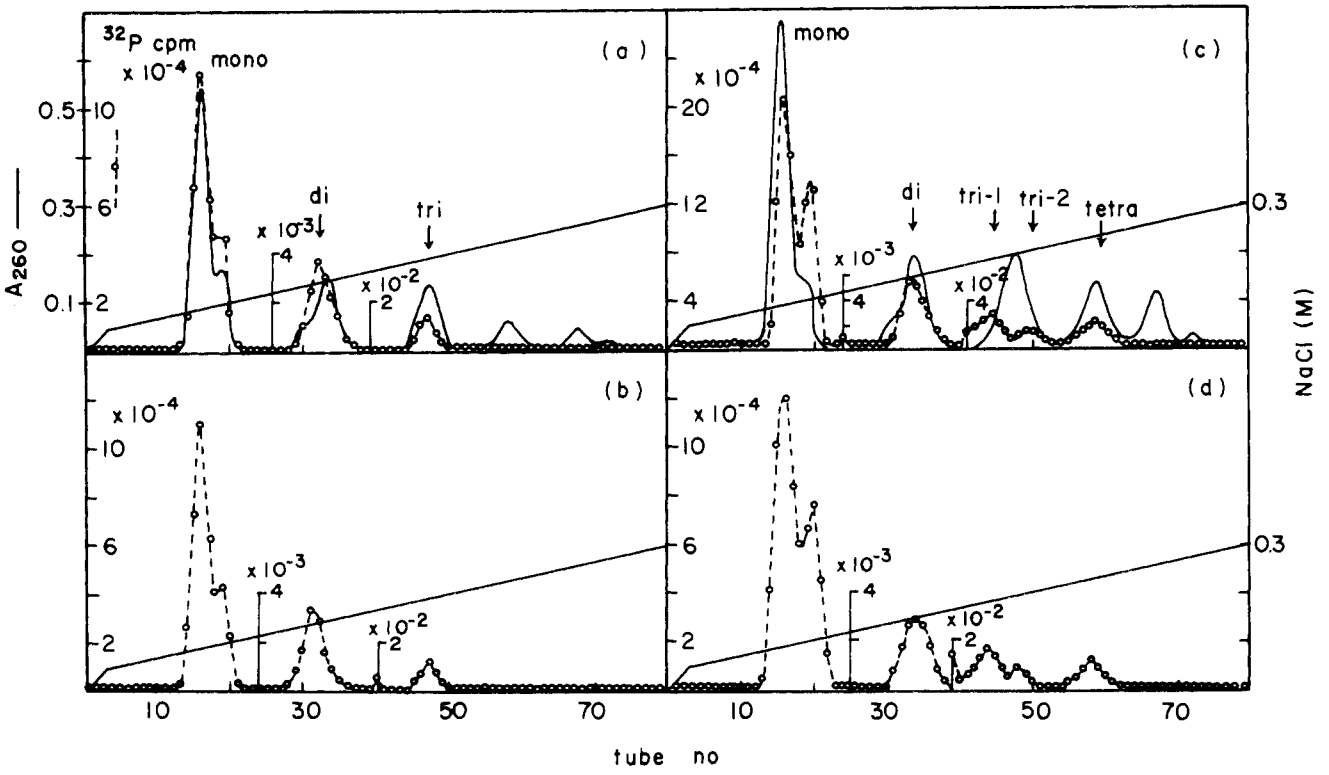


FIGURE 1: DEAE-Sephadex chromatography of alkaline (a,c) or RNase $T_2$ (b,d) hydrolysate of $^{32}$P-labeled 18S (a,b) and 28S (c,d) RNA. Each hydrolysate (0.2–2 mg) was neutralized and diluted with 0.03 *M* Tris-HCl (pH 7.6), containing 7 *M* urea and loaded on a column (0.4 × 20 cm) equilibrated with the same buffer. Elution was performed with a linear gradient of 0.05–0.3 *M* NaCl formed in 0.03 *M* Tris-HCl (pH 7.6) containing 7 *M* urea (60 ml each). Flow rate was adjusted to 6 ml/hr and 1.5-ml fractions were collected. The absorbance at 260 nm was measured and 30 μl of each fraction was counted. The alkaline hydrolysates were chromatographed together with approximately 5 $A_{260}$ units of pancreatic RNase digest of yeast RNA as a marker: (—) absorbance at 260 nm; (O- - -O) radioactivity; (a) alkaline hydrolysate of $^{32}$P-labeled 18S RNA; (b) RNase $T_2$ hydrolysate of $^{32}$P-labeled 18S RNA; (c) alkaline hydrolysate of $^{32}$P-labeled 28S RNA; (d) RNase $T_2$ hydrolysate of $^{32}$P-labeled 28S RNA.

Table I: Distribution of Radioactivity over Mono-, Di-, Tri-, and Tetranucleotides in Alkaline and RNase $T_2$ Hydrolysates of $^{32}$P- or methyl-$^3$H-Labeled rRNA.[a]

| | 18S | | | 28S | | |
|---|---|---|---|---|---|---|
| | $^{32}$P, % | | Methyl-$^3$H, % | $^{32}$P, % | | Methyl-$^3$H, % |
| | Alkaline | RNase $T_2$ | RNase $T_2$ | Alkaline | RNase $T_2$ | RNase $T_2$ |
| Mono | 96.45 | 96.38 | 17.0 | 97.40 | 97.39 | 8.4 |
| Di | 3.44 | 3.52 | 83.0 | 2.32 | 2.38 | 82.0 |
| Tri | 0.11 | 0.10 | | 0.14 | 0.11 | 5.7 |
| Tri-2 | | | | 0.05 | 0.04 | |
| Tetra | | | | 0.09 | 0.08 | 3.9 |

[a] The percentage was calculated on the basis of the total radioactivity recovered from the DEAE-Sephadex columns (Figures 1 and 2). The values are averages of three to five experiments.

proximately 20 mg of the RNA was hydrolyzed with 1 mg of pancreatic ribonuclease and chromatographed on a DEAE-Sephadex column in 7 *M* urea at pH 7.6. Approximately 60 $A_{260}$ units of the dinucleotide fraction was desalted and digested with 1 mg of snake venom phosphodiesterase in 1 ml of 0.05 *M* Tris buffer (pH 8.3). The digest was chromatographed on a Dowex-1 column (0.3 × 30 cm) to obtain pCp and pUp. The pAp and pGp were isolated from the ribonuclease $U_2$ digest of yeast RNA in the same way. In this case, approximately 10 mg of the RNA was digested with 5 units of ribonuclease $U_2$ which may be used as a purine specific ribonuclease at this concentration (Uchida et al., 1970). The resulting dinucleotides, which were mostly Py-Pup, were digested with snake venom phosphodiesterase to obtain pAp and pGp, which were isolated on a Dowex-1 column as described above.

## Results

*Chromatography of Alkaline or RNase $T_2$ Hydrolysates of 18S and 28S RNA.* In Figure 1, DEAE-Sephadex column chromatograms are shown of alkaline or RNase $T_2$ digests of $^{32}$P-labeled 18S and 28S RNA. Those of RNase $T_2$ digests of $^3$H-labeled RNAs are shown in Figure 2. Of the total isotope applied onto the column, over 98% was recovered in the mono-, di-, tri-, and tetranucleotide fractions and no further radioactivity was eluted with 0.5 *M* NaCl. The contents of RNase $T_2$ stable oligonucleotides in $^{32}$P-labeled and $^3$H-labeled 18S and 28S RNA are shown in Table I. Almost identical results were obtained when alkaline hydrolysates were used. Of the total $^{32}$P-labeled oligonucleotides, approximately 96.4 and 97.4% were recovered in the mononucleotide peak of 18S and 28S RNA, respectively. Dinucleotides comprised 3.52 and 2.38% of the total $^{32}$P radioactivity recovered in 18S and 28S RNA, respectively.

The peak corresponding to trinucleotides which was present in the $^{32}$P-labeled 18S RNA was absent in methyl-labeled 18S RNA (Figure 2a) suggesting that this peak from 18S RNA did not contain trinucleotides but was in fact the nucleoside 3′–5′-diphosphate originated from the 5′ terminus.

In the digests of $^{32}$P-labeled 28S RNA, there were two peaks corresponding to trinucleotides (tri-1, tri-2) and one

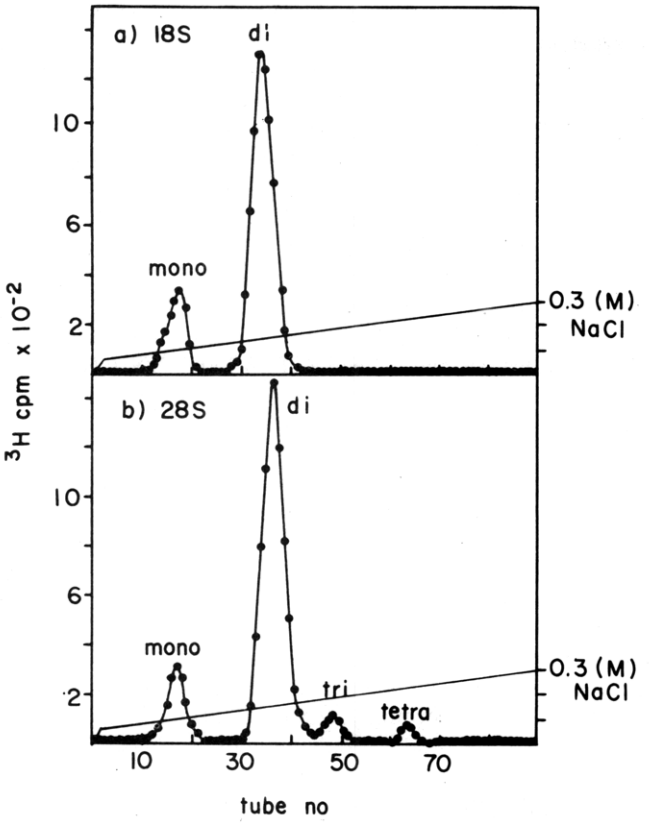


FIGURE 2: DEAE-Sephadex chromatography of RNase $T_2$ hydrolysate of methyl-$^3$H-labeled 18S (a) and 28S (b) RNA. The experimental procedures were the same as Figure 1: (●–●) $^3$H radioactivity; (—) NaCl concentration; (a) RNase $T_2$ hydrolysate of methyl-$^3$H-labeled 18S RNA; (b) RNase $T_2$ hydrolysate of methyl-$^3$H-labeled 28S RNA.

peak corresponding to tetranucleotides. However, only one peak was found in the region of trinucleotide when methyl-$^3$H-labeled 28S RNA was used (Figure 2b). This suggested that the tri-2 fraction in $^{32}$P-labeled 28S RNA digests was also the nucleoside 3′,5′-diphosphate originating from the 5′ terminus of this molecule. Tri-1 and tetranucleotide fractions of $^{32}$P-labeled or methyl-$^3$H-labeled 28S RNA digests were demonstrated to be 2′-O-methylated tri- and tetranucleotides having two and three adjacent 2′-O-methyl nucleosides, respectively (vide infra).

*Two-Dimensional Thin-Layer Chromatography and*

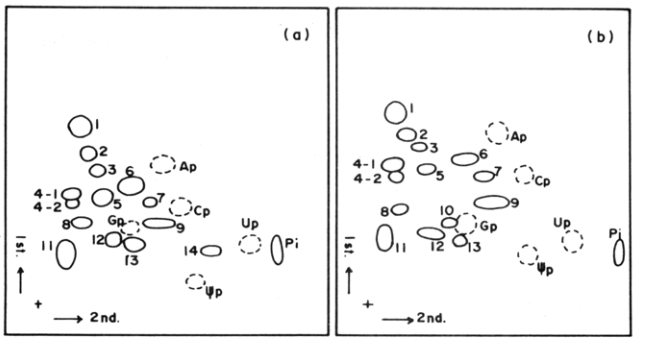


FIGURE 4: Diagrams of the autoradiographs of the two-dimensional thin-layer chromatography shown in Figure 3. The numbers correspond to the nucleotides whose sequences are given in Table II. Dotted circles on the diagrams show nucleotide markers: (a) dinucleotides from 18S RNA; (b) dinucleotides from 28S RNA.

*Identification of the 2′-O-Methylated Dinucleotides.* The two-dimensional thin-layer chromatographic patterns of the 2′-O-methylated dinucleotides of 18S and 28S RNA are shown in Figure 3.

The diagrams with numbering of the spots are presented in Figure 4. Characteristic patterns were obtained reproducibly for 18S and 28S RNA. As seen in Table II, the $^3$H/$^{32}$P ratio for each spot derived from doubly labeled RNA was almost the same indicating that each dinucleotide contained the same amount of methyl residues per one nucleotide molecule. The sequence of each dinucleotide was determined in such a manner as described below. Figure 5 shows Dowex-1 chromatographic patterns of spleen phosphodiesterase and snake venom phosphodiesterase digests of spots 4-1 and 4-2. As shown in Figure 5a, Ap and Gp were found in a ratio of nearly 1:1, when either of these spots was digested with spleen phosphodiesterase, indicating these spots to be either Am-Gp or Gm-Ap. Next, these spots were treated with snake venom phosphodiesterase to determine the 3′ side of the dinucleotide. Spot 4-1 yielded only pGp and spot 4-2 yielded pAp (Figure 5b,c). These findings established the sequences of spot 4-1 and 4-2 to be Am-Gp and Gm-Ap, respectively. The sequences of the other spots were determined in the same way.

There was some inorganic phosphate in the digests of the phosphodiesterase especially when a large amount of snake venom phosphodiesterase was used. Although this is most

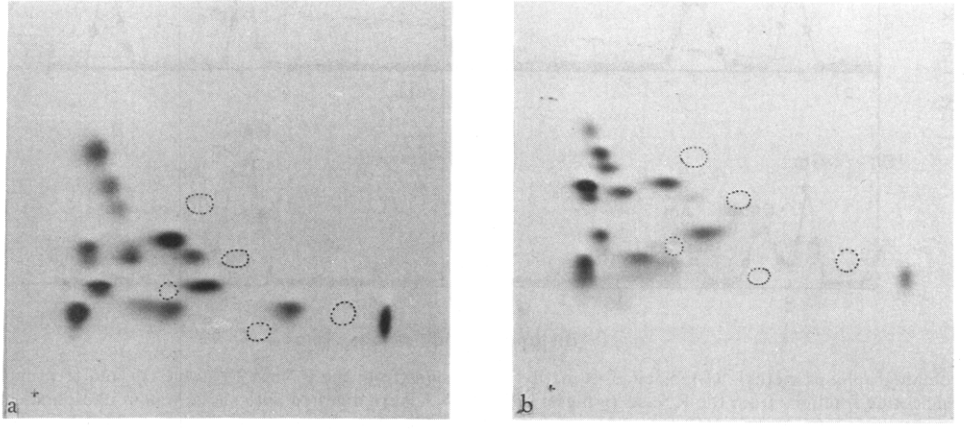


FIGURE 3: Typical autoradiographs of the two-dimensional thin-layer chromatography of RNase $T_2$ resistant dinucleotides from 18S (a) and 28S RNA (b). The $^{32}$P-labeled or $^{32}$P and methyl-$^3$H doubly labeled dinucleotides were applied onto an Avicel SF cellulose plate (20 cm × 20 cm) with 0.1 $A_{260}$ unit each of Cp, Ap, Ψp, Up, and Gp as markers. The solvent systems are described in Materials and Methods. Dotted circles show nucleotide markers detected by an ultraviolet lamp.

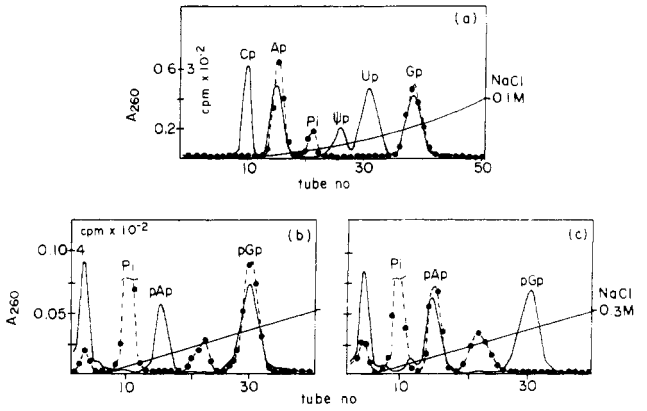


FIGURE 5: Sequence analysis of RNase $T_2$ stable dinucleotides, spots 4-1 and 4-2. Spots 4-1 and 4-2 from one plate were eluted and digested with 1 unit of spleen phosphodiesterase in 0.5 ml of 0.05 *M* sodium acetate buffer (pH 4.5) at 37° for 16 hr. The digests were applied on a Dowex-1 column with 0.2–0.5 $A_{260}$ unit each of Cp, Ap, Ψp, Up, and Gp. After equilibration of the column with 0.004 *N* HCl, elution was effected with a concave gradient formed by connecting a 30-ml mixing chamber containing 0.004 *N* HCl to a 20-ml reservoir containing 0.1 *M* NaCl in 0.01 *N* HCl (a). The pots from the other plate were digested with snake venom phosphodiesterase in 0.05 *M* Tris-HCl (pH 8.3) and chromatographed on a Dowex-1 column with 0.2 $A_{260}$ unit each of pAp and pGp as markers. Elution was performed as described above except that the linear gradient was formed with 30 ml of 0.004 *N* HCl and 30 ml of 0.3 *M* NaCl containing 0.012 *N* HCl (b,c): (●- - -●) $^{32}P$ radioactivity; (—) $A_{260}$; (a) spleen phosphodiesterase digests of spot 4-1; (b) snake venom phosphodiesterase digest of spot 4-1; (c) snake venom phosphodiesterase digest of spot 4-2.

likely due to the trace contamination of phosphomonoesterase in the enzyme preparation—the alternative possibility of monoesterase action of this enzyme is not ruled out (McLennan and Lane, 1968)—it did not affect the unequivocal determination of the sequences.

Spot 9 consisted of Cm-Up and Um-Cp, the only 2′-O-methylated dinucleotides that could not be separated by each other by this thin-layer chromatography. This spot was analyzed, without separation, by snake venom phosphodiesterase and *E. coli* alkaline phosphatase digestion followed by paper chromatography as described in Materials and Methods.

The molar ratio of the two dinucleotides was estimated from the $^3H$ radioactivity ratio of Cm to Um and found to be 0.36:1.00 for 18S RNA and 1.43:1.00 for 28S RNA. From these ratios the content of each Cm-Up and Um-Cp was calculated and is shown in Table II. Assignments of the sequences to all the spots are as shown in Table II.

*Distribution of the 2′-O-Methylated Dinucleotides in 18S and 28S RNA.* The percentage distribution of radioactivity among various RNase $T_2$ stable dinucleotides is presented in Table II, together with calculated numbers of the dinucleotides per one molecule of 18S and 28S RNA. The data from five different preparations of ribosomal RNA fell into a rather narrow range as indicated by the small standard error.

If one assumes that 18S and 28S RNA are polynucleotide chains with 2000 and 5000 nucleotide residues, respectively (Staehelin et al., 1964; Loening, 1968), then single dinucleotides should each contain 0.1 and 0.04% of the total $^{32}P$ radioactivity. Since average yields of dinucleotide of the 18S and 28S RNA were 3.52 and 2.38%, respectively, there must be a total of 35.2 methyl residues on the sugar moiety per 18S RNA and 59.5 methyl residues on the sugar moiety of 28S RNA. Accordingly, 2.86% (100/35.2) and 1.68% (100/59.5) of the total $^{32}P$ radioactivity in RNase $T_2$ stable dinucleotide fractions represent 1 mol of dinucleotide in 18S and 28S RNA molecules, respectively. The moles/mole values in Table II were calculated on this basis.

It is apparent from Table II that, while most of the 16 possible RNase $T_2$ stable sequences of the type Nm-Np are present in both 18S and 28S RNA, there are significant differences in the relative amounts of the sequences. For instance, the sequence Um-Up which was found in the 18S RNA was absent in the 28S RNA, whereas the sequence Gm-Cp was present only in non-heat-quenched 28S RNA. This sequence must have derived from the 5.8S RNA which was attached to 28S RNA by H bonding (Maden and Robertson, 1974).

*Sequence Determination of the 2′-O-Methylated Tri-*

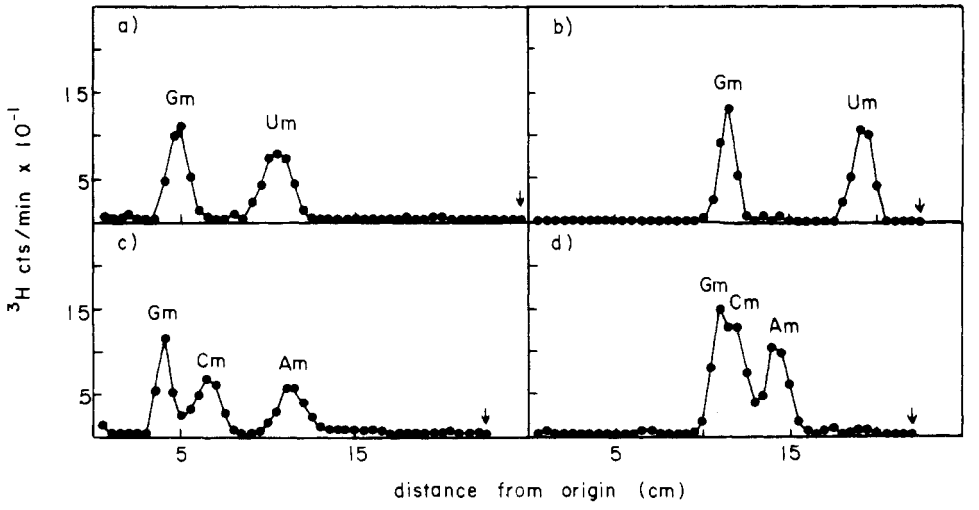


FIGURE 6: Paper chromatography of methyl-$^3H$-labeled 2′-O-methyl nucleosides from the RNase $T_2$ stable tri- and tetranucleotides. Methyl-$^3H$-labeled tri- and tetranucleotide fractions from the RNase $T_2$ digest of 28S RNA were digested with snake venom phosphodiesterase and *E. coli* alkaline phosphatase completely in 0.05 *M* Tris-HCl (pH 8.3) at 37° for 16 hr. The digests were developed with the following two different solvent systems: (I) 1-butanol–0.8 *M* boric acid–ammonia (2000:270:8, v/v/v) (a,c); (II) 2-propanol–HCl–water (680:176:144, v/v/v) (b,d). The paper strips were cut into 0.5-cm pieces and counted in a toluene scintillator. The 2′-O-methyl nucleosides were identified by $R_f$ values (Al-Arif and Sporn, 1972; Hall, 1965): (a) digest from tri fraction with solvent I; (b) digest from tri fraction with solvent II; (c) digest from tetra fraction with solvent I; (d) digest from tetra fraction with solvent II. The arrows indicate the front of the solvent.

Table II: Distribution of RNase $T_2$ Stable Dinucleotides in 18S and 28S rRNA.[a]

| Spot no. | Sequence | 18S $^3H/^{32}P$ | 18S % of Total Di | 18S mol/mol of RNA ± SE | 28S $^3H/^{32}P$ | 28S % of Total Di | 28S mol/mol of RNA ± SE |
|---|---|---|---|---|---|---|---|
| 1 | AmpAp | 0.32 | 9.4 | 3.3 ± 0.2 | 1.23 | 4.6 | 2.7 ± 0.2 |
| 2 | AmpCp | 0.30 | 7.0 | 2.4 ± 0.2 | 1.18 | 7.1 | 4.2 ± 0.3 |
| 3 | CmpAp | 0.27 | 3.2 | 1.1 ± 0.1 | 1.31 | 5.6 | 3.4 ± 0.1 |
| 4-1 | AmpGp | 0.30 | 6.5 | 2.3 ± 0.1 | 1.32 | 13.7 | 8.1 ± 0.4 |
| 4-2 | GmpAp | 0.36 | 2.0 | 0.7 ± 0.1 | 1.17 | 3.9 | 2.2 ± 0.1 |
| 5 | CmpCp | 0.29 | 8.2 | 2.9 ± 0.1 | 1.12 | 8.3 | 5.0 ± 0.2 |
| 6 | AmpUp | 0.28 | 13.6 | 4.8 ± 0.2 | 1.17 | 8.6 | 5.1 ± 0.1 |
| 7 | UmpAp | 0.25 | 4.2 | 1.5 ± 0.2 | 1.22 | 1.4 | 0.8 ± 0.1 |
| 8 | CmpGp | 0.34 | 8.6 | 3.0 ± 0.1 | 1.46 | 6.3 | 3.7 ± 0.1 |
| 9[b] | CmpUp | 0.30 | 9.3 | 0.9 ± 0.1 | 1.11 | 10.5 | 3.7 ± 0.2 |
| | UmpCp | | | 2.4 ± 0.1 | | | 2.7 ± 0.2 |
| 10 | GmpCp | | 0 | 0 | 1.07 | 1.9 | 1.0 ± 0.1 |
| 11 | GmpGp | 0.28 | 14.1 | 5.0 ± 0.2 | 1.29 | 14.0 | 8.3 ± 0.1 |
| 12 | GmpUp | 0.32 | 3.5 | 1.2 ± 0.1 | 1.13 | 10.4 | 6.2 ± 0.2 |
| 13 | UmpGp | 0.28 | 6.0 | 2.1 ± 0.2 | 1.50 | 3.6 | 2.2 ± 0.2 |
| 14 | UmpUp | 0.25 | 4.4 | 1.6 ± 0.1 | | 0 | 0 |
| Total | | | 100.0 | 35.2 | | 100.0 | 59.3 |

[a] The percentage was calculated on the basis of the total radioactivity recovered from the thin-layer plate. Inorganic phosphate was not included which accounted for less than 5% of the total radioactivity. The number of each 2′-O-methylated dinucleotide was calculated on the assumption that total radioactivity recovered from the thin-layer plate corresponded to 35 and 60 mol of dinucleotides, respectively: SE = $[\Sigma(x - \bar{x})^2/n(n-1)]^{1/2}$. [b] Spot 9 was analyzed, without separation, by *E. coli* alkaline phosphatase and snake venom phosphodiesterase digestion of methyl-$^3$H-labeled spot 9, followed by paper chromatography. The molar ratio of the two dinucleotides was estimated from the $^3$H radioactivity of Cm and Um.

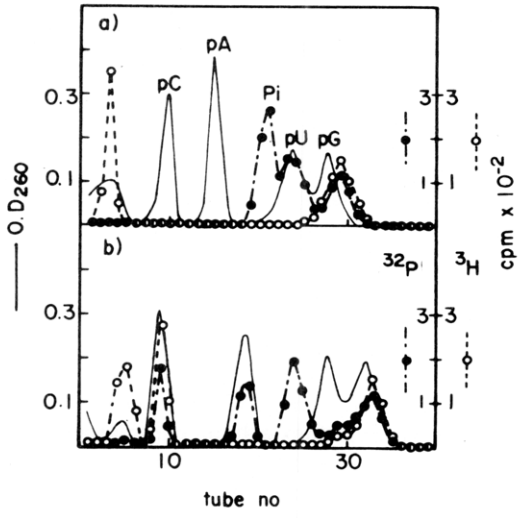


FIGURE 7: Dowex-1 column chromatography of digestion products with snake venom phosphodiesterase and nuclease $P_1$ of $^{32}P$-labeled and methyl-$^3$H-labeled tri- and tetranucleotides. $^{32}$P-Labeled 2′-O-methylated tri (a) or tetra (b) fractions were mixed with methyl-$^3$H-labeled homologous fractions, and completely digested with snake venom phosphodiesterase and small amount of nuclease $P_1$. The digests were chromatographed on a Dowex-1 column with about 0.5 $A_{260}$ unit each of four nucleoside 5′-monophosphates (pC, pA, pU, and pG) as the markers in same manner as Figure 5a: (—) $A_{260}$ nm; (O- - -O) $^3$H radioactivity; (●---●) $^{32}$P radioactivity; (a) 2′-O-methylated trinucleotide; (b) 2′-O-methylated tetranucleotide.

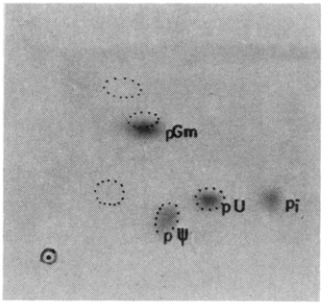


FIGURE 8: Thin-layer chromatography of snake venom phosphodiesterase and nuclease $P_1$ digestion product of 2′-O-methylated trinucleotides. The $^{32}$P-labeled 2′-O-methyl trinucleotide was digested with snake venom phosphodiesterase and nuclease $P_1$ as described in Figure 7. The digest was analyzed on two-dimensional thin-layer chromatography as shown in Figure 3 with pA, pC, pG, pΨ, and pU as markers. The dotted circles indicate pN markers detected by an ultraviolet lamp.

*and Tetranucleotides.* Tri-1 and tetranucleotide fractions of 28S RNA digests (Figures 1c, 1d, and 2b) were 2′-O-methylated tri- and tetranucleotides, having two or three adjacent 2′-O-methyl nucleosides, respectively. The $^{32}$P radioactivity recovered in tri- and tetranucleotide fractions on the DEAE-Sephadex column indicated that 2 mol of 2′-O-methylated trinucleotides and 1 mol of 2′-O-methylated tetranucleotide were present in 28S RNA assuming a chain length of 5000 nucleotides (Table I).

(i) ANALYSIS OF 2′-O-METHYLATED TRINUCLEOTIDES. For analysis of the 2′-O-methyl nucleoside composition, paper chromatography with two different solvent systems was performed as described in Materials and Methods. Figures 6a and 6b show that the same amount of Gm and Um is present in 2 mol of 2′-O-methylated trinucleotides. The $^{32}$P-labeled and methyl-$^3$H-labeled trinucleotides were mixed and digested completely with snake venom phosphodiesterase and a small amount of nuclease $P_1$. The digests were applied on a Dowex-1 column with pN's as a marker (Figure 7a). Nonadsorbed $^3$H radioactivity contained only Um as identified by paper chromatography. The $^3$H and $^{32}$P radioactivity peak in Figure 7a, which eluted slower than the pG marker, was further analyzed and identified as pGm by paper chromatography after dephosphorylation with *E. coli* alkaline phosphatase. These results showed that 2 mol of the trinucleotides had a common sequence, Um-Gmp, at the 5′ side. In Figure 7a, the peak containing only $^{32}$P radioactivity must be the 3′-end nucleotides of both trinucleotides. Inorganic phosphate was also

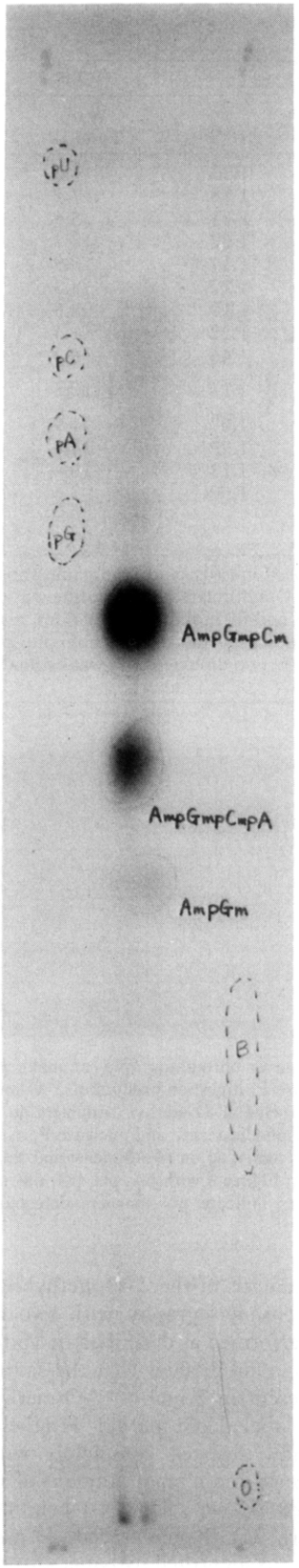


FIGURE 9: Partial digestion of 2′-O-methylated tetranucleotide with snake venom phosphodiesterase. The $^{32}$P-labeled 2′-O-methylated tetranuceotide was partially digested with snake venom phosphodiesterase and small amount of nuclease $P_1$ as described in Materials and Methods. The digests were separated by electrophoresis on DEAE-paper at 30 V/cm for 4 hr. Each spot was analyzed with spleen and/or snake venom phosphodiesterase digestion followed by Dowex-1 column chromatography as described in the text. B and O represent blue and orange markers, respectively.

eluted just before this peak. In order to determine the 3′ end of both trinucleotides, snake venom phosphodiesterase plus nuclease $P_1$ digests of the $^{32}$P-labeled trinucleotides were separated on thin-layer chromatography (Figure 8). Three spots were detected on the plate, which were identified as pGm, pΨ, and pU by Dowex-1 column chromatography. The ratio of the radioactivity was almost 2:1:1 for pGm, pΨ, and pU. These results established the sequences of 2′-O-methylated trinucleotides to be Um-Gm-Ψp and Um-Gm-Up, and they were present 1 mol each in 28S RNA.

(ii) ANALYSIS OF 2′-O-METHYLATED TETRANUCLEOTIDE. Figures 6c and 6d show that the 2′-O-methylated tetranucleotide is composed of Am, Gm, and Cm as the three nucleosides at the 5′ side. The Dowex-1 column chromatographic pattern of the snake venom phosphodiesterase plus nuclease $P_1$ digest is shown in Figure 7b.

The $^3$H radioactive peak was identified as Am by paper chromatography. The $^{32}$P radioactive peak without $^3$H radioactivity coincided exactly with pA. The $^3$H and $^{32}$P doubly radioactive peaks were identified as pCm and pGm also by paper chromatography after treatment with *E. coli* alkaline phosphatase. These results suggested that the 5′ end of the tetranucleotide was Am and the 3′ end was Ap. For determination of the complete sequence of the 2′-O-methylated tetranucleotide, partial digestion with snake venom phosphodiesterase was performed as described in Materials and Methods. The digests were separated with DEAE-paper electrophoresis in 7% formic acid (Figure 9). Each spot was analyzed on a Dowex-1 column after the complete digestion with snake venom and/or spleen phosphodiesterase. As shown in Figure 9 three relatively slow moving spots were obtained besides mononucleotides pCm, pA, and pGm. The slowest moving spot produced pGm when digested with snake venom phosphodiesterase, and also produced Amp when digested with spleen phosphodiesterase revealing the sequence to be Am-Gm. The fastest of these was a trinucleotide containing pGm and pCm. The middle spot was a tetranucleotide containing pA, pCm, and pGm.

These results established the sequence of the 2′-O-methylated tetranucleotide to be Am-Gm-Cm-Ap.

*5′-Termini of the 18S and 28S Ribosomal RNA.* The tri fraction in DEAE-Sephadex column chromatography of the RNase $T_2$ digest of 18S RNA was chromatographed on a column of Dowex-1 with nucleoside 3′,5′-diphosphate markers. The tri-2 fraction was used in the case of 28S RNA. As shown in Figure 10, more than 95% of the radioactivity from 18S and 28S RNA coincided with pUp and pCp, respectively. From the percentage of isotope in mononucleoside diphosphate lengths of about 2000 and 5000 may be calculated for 18S and 28S RNA species, respectively (Table I). These values agree extremely well with the molecular weights determined by physicochemical procedures and attest to the homogeneity of 5′ ends of these RNAs.

## Discussion

We have described a system with which almost all of the 16 possible 2′-O-methylated dinucleotides of ribosomal RNA can be separated and quantitated with the use of DEAE-Sephadex column chromatography followed by two-dimensional thin-layer chromatography on a cellulose powder plate. The method is especially useful for the analysis of minute amounts of RNA digests provided that they are sufficiently labeled.

With this procedure, we have determined all of the 2′-O-

methylated dinucleotides of 18S and 28S RNA of mouse hepatoma MH 134 cells. In these experiments, RNase $T_2$ digestion was preferentially utilized rather than alkaline hydrolysis to avoid the unfavorable artifacts that may occur at high pH values. It has been shown that alkaline conditions frequently induce partial deamination of Cp into Up and conversion of 1-methyladenosine to $N^6$-methyladenosine and of 7-methylguanine to 2,6-diamino-4-hydroxy-5-methylformamidopyrimidine. The total yield of mono-, di-, and other oligonucleotides was not different between RNase $T_2$ digest and alkaline hydrolysate indicating that RNase $T_2$ had acted completely and no partially digested material was present when the RNA was treated with the enzyme under the present conditions.

The ribosomal RNA was labeled with $^{32}P$ for such a long period that the specific activity of each precursor pool did not affect the calculation of nucleotide distribution (Sitz et al., 1973; Kominami, unpublished observation). Double labeling with $^{32}P$ and $^{3}H$, which labeled specifically methyl groups of the nucleotides, has also been employed to confirm and extend the results obtained with $^{32}P$. Patterns of 2′-O-methylation in 18S and 28S RNA of MH 134 cells thus determined were in many ways similar to but in some respects different from those of L cells reported by Lane and Tamaoki (1969). Other workers have reported somewhat different patterns for different cells (Egawa et al., 1971; Wagner et al., 1967). Whether these differences are really due to the species difference remains to be seen.

An extraordinary RNase $T_2$ or alkali stable tetranucleotide was found to be present in 28S RNA of the mouse hepatoma cells. The compound was identified as Am-Gm-Cm-Ap by several methods described in the Results section. Also we might add that we have detected both this and the other trinucleotide sequences in RNase $T_1$ isopliths longer than 10. The same sequence has recently been reported by Nazar and Busch (1974) in Novikoff hepatoma. Since HeLa cells appear to have the same sequence (Maden and Salim, 1974), this three consecutively sugar methylated sequence must be present equally in at least higher animals. It should be mentioned in this connection that this sequence is not detected in yeast 28S RNA (Klootwijk and Planta, 1973). The trinucleotides found in these mouse cells were the same as those found in HeLa cells (Lane, 1965; Salim and Maden, 1973). One of these trinucleotides has also been found in Novikoff hepatoma (Nazar and Busch, 1974). Four trinucleotides were reported in yeast, but only one of them (Um-Gm-Up) agreed with that of mammalian 28S RNA. Patterns for 2′-O-methylated dinucleotides were also considerably different between the yeast and the mammals. These findings indicate that there must have been considerable alterations in the methylation pattern during the course of evolution of ribosomal RNA in eukaryotes.

In the MH 134 cells of the mouse, the 5′-termini of the 18S and 28S RNA were found to be pUp and pCp, respectively, in agreement with the data of Lane and Tamaoki (1967) for L cells. In other experiments which will be reported elsewhere, we have determined the 5′-termini of ribosomal RNA by labeling with [$\gamma$-$^{32}P$]ATP and polynucleotide kinase, and analyzing the labeled nucleotides after enzymatic or alkaline hydrolysis. These data also indicated that the major 5′-termini of 18S and 28S RNA were pUp and pCp, respectively, for MH 134 cells, the liver of C3H/He mice, as well as for other mammalian cells including rat liver and human tissue culture cells (Sakuma, Kominami, Muramatsu, and Sugiura, manuscript in preparation).

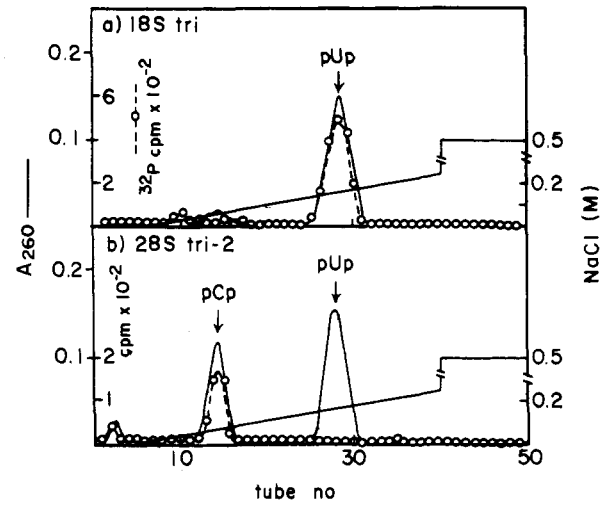


FIGURE 10: Dowex-1 column chromatography of RNase $T_2$ stable tri fraction of 18S RNA (a) and tri-2 fraction of 28S RNA (b). The tri fraction of 18S in Figure 1b and the tri-2 fraction of 28S RNA in Figure 1d were pooled, diluted fivefold with water, and loaded on Dowex-1 columns (0.3 × 15 cm) with pCp and pUp as markers. Elution was performed as described in the legends for Figures 5b and 5c: (—) absorbance at 260 nm; (O- - -O) radioactivity; (a) tri fraction of 18S RNA; (b) tri-2 fraction of 28S RNA.

From the recovery of 5′-termini, the chain lengths of 18S and 28S RNA have been calculated to be approximately 2000 and 5000, respectively. The molecular weights of these RNAs may be calculated from the chain lengths and the base compositions (Hashimoto and Muramatsu, 1973) to be $0.65 \times 10^6$ and $1.7 \times 10^6$, respectively, which are in close agreement with previously published data obtained by physicochemical analyses (Staehelin et al., 1964; Loening, 1968). Although Lane and Tamaoki (1967) estimated the chain length of mouse L cell 28S RNA to be approximately 2000, this difference may probably be attributed to the fact that 5.8S RNA had not been removed from their 28S RNA. Recently, Birnboim (1972) has estimated the chain length of 18S RNA to be 2000 nucleotides from the analysis of adenylate rich oligonucleotides. If, on the other hand, these values are assumed to be correct, the 5′-termini of these RNAs must be homogeneous and the recovery must have been nearly quantitative.

In the same vein, the newly found tetranucleotide sequence must exist only one in each molecule of 28S RNA.

Acknowledgments

The authors wish to thank Drs. S. Takemura, Nagoya University, T. Uchida, Mitsubishi Kasei Institute for Life Sciences, and A. Kuninaka, Yamasa Shoyu Co., for their kind gifts of purified spleen phosphodiesterase, RNase $U_2$, and nuclease $P_1$, respectively. We are also indebted to Dr. Kuninaka for the marker ΨMP.

References

Al-Arif, A., and Sporn, M. B. (1972), *Anal. Biochem. 48,* 386.

Attardi, G., and Amaldi, F. (1970), *Annu. Rev. Biochem. 39,* 183.

Birnboim, H. C. (1972), *Can. J. Biochem. 50,* 993.

Brown, G. M., and Attardi, G. (1965), *Biochem. Biophys. Res. Commun. 20,* 298.

Choi, Y. C., and Busch, H. (1970), *J. Biol. Chem. 245,* 1954.

Egawa, K., Choi, Y. C., and Busch, H. (1971), *J. Mol. Biol. 56,* 565.
Fujimoto, M., Kuninaka, A., and Yoshino, H. (1969), *Agric. Biol. Chem. 33,* 1517.
Greenberg, H., and Penman, S. (1966), *J. Mol. Biol. 21,* 527.
Hall, R. H. (1965), *Biochemistry 4,* 661.
Hashimoto, S., Kawata, M., and Takemura, S. (1972), *J. Biochem. (Tokyo) 72,* 1339.
Hashimoto, S., and Muramatsu, M. (1973), *Eur. J. Biochem. 33,* 446.
King, H. W. S., and Gould, H. (1970), *J. Mol. Biol. 51,* 687.
Klootwijk, J., and Planta, R. J. (1973), *Eur. J. Biochem. 39,* 325.
Lane, B. G. (1965), *Biochemistry 4,* 212.
Lane, B. G., and Tamaoki, T. (1967), *J. Mol. Biol. 27,* 335.
Lane, B. G., and Tamaoki, T. (1969), *Biochim. Biophys. Acta 179,* 332.
Loening, U. E. (1968), *J. Mol. Biol. 38,* 355.
Maden, B. E. H., and Robertson, J. S. (1974), *J. Mol. Biol. 87,* 227.
Maden, B. E. H., and Salim, M. (1974), *J. Mol. Biol. 88,* 133.
Maden, B. E. H., Salim, M., and Summers, D. F. (1972), *Nature (London), New Biol. 237,* 5.
McLennan, B. D., and Lane, B. G. (1968), *Can. J. Biochem. 46,* 93.
Muramatsu, M., and Fujisawa, T. (1968), *Biochim. Biophys. Acta 157,* 476.
Muramatsu, M., Shimada, N., and Higashinakagawa, T. (1970), *J. Mol. Biol. 53,* 91.
Nazar, R. N., and Busch, H. (1974), *J. Biol. Chem. 249,* 919.
Nishimura, S. (1972), *Prog. Nucleic Acid Res. Mol. Biol. 12,* 49.
Salim, M., and Maden, B. E. H. (1973), *Nature (London) 244,* 334.
Singh, H., and Lane, B. G. (1964a), *Can. J. Biochem. 42,* 87.
Singh, H., and Lane, B. G. (1964b), *Can. J. Biochem. 42,* 1011.
Sitz, T. O., Nazar, R. N., Spohn, W. H., and Busch, H. (1973), *Cancer Res. 33,* 3312.
Staehelin, T., Wettstein, F. O., Oura, H., and Noll, H. (1964), *Nature (London) 201,* 264.
Tamaoki, T., and Lane, B. G. (1968), *Biochemistry 7,* 3431.
Uchida, T., Arima, T., and Egami, F. (1970), *J. Biochem. (Tokyo) 67,* 91.
Vaughan, M. H., Soeiro, R., Warner, J. R., and Darnell, J. E. D. (1967), *Proc. Natl. Acad. Sci. U.S.A. 58,* 1527.
Wagner, E. K., Penman, S., and Ingram, V. M. (1967), *J. Mol. Biol. 29,* 371.

# Endoribonuclease from Bovine Adrenal Cortex Cytosol†

Henry I. Miller, Lucille A. Perkins, and Michael G. Rosenfeld*,‡

ABSTRACT: An endoribonuclease which digests a variety of synthetic homoribopolymers and poly(A)-rich mRNA has been identified and purified >500-fold with respect to specific activity from bovine adrenal cortex cytosol. Enzymatic digestion of synthetic poly(riboadenylic acid) was stimulated by $Mn^{2+}$ and $Mg^{2+}$ and the enzyme exhibited broad pH and salt optima. Poly(cytidylic acid) and poly(uridylic acid), but not poly(guanylic acid), served as substrates for the enzyme preparation; double-stranded RNA, DNA, and DNA-RNA hybrids were not digested by the enzyme. Digestion generated oligonucleotides with 3′-hydroxyl and 5′-monophosphoester termini. On isoelectric focusing, the enzymatic activity banded at pH 8.3 ± 0.2. An initial preferential cleavage of the poly(A) tract of poly(A)-rich RNA is suggested by the rapid appearance of a 4-6S digestion product highly enriched for adenylic acid; however, progressive digestion of the RNA occurs with additional incubation.

A region rich in poly(riboadenylic acid) has been identified in many eukaryotic and prokaryotic mRNAs (Lee et al., 1971; Darnell et al., 1971a,b; Edmonds et al., 1971; Lim and Canellakis, 1971; Aviv and Leder, 1972; Comstock et al., 1972; Lai and Duesberg, 1972; Prescott et al., 1971; Armstrong et al., 1972), and it has been proposed that most mRNAs in the eukaryotic cell contain a poly(A)[1] tract (Adesnik et al., 1972) at the 3′ terminus (Yogo and Wimmer, 1972; Molloy et al., 1972). The poly(A) tract may serve a regulatory role (Darnell et al., 1973), and regulation of its synthesis and degradation have been widely studied. Several eukaryotic ribonucleases which can digest poly(A) have been identified including a processive nuclear exoribonu-

† From the Department of Medicine, Division of Endocrinology, University of California, San Diego, School of Medicine, La Jolla, California 92037. *Received October 25, 1974.* This investigation was supported by Public Health Service Research Grant No. AM13149 from the National Institute of Arthritis, Metabolism and Digestive Diseases and by Grant No. BC169 from the American Cancer Society, Inc. In a previous publication (Perkins et al, 1974), the enzyme described herein was referred to as poly(A) ribonuclease.

‡ Teaching and Research Scholar of the American College of Physicians

[1] Abbreviations used are: MEM, minimal essential medium; SDS, sodium dodecyl sulfate; poly(A), poly(riboadenylic acid); poly(C), poly(ribocytidylic acid); poly(G), poly(riboguanylic acid); poly(U), poly(ribouridylic acid).