Oligonucleotide Sequences of Pancreatic and T_1 Ribonuclease Digests of 5S Ribosomal RNA from Mouse Cells†

Kenichi Takai,‡ Shuichi Hashimoto, and Masami Muramatsu*

ABSTRACT: Oligonucleotides produced by complete pancreatic and T_1 RNase digestion of 5S ribosomal RNA from a mouse hepatoma, MH 134, have been separated with two-dimensional electrophoresis and their nucleotide sequences determined. Except for the presence of a 5′-terminal diphosphate, these nucleotide sequences were identical with those of KB cells, confirming the identity of the primary structure of 5S RNA between these animals. Both oligonucleotide patterns produced with these enzymes from 5S RNA of the liver were also identical with those of the hepatoma. All these agree with the strong conservation of 5S RNA genes in animal species. However, when 5S ribosomal RNA was extracted from ribosomes which were prepared from microsomal pellet, pancreatic RNase digest contained two trinucleotides (A-G-Cp and G-A-Cp) that were not found in 5S RNA prepared with a one-step procedure. It was concluded that different isolation procedure might indeed cause artifactual fragments on enzymatic digestion due to internal nicks produced during isolation. The significance of 5′-terminal diphosphate in relation to the biosynthesis of 5S ribosomal RNA is also discussed.

Although the precise function of the 5S ribosomal RNA is quite obscure, it appears to have a vital role in the protein synthesizing machinery in view of the fact that one molecule of this RNA is always present in any type of the large ribosomal subunit. Because of the small size and availability of this molecule, the primary structure of 5S RNA has been determined for a number of organisms including *Escherichia coli* (Brownlee *et al.*, 1968), human KB cells (Forget and Weissman, 1969), *Pseudomonas fluorescens* (DuBuy and Weissman, 1971), *Saccharomyces carlsbergensis* (Hindley and Page, 1972), and *Torulopsis utilis* (Nishikawa and Takemura, 1974). Oligonucleotide mapping patterns have also been studied with the result that 5S RNAs from human, mouse, rabbit, and rat cells most probably have an identical nucleotide sequence (Williamson and Brownlee, 1969; Labrie and Sanger, 1969). Furthermore, analysis of oligonucleotide sequences of *Xenopus laevis* revealed differences at only eight positions from the mammalian sequence indicating the strong conservation of the 5S RNA sequence during evolution (Brownlee *et al.*, 1972).

On the other hand, conspicuous differences of the primary structure have been reported between somatic and oocyte 5S RNA (Wegnez *et al.*, 1972; Ford and Southern, 1973). Surprisingly, the difference between them was no less than that between *Xenopus* and man. Under these circumstances, it appears worth re-checking the primary structure of mammalian 5S RNA from different cells of the same species.

In the present study, mapping patterns and oligonucleotide sequences of the pancreatic and RNase T_1 digests of 5S RNA were compared between normal liver of C3H/He mice and an ascites hepatoma maintained on the same strain of mice.

Experimental Procedure

(*A*) *Animals and the Tumor.* Mice of C3H/He strain and the ascites hepatoma MH 134 used throughout the experiments were described previously (Hashimoto and Muramatsu, 1973).

(*B*) *Labeling with [^{32}P]Orthophosphate.* The method of labeling with [^{32}P]orthophosphate was described in a previous paper (Hashimoto and Muramatsu, 1973). The specific activity of purified 5S RNA was 1–1.5 × 10^7 cpm/mg of RNA for MH 134 cells and 3–4 × 10^6 cpm/mg RNA for normal liver, respectively.

(*C*) *Isolation of Ribosomal Fraction and Extraction of 5S RNA.* The procedure used for isolation of the ribosomal fraction was a modification of that described previously (Hashimoto and Muramatsu, 1973). The MH 134 cells from two mice were collected from ascites, immediately suspended in 20 ml of buffer A (0.01 M Tris-HCl (pH 7.4)–0.01 M NaCl–1.5 mM $MgCl_2$) and centrifuged for 5 min at 1000*g*. The cells were resuspended in 20 ml of buffer A and allowed to stand at 0° for 10 min. Then 0.2 ml of 10% Nonidet P-40 was added and homogenized in a Potter-Elvehjem homogenizer with seven to ten strokes. The homogenate was centrifuged at 12,000*g* for 10 min; the supernatant was adjusted to 0.6% with respect to sodium deoxycholate and centrifuged at 105,000*g* for 1.5 hr. The pellet was treated with buffer B (0.05 M sodium acetate (pH 5.1)–0.14 M NaCl–0.3% sodium dodecyl sarcosinate) and phenol to extract ribosomal RNA as previously described (Hashimoto and Muramatsu, 1973). Livers from two mice (~2 g) were homogenized with 10 ml of 0.25 M sucrose in buffer C (0.05 M Tris-HCl (pH 7.4)–0.025 M KCl–5 mM $MgCl_2$) in a Potter-Elvehjem homogenizer with seven to ten strokes. The homogenate was centrifuged at 10,000*g* for 10 min to sediment unbroken cells, nuclei, mitochondria, and other debris. The resulting supernatant was adjusted to 0.6% with respect to sodium deoxycholate, gently homogenized, and centrifuged at 105,000*g* for 1.5 hr. The ribosomal pellet was homogenized with 15 ml of buffer B and phe-

† From the Department of Biochemistry, Tokushima University School of Medicine, Tokushima, Japan. *Received August 8, 1974.*

‡ Present address: Department of Microbiology, Okayama University School of Medicine, Okayama, Japan.

nol to extract ribosomal RNA. The ribosomal RNA was separated by 10–30% linear sucrose density gradient centrifugation and the fractions corresponding to low molecular weight RNA were pooled. Pooled RNA fractions, about 0.1 mg, were co-precipitated with 0.5 mg of tRNA as a carrier at −20° overnight. For further purification of 5S RNA, the RNA was applied to a 0.5 × 110 cm column of Sephadex G-100, equilibrated 0.1 M NaCl at room temperature. The flow rate was 1.2 ml/hr and 0.6-ml fractions were collected. The fractions were analyzed for radioactivity by counting a small aliquot (2–5 μl) in 10 ml of Bray's scintillator and for the optical density by reading at 260 nm. Ribosomal 5S RNA was separated in a discrete peak from contaminating tRNA and degraded high molecular weight RNA.

(*D*) *Digestion of 5S RNA and Fractionation of Oligonucleotides.* The RNA was dissolved in 10 μl of 0.01 M Tris-HCl (pH 7.5)–1 mM EDTA with pancreatic RNase (Worthington Biochemical Corp.) or RNase T_1 (Sankyo Co.) at an enzyme to substrate ratio of 1:20 for 1 hr at 37°. For the combined digestion with RNase T_1 plus *E. coli* alkaline phosphatase (Worthington Biochemical Corp.), the RNA was dissolved in 5 μl of 0.01 M Tris-HCl (pH 7.5)–1 mM EDTA with RNase T_1 at an enzyme to substrate ratio of 1:20, and 5 μl of alkaline phosphatase (1 mg/ml) was added (enzyme to substrate 1:10). Incubation was continued for 70 min at 37°.

Fractionation of the complete digests was carried out by two-dimensional electrophoresis as described previously (Sanger *et al.*, 1965). Digest (10 μl) containing 0.05 mg of material was developed in the first dimension on a strip (90 × 3 cm) of cellulose acetate (Separax, Fuji Film Co.) in 5% acetic acid–7 M urea (pH 3.5) for 1 hr at 4.5 kV. Electrophoresis in the second dimension was carried out on the DEAE paper (Whatman Chromedia, DE-81) in 7% formic acid (pH 1.9) for 7 hr at 1.5 kV. After drying, the paper was marked with radioactive dye and was placed against a sheet of X-ray film. After 1–3 days, the resulting autoradiographs were cut out and after counting in polyethylene vials by the Čerenkov scintillation or in a toluene scintillator, each spot was eluted with triethylamine–carbonate as described previously (Sanger *et al.*, 1965). The eluted material was allowed to evaporate for further analysis.

(*E*) *Analysis of Oligonucleotides.* The oligonucleotides were further digested with T_1 or pancreatic RNase for 1 hr at 37° in 5 μl of 0.01 M Tris-HCl (pH 7.5)–1 mM EDTA containing 0.05 mg/ml of enzyme. The products were fractionated by electrophoresis on DEAE paper for 7 hr at 1.5 kV in the pH 1.9 buffer system. After autoradiography, the nucleotide composition of each oligonucleotide was determined by RNase T_2 digestion followed by Dowex-1 column chromatography or the two-dimension thin-layer chromatography as previously described (Hashimoto and Muramatsu, 1973). RNase U_2 digestion of oligonucleotides from RNase T_1 or pancreatic RNase digestion was carried out for 1 hr at 37° in 5 μl of 0.05 M sodium acetate (pH 4.5) containing 2 μl of 25 units/ml of enzyme and 0.05 mg of tRNA. The resulting oligonucleotides were fractionated by electrophoresis on DEAE paper at pH 1.9. Complete snake venom phosphodiesterase (Worthington Biochemical Corp.) digestion of oligonucleotides (from combined RNase T_1 plus alkaline phosphatase) was done with 0.01 ml of a 0.1 mg/ml solution of the enzyme in 0.01 M Tris-HCl (pH 8.5)–2 mM $MgCl_2$ for 1.5 hr at 37°. In the case of 3′-phosphorylated oligonucleotide, the concentration of the enzyme was ten times that in the dephosphorylated oligonucleotides. The products were fractionated by electrophoresis on DEAE paper at pH 1.9. Partial digestion of oligonucleotides with spleen phosphodiesterase was done with 10 μl of solution containing variable concentrations of the enzyme, *i.e.*, 18–90 units/ml of 0.15 M acetate buffer (pH 4.8). Incubation time also varied from 30 to 180 min at 37°. These partial digestion products were fractionated by electrophoresis on DEAE paper at pH 1.9.

(*F*) *Determination of 5′-Terminal Nucleotide.* Purified ^{32}P-labeled 5S RNA (0.05 mg) was digested with 0.1 unit of RNase T_2 in 10 μl of 0.01 M sodium acetate (pH 4.6) at 37° for 1 hr. The digest was treated with hydrochloric acid at pH 1.0 at 0° for 5 hr to cleave cyclic phosphodiester linkages. After neutralization, the digest was applied on a column (0.3 × 30 cm) of Chromedia DE-32 (Whatman Biochemical Co.) equilibrated with 7 M urea–0.01 M Tris-HCl (pH 7.6), and several column volumes of the same buffer were allowed to flow before applying about 10 OD units of tRNA pancreatic digest. Chromatography on Chromedia DE-32 was done as described previously (Hashimoto and Muramatsu, 1973). Aliquots (50 μl) were used for counting of radioactivity and, after optical density measurement, corresponding trinucleotide and tetranucleotide fractions were utilized for the analysis of 5′-terminal nucleotide. Chromatography on Dowex-1 (chloride form) was carried out essentially as described previously (Hatlen *et al.*, 1969).

Results

Oligonucleotide Patterns of Pancreatic RNase Digest. An oligonucleotide pattern produced by pancreatic RNase digestion of 5S RNA is shown in Figure 1a,b. Table I summarizes the results of sequence analysis of these oligonucleotides together with those by RNase T_1 digestion. All the sequences of pancreatic RNase digestion products were established by further digestion with RNase T_1, T_2, and U_2. Spot 12 released G-Gp after partial degradation with RNase U_2. This nucleotide was identified by its mobility on electrophoresis on DEAE paper at pH 1.9 and its sequence was confirmed by base composition. Partial digestion of spot 18 with RNase U_2 gave a nucleotide with the electrophoretic mobility and base composition of A-G-Cp, which indicate that spot 18 has the sequence G-G-A-A-G-Cp. The sequence of spot 19 was established by the result of partial digestion with RNase U_2 which produced fragments G-G-Gp, (G,A_2), and G-A-Cp. Spot 21 yielded G-G-G-Up when partially digested with RNase U_2, which was established by the electrophoretic mobility and base composition, indicating the sequence to be A-G-G-G-Up. Spot 23 was the 5′-terminal oligonucleotide. Complete RNase T_2 digestion of this spot produced two radioactive peaks on a Dowex-1 column (data not shown). One coincided with the cold Up marker and the other eluted immediately after GTP, which agreed with the positions previously reported for the elution of ppGp (Hatlen *et al.*, 1969). Although the ratio of radioactivity recovered in ppGp and Up was a little lower than the theoretical value, its sequence was determined to be ppG-Up. The characterization of the 5′-terminus will be discussed later.

Oligonucleotide Patterns of RNase T_1 Digest. A typical oligonucleotide pattern obtained by RNase T_1 digestion of 5S RNA is also shown in Figure 1c,d. There are several overlapping spots which could not be separated with this procedure. These are spots 9, 10, 11, and 50-B, spots 35 and 37, and spots 53 and 56, respectively. However, these spots

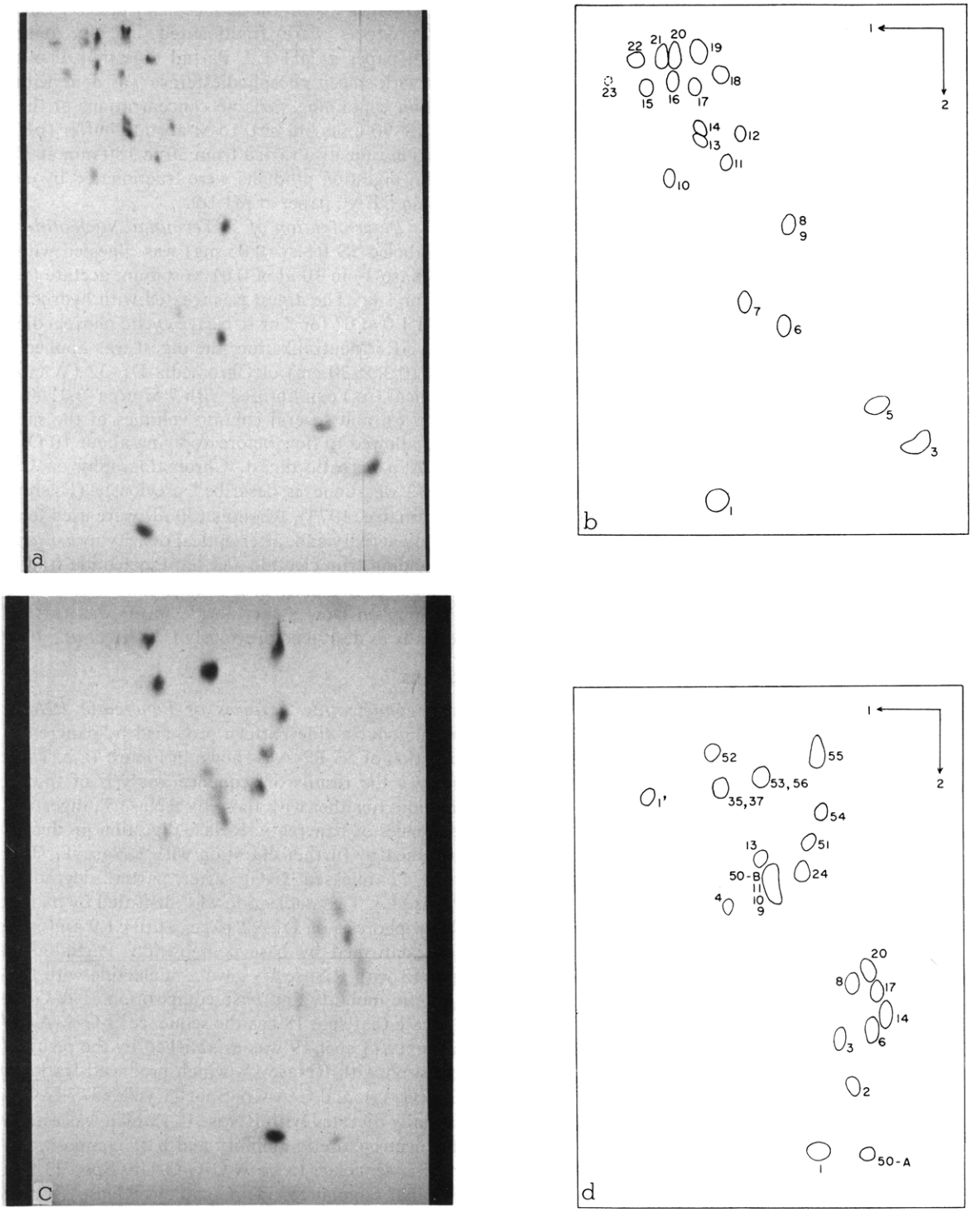

FIGURE 1: Typical autoradiographs of two-dimensional electrophoresis of pancreatic RNase (a) and RNase T_1 (c) digests of ^{32}P-labeled 5S RNA from MH 134 cells: upper and lower left. Electrophoresis was from right to left on cellulose acetate at pH 3.5, and from top to bottom on DEAE-cellulose paper at pH 1.9. Diagrams of autoradiographs of pancreatic RNase (b) and RNase T_1 (d) digests of ^{32}P-labeled 5S RNA from MH 134 cells on the two-dimensional system. The numbers correspond to the oligonucleotides whose sequences are given in Table I.

could be separated either by re-electrophoresis or by simultaneous digestion with RNase T_1 plus alkaline phosphatase as will be described later. In Table I are shown the deduced sequences of these oligonucleotides. Each spot was analyzed further by digestion with either pancreatic RNase, RNase U_2, snake venom phosphodiesterase or spleen phosphodiesterase. An overlapping spot which was located approximately at the center of the fingerprint (Figure 1c,d) was separated into three bands by re-electrophoresis on a DEAE paper strip at pH 1.9, each representing spots 9, 10, and 11 plus 50-B. The sequences of spots 9 and 10 were determined by the analysis of RNase T_2 and snake venom phosphodiesterase digestion products. Spot 11 plus 50-B gave, after RNase U_2 digestion, three spots on electrophoresis. Two of them were Gp and U-Ap and the remaining spot had the same electrophoretic mobility as original spot 11. RNase T_2 digestion of this spot gave the products Cp and Up in a ratio of 1.0:2.1 and snake venom phosphodiesterase produced pU. Therefore, the sequence was C-U-U-Uoh, representing one of the 3′-terminal nucleotides. The other 3′-terminal nucle-

Table I: Oligonucleotides Obtained by Complete Digestion of MH 134 5S RNA with Either Pancreatic or T_1 RNase.[a]

Pancreatic RNase Digestion Products (A)				RNase T_1 Digestion Products (B)			
Spot No. in Fig. 1	Sequence	Molar Yield Expt.[b]	Molar Yield Theor.	Spot No. in Fig. 2	Sequence	Molar Yield Expt.[c]	Molar Yield Theor.
1	Up	11.53	13–14	1	Gp	14.98	15
3	Cp	14.00	17	2	C-Gp	1.16	1
5	A-Cp	5.40	5	3	A-Gp	1.07	1
6	G-Cp	4.37	4	4	U-Gp	1.04	1
7	A-Up	1.17	1	6	C-A-Gp	1.05	1
8	A-A-G-Cp	0.83	1	8	A-A-Gp	1.02	1
9	G-A-A-Cp	1.21	1	9	U-C-Gp	0.90	1
10	G-Up	2.53	2	10	C-U-Gp	1.14	1
11	G-G-Cp	1.00	1	11	U-A-Gp	0.95	1
12	A-G-G-Cp	0.97	1	50-B	C-U-U-Uoh	0.38	0.5
13	G-A-Up	2.18	2	13	A-U-Gp	1.13	1
14	A-G-Up	1.07	1	14	C-C-C-Gp	0.97	1
15	G-G-Up	0.96	1	17	A-C-C-Gp	1.01	1
16	G-G-A-Up	1.04	1	20	A-A-C-Gp	0.92	1
17	G-G-G-Cp	0.94	1	24	C-C-U-Gp	2.00	2
18	G-G-A-A-G-Cp	0.98	1	35	U-C-U-Gp	1.10	1
19	G-G-G-A-G-A-Cp	0.84	1	37	U-U-A-Gp	1.06	1
20	G-G-G-A-A-Up	1.03	1	51	C-U-A-A-Gp	1.07	1
21	A-G-G-G-Up	0.86	1	52	U-A-C-U-U-Gp	1.15	1
22	G-G-G-Up	1.15	1	53	A-U-C-U-C-Gp	1.92	2
23[d]	ppG-Up	0.17	1	54	A-A-U-A-C-C-Gp	0.97	1
				55	C-C-A-U-A-C-C-A--C-C-C-U-Gp	0.91	1
				56	U-C-U-A-C-Gp	0.92	1
				50-A	C-U-Uoh	0.42	0.5
				1[d]	ppGp	0.19	1

[a] (A) pancreatic and (B) T_1 RNase digestion products of MH 134 5S RNA. Determination of the sequences was done as described in the text. Experimental molar yield (Expt) was obtained by counting the radioactivity in the area of the paper. [b] Averages of three experiments. [c] Averages of four experiments. [d] Averages of two experiments. Theoretical molar yields (Theor.) are calculated from the sequence of human 5S rRNA according to Forget and Weissman (1969).

otide was found in spot 50-A which gave equal amounts of Cp and Up on RNase T_2 digestion and pU on complete digestion with snake venom phosphodiesterase. Thus, 3′-terminal nucleotide was shown to be the same alternative sequence as in KB cells (Forget and Weissman, 1969). The sequence of spot 24 was determined from oligonucleotide produced by partial digestion with spleen phosphodiesterase. A partial digestion product, C-U-Gp, was determined from the data of RNase T_2 and snake venom phosphodiesterase digestion. Spots 35, 37, 53, and 56 could not be separated by fingerprinting RNase T_1 digest. However, they could be separated after simultaneous digestion of 5S RNA with RNase T_1 and alkaline phosphatase (pattern omitted). Spots 35 and 37 were found to be tetranucleotides and spots 53 and 56 were found to be hexanucleotides according to the results from RNase T_2 and snake venom phosphodiesterase digestion. Complete pancreatic RNase digestion was also performed. To determine these sequences, the overlapping spots were subjected to RNase U_2 digestion followed by electrophoresis on DEAE-cellulose paper at pH 1.9. The sequences of these split products were determined by RNase T_2 and by partial digestion with spleen phosphodiesterase. The sequences of spots 35, 37, 53, and 56 were thus determined by these combined data to be U-C-U-Gp, U-U-A-Gp, A-U-C-U-C-Gp, and U-C-U-A-C-Gp, respectively. Partial spleen phosphodiesterase digestion was required to determine the sequence of spot 55. Since spot 55 is a long oligonucleotide, spleen phosphodiesterase did not digest this oligonucleotide completely. By varying the condition of enzymatic digestion (incubation time and enzyme concentration), we could detect several split products. These split products were subjected to RNase T_2 and snake venom phosphodiesterase digestion to determine the sequence. The sequence of spot 55 was thus determined as C-C-A-U-A-C-C-A-C-C-C-U-Gp. Table I also shows that the kinds and molar yields of oligonucleotides produced by these two enzymes were exactly as expected from the primary structure obtained for KB cells (Forget and Weissman, 1969), except for spot 1′ which represented 5′-terminal nucleotide. This spot, when chromatographed on a Dowex-1 column after RNase T_2 digestion, eluted immediately after GTP at the position of ppGp. Dowex-1 column chromatography of snake venom phosphodiesterase digest of spot 1′ yielded two radioactive peaks, one agreeing with cold pGp marker and the other with inorganic phosphate. As snake venom phosphodiesterase is known to hydrolyze the bond between the α- and β-phosphate residues of a nucleotide 5′-diphosphate liberating inorganic phosphate (Zeller, 1950), spot 1′ was determined to be ppGp. Additional evidence as to the 5′-terminus was pro-

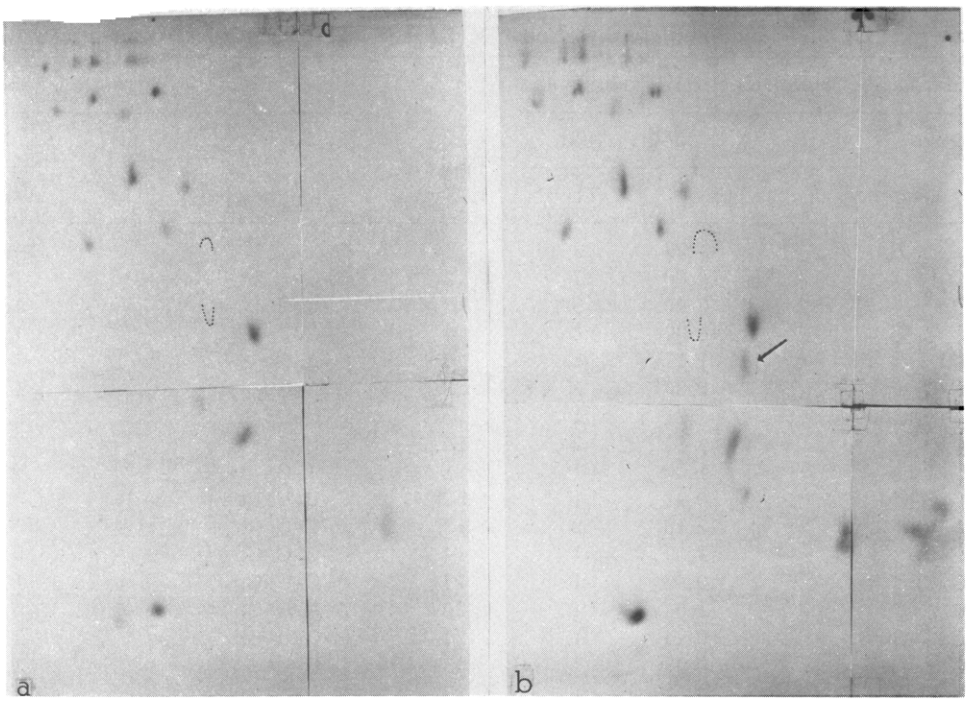

FIGURE 2: Autoradiographic comparison of pancreatic RNase digests of liver 5S RNA prepared with different procedures: (a) 5S RNA was extracted from ribosomes which had been prepared from postmitochondrial supernatant with deoxycholate (one-step procedure); (b) 5S RNA was extracted from ribosomes which had been prepared from once-pelleted microsomes with deoxycholate washing (two-step procedure). Numbers correspond to those in Figure 1. Newly appeared spot (24) is indicated by an arrow. Dotted circles show the blue marker; first dimension, from right to left; second dimension, from top to bottom.

vided by an analysis of the RNase T_2 digest of 5S RNA on a DEAE-cellulose column, in which the radioactivity was eluted in the region of the marker tetranucleotide. Dowex-1 column chromatography of this component revealed only one peak eluting immediately after the GTP marker. Since 5S RNA does not contain any 2′-O-methylated nucleotide, this peak which is eluted at the −4 region must be ppGp representing the 5′-terminus.

Comparison with Liver 5S RNA and a Possible Artifact. When the same fingerprinting technique was applied on 5S RNA of mouse liver, no significant differences were detected by either RNase between MH 134 and mouse liver. The molar yields were also very similar bettween them. It is therefore highly likely that the total sequence is identical in both MH 134 and mouse liver. Evidence has been presented which suggests some sequence heterogeneity in HeLa cell 5S RNA (Hatlen *et al.*, 1969). They have detected a trinucleotide, A-G-Cp, in the digestion products with pancreatic RNase which has not been found in human KB cells. We have encountered a similar pattern when the 5S RNA of the liver was extracted from a ribosomal preparation which had been purified from once-sedimented microsomal fraction with deoxycholate washing. In this procedure, the postmitochondrial supernatant was first centrifuged at 105,000*g* for 1.5 hr to sediment microsomes. The microsomal pellet was then suspended in buffer C and the solution adjusted to 0.6% with respect to deoxycholate. The ribosomes were obtained by re-centrifuging the solution at 105,000*g* for 1.5 hr. This procedure is designated as a *two-step* procedure (involving two ultracentrifugations) in contrast to the *one-step* standard procedure described under Materials and Methods. For 5S RNA of MH 134 cells, the oligonucleotides obtained with either enzyme and their molar yields are almost identical with those shown in Figure 1 and Table I irrespective of the isolation procedure of ribosomes. On the contrary, the oligonucleotide pattern of pancreatic RNase digest revealed distinct differences when 5S RNA was extracted from ribosomes prepared by the *two-step* procedure from mouse liver. First, a new spot appeared as shown in Figure 2. Second, some of the oligonucleotides showed different molar yields as shown in Tables II and III. The new spot, designated as spot 24, consisted of A-G-Cp and G-A-Cp and comprised approximately 0.5 mol each. Concomitantly with the appearance of these sequences, the molar yields of certain longer nucleotides, such as G-G-G-A-G-A-Cp (spot 19), were found to decrease considerably. It may be seen that the molar yields of these oligonucleotides obtained with the *two-step* procedure resemble well those of HeLa cells determined by column chromatography (Hatlen *et al.*, 1969). The relationship between these molar yields has been elegantly explained by the postulation that there may be alternative sequences in spot 18, G-G-A-A-G-Cp, in which the third base from the 5′ end has mutated from a purine to a pyrimidine. This would produce a new sequence, A-G-Cp, and at the same time an increase in the yield of the G-G-Cp or G-G-Up sequence. However, in mouse cells, the fact that the sequences A-G-Cp and G-A-Cp appear only when 5S RNA is extracted from ribosomes prepared by the *two-step* procedure almost excludes that reasoning, because from the nature of the procedure there is no possibility that the *two-step* procedure alone picks up 5S RNA population which has a Py-A-G-Cp or Py-G-A-Cp sequence in it and the *one-step* procedure does not. Contamination of 5S RNA with messenger or other unknown species of RNA cannot explain these changes since it should be even smaller in the ribosomes prepared by the *two-step* procedure than those prepared by the *one-step* procedure. Thus, we are led to seek the cause of these changes in the artifacts produced by the isolation procedure of ribosomes. For instance, a nick occurring between the

Table II: Comparison of Pancreatic RNase Digest of 5S RNA with Different Isolation Procedure of Ribosomes.[a]

Spot No. in Fig. 1	Sequence	Experimental Molar Yield			
			C3H Liver		
		MH 134[b]	1[c]	2[d]	HeLa[e]
11	G-G-Cp	1.00	0.89	1.84	1.2
15	G-G-Up	0.96	0.91	1.35	1.4
17	G-G-G-Cp	0.94	0.78	0.71	0.60
18	G-G-A-A-G-Cp	0.98	0.87	0.82	1.0[f]
21	A-G-G-G-Up	0.86	0.66	0.58	
19	G-G-G-A-G-A-Cp	0.84	0.83	0.46	0.63[g]
20	G-G-G-A-A-Up	1.03	0.94	0.76	0.55
24[i]	A-G-Cp + G-A-Cp	<0.1	<0.1	0.90	0.81[h]

[a] Only spots showing major changes are presented. [b] 5S RNA was extracted from ribosomes which had been prepared by the two-step procedure described in the text. The values are averages of three experiments. [c] 5S RNA was extracted from ribosomes which had been prepared by the one-step procedure described in the text. The values are averages of three experiments. [d] 5S RNA was prepared as in footnote *b*. The values are averages of four experiments. [e] From Hatlen *et al.* (1969). [f] (A-G-G-G)Up + (G-G-A-A-G)Cp. [g] (G-G-G,A-G)A-Cp. [h] Only A-G-Cp was detected in HeLa cells. [i] Shown in Figure 2b.

Table III: Comparison of RNase T_1 Digest of 5S RNA with Different Isolation Procedure of Ribosomes.

Spot No. in Fig. 1	Sequence	Experimental Molar Yield		
			C3H Liver	
		MH 134[a]	1[b]	2[c]
2	C-Gp	1.16	1.24	2.17
3	A-Gp	1.07	1.12	2.03
4	U-Gp	1.04	1.01	1.66
17	A-C-C-Gp	1.01	0.96	0.88
24	C-C-U-Gp	2.00	1.90	1.80
53,56	A-U-C-U-C-Gp + U-C-U-A-C-Gp	2.84	2.79	2.35
55	C-C-A-U-A-C-C-A-C-C-C-U-Gp	0.91	1.05	0.78

[a] From Table I. [b,c] See footnotes *c* and *d* of Table II.

fourth and fifth nucleotides from the 5′ end of G-G-G-A-G-A-Cp (spot 19) would release a new oligonucleotide, G-A-Cp. Although the amount of newly appeared spot 24 cannot be accounted for by the decrease in the amount of spot 19, another possible origin of spot 24 may be found in spot 18, which could release the A-G-Cp sequence by a nick at the middle of the oligonucleotide. Thus, it seems possible that the oligonucleotides in the new spot came from more than one site of 5S RNA by a few internal nicks. The remaining portion of the original oligonucleotide, *e.g.*, the G-G-G-Ap sequence in spot 19, may have been degraded into smaller pieces by further nicks and escaped the detection because of the low radioactivity. Indeed, there were several very faint spots that were not registered in the fingerprints of 5S RNA especially when it was derived from ribosomes prepared by the *two-step* procedure (Figure 2b). The origin of the increased molar yields of spot 11 (G-G-Cp) and spot 15 (G-G-Up) is not clear although small decrease in spots 17 (G-G-G-Cp) and 21 (A-G-G-G-Up) may account for it only in part. It is worth mentioning that the sequences which reduced the molar yields by the *two-step* procedure were mostly present as a loop in the postulated secondary structure of 5S RNA (Raacke, 1968; Forget and Weissman, 1969). These include spots 18, 19, and 20.

Taken altogether, we may reasonably conclude that the occurrence of spot 24 accompanied by the change in molar yields of certain oligonucleotides is the result of nucleolytic attack of 5S RNA during the course of ribosome preparation. The increase in shorter fragments (dinucleotides) in RNase T_1 digest concomitant with a decrease in some longer oligonucleotides (Table III) also argues for this contention.

Discussion

T_1 and pancreatic RNase "fingerprints" of 5S RNA from two mouse cell lines have been studied previously (Williamson and Brownlee, 1969). Both fingerprints were reported to have identical patterns with those from human KB cells (Forget and Weissman, 1969), suggesting strongly that the primary structure of 5S RNA from mouse cells was the same as that of human 5S RNA, although detailed data for sequence analysis have not been published since. Furthermore, fingerprint analysis of other animals including rat, rabbit (Labrie and Sanger, 1969), and rat kangaroo (Averner and Pace, 1972) suggests strongly the identity of 5S RNA structure in mammals.

On the other hand, some sequence heterogeneity was suggested in HeLa cells (Hatlen *et al.*, 1969) as well as in *Xenopus laevis* (Wegnez *et al.*, 1972; Ford and Southern, 1973) on the basis of oligonucleotide analysis. Indeed, even in *Escherichia coli* there are at least two major 5S RNA species with one base substitution (Brownlee *et al.*, 1968; Brownlee, 1972). Given much higher redundancy of 5S RNA genes in animal cells (Brown and Weber, 1968; Hatlen and Attardi, 1971), it may be natural to suspect some heterogeneity among them. If this be the case, it may possibly be reflected in the fingerprint of 5S RNA.

In the present study, all the oligonucleotides produced by either pancreatic RNase or RNase T_1 from 5S RNA of two types of mouse cells have been separated and their sequence determined. It was found, as Williamson and Brownlee (1969) had already found, that the overall mapping patterns and molar ratios were almost identical with those of KB cells and there was no difference between the MH 134 and liver cells. The molar yields of most oligonucleotides were close to unity, suggesting the near homogeneity of 5S RNA in these cells.

We have also found that when 5S RNA was extracted from ribosomes that had been prepared from once-pelleted microsomes of the liver (*two-step* procedure), a different fingerprint could really be obtained reproducibly, in which a new spot containing A-G-Cp and G-A-Cp appeared. However, significant amounts of these sequences appear only when 5S RNA is extracted from ribosomes which were subjected to more drastic treatment and to a longer chance of nucleolytic attack strongly indicating that the occurrence of these sequences together with some change in the molar

yields of certain oligonucleotides are artifacts of nuclease degradation. Similar consideration may apply to the case of HeLa cells (Hatlen *et al.*, 1969), since the molar yields of various oligonucleotides including A-G-Cp in HeLa cells agree rather closely with those of mouse liver 5S RNA isolated by the *two-step* procedure. In fact, in a fingerprint of HeLa cell 5S RNA made for other purposes, we have recently found a very faint spot corresponding to A-G-Cp and G-A-Cp, which is just barely detectable (Hayashi, Kominami, and Muramatsu, manuscript in preparation).

Our results are thus compatible with the notion that the 5S RNA in mammalian cells is mostly homogeneous and its primary structure conserved in all the mamlian species. However, one cannot conclude from these data that all the several thousand 5S RNA genes are *literally* identical since minor heterogeneity or divergence of sequences may not be detected by the present fingerprinting technique.

It is noteworthy that ppGp was detected as the 5′-terminus of mouse 5S RNA. Contradictory results have been reported as to the extent of phosphorylation at the 5′-terminus of 5S RNA in various organisms. Bacterial 5S RNAs apparently have a monophosphate at their 5′-termini (Forget and Weissman, 1969; DuBuy and Weissman, 1971; Soave *et al.*, 1973). This is compatible with the finding that they are cleaved from longer precursor molecules having extra sequences at the 5′ end of mature 5S RNA (Monier *et al.*, 1969; Pace *et al.*, 1973). On the other hand, a different extent of phosphorylation or a mixture of mono-, di-, and triphosphates was found at the 5′-termini of eukaryotes, such as HeLa cells (Hatlen *et al.*, 1969), *Saccharomyces carlsbergensis* (Hindley and Page, 1972), *Saccharomyces cerevisiae* (Soave *et al.*, 1973; Miyazaki, 1974), *Xenopus laevis* (Wegnez *et al.*, 1972), *Euglena gracilis*, wheat germ, rat liver, rabbit reticulocytes (Soave *et al.*, 1973), and *Torulopsis utilis* (Nishikawa and Takemura, 1974), although only one phosphate group was reported for KB cells (Forget and Weissman, 1969), *Potorous tridactylis* (Averner and Pace, 1972), and *Xenopus laevis* (Brownlee *et al.*, 1972). Considering the possible variation in the phosphatase activity in different cells, these inconsistent data may not be surprising. However, the presence of di- or triphosphate at the 5′-termini of eukaryotic 5S RNAs strongly indicates that these 5S RNAs are transcribed from the very 5′ end of the mature 5S molecule without any leader sequence, as discussed by Hatlen *et al.* (1969). On the other hand, should eukaryotic 5S RNAs be formed from a longer precursor by a similar mechanism as prokaryotes, there must be an unknown phosphorylation mechanism for this RNA which produces di- or triphosphate at the 5′-terminus of a polynucleotide.

Acknowledgments

We thank Dr. T. Uchida, at Mitsubishi Life Science Research Institute, for furnishing us with RNase U_2 and Dr. M. Kawada, Faculty of Science, Nagoya University, for the supply of spleen phosphodiesterase. We also wish to thank Drs. M. Sakai, R. Kominami, and K. Nii in this laboratory for their help in some aspects of these experiments and stimulating discussion. The investigation was supported in part by grants from the Ministry of Education of Japan.

References

Averner, M. J., and Pace, N. R. (1972), *J. Biol. Chem. 247,* 4491.

Brown, D. D., and Weber, C. S. (1968), *J. Mol. Biol. 34,* 661.

Brownlee, G. G. (1972), Determination of Sequences in RNA, North-Holland Publishing Co., Amsterdam, p 180.

Brownlee, G. G., Cartwright, E., McShane, T., and Williamson, R. (1972), *FEBS Lett. 25,* 8.

Brownlee, G. G., Sanger, F., and Barrell, B. G. (1968), *J. Mol. Biol. 34,* 379.

DuBuy, B., and Weissman, S. M. (1971), *J. Biol. Chem. 246,* 747.

Ford, P. J., and Southern, E. M. (1973), *Nature (London), New Biol. 241,* 7.

Forget, B. G., and Weissman, S. M. (1969), *J. Biol. Chem. 244,* 3148.

Hashimoto, S., and Muramatsu, M. (1973), *Eur. J. Biochem. 33,* 446.

Hatlen, L. E., Amaldi, F., and Attardi, G. (1969), *Biochemistry 8,* 4989.

Hatlen, L. E., and Attardi, G. (1971), *J. Mol. Biol. 56,* 535.

Hindley, J., and Page, S. M. (1972), *FEBS Lett. 26,* 157.

Labrie, F., and Sanger, F. (1969), *Biochem. J. 114,* 29P.

Miyazaki, M. (1974), *J. Biochem. 75,* 1407.

Monier, R., Feunteun, J., Forget, B., Jordan, B., Reynier, M., and Varricchio, F. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34,* 139.

Nishikawa, K., and Takemura, S. (1974), *FEBS Lett. 40,* 106.

Pace, N. R., Pato, M. L., Mckibbin, J., and Radcliffe, C. W. (1973), *J. Mol. Biol. 75,* 619.

Raacke, I. D. (1968), *Biochem. Biophys. Res. Commun. 31,* 528.

Sanger, F., Brownlee, G. G., and Barrell, B. G. (1965), *J. Mol. Biol. 13,* 373.

Soave, C., Nusca, R., Sala, E., Viotti, A., and Galante, E. (1973), *Eur. J. Biochem. 32,* 392.

Wegnez, M., Monier, R., and Denis, H. (1972), *FEBS Lett. 25,* 13.

Williamson, R., and Brownlee, G. G. (1969), *FEBS Lett. 3,* 306.

Zeller, E. A. (1950), *Arch. Biochem. 28,* 138.