Modified Nucleotides in T_1 RNase Oligonucleotides of 18S Ribosomal RNA of the Novikoff Hepatoma†

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ABSTRACT: The primary structure of 18S rRNA of the Novikoff hepatoma cells was investigated. Regardless of whether the primary sequence of 18S rRNA is finally determined by RNA sequencing methods or DNA sequencing methods, it is important to identify numbers and types of the modified nucleotides and accordingly the present study was designed to localize the modified regions in T_1 RNase derived oligonucleotides. Modified nucleotides found in 66 different oligonucleotide sequences included 2 $m^6{}_2A$, 1 m^6A, 1 m^7G, 1 m^1 cap$^3\psi$, 7 Cm, 13 Am, 9 Gm, 11 Um, and 38 ψ residues. A number of these modified nucleotides are now placed in defined sequences of T_1 RNase oligonucleotides which are now being searched for in larger fragments derived from partial T_1 RNase digests of 18S rRNA. Improved homochromatography fingerprinting (Choi et al. (1976) *Cancer Res. 36,* 4301) of T_1 RNase derived oligonucleotides provided a distinctive pattern for 18S rRNA of Novikoff hepatoma ascites cells. The 116 spots obtained by homochromatography contain 176 oligonucleotide sequences.

Recent progress in nucleic acid chemistry shows that nucleotide sequence of high molecular weight RNA can be obtained by direct sequence analysis of RNA (Brownlee, 1972) as with 16S rRNA[1] of *E. coli* (Fellner, 1974) and MS2 RNA (Fiers et al., 1976) or by deduction of RNA sequences from DNA sequences (Sanger & Coulson, 1975; Maxam & Gilbert, 1977) as for ϕX174 (Sanger et al., 1977) and the 5S RNA gene of yeast (Valenzuela et al., 1977; Maxam et al., 1977). For final structures, both sequence methods necessitate the determination of modified nucleotides which are products of posttranscriptional modification. Studies of rDNA are now providing important additions to rRNA sequence analysis. A cDNA of 28S rRNA of rat liver has been prepared with *E. coli* DNA polymerase I (Daubert & Dahmus, 1976). With plasmids, it has become possible to obtain a large quantity of rDNA (Morrow et al., 1974; McClements & Skalka, 1977). Accordingly, the methods of DNA sequencing (Sanger & Coulson, 1975; Maxam et al., 1977) can be applied to rDNA for study of rRNAs and pre-rRNAs.

Recent studies have provided much valuable structural information on rRNA and pre-rRNAs of eukaryotic cells (Choi et al., 1974; Hadjlov & Nikolaev, 1976). Analyses of modified nucleotides have clarified the patterns of posttranscriptional modification during synthesis of 45S pre-rRNA and the maturation of rRNA (Lane & Tamaoki, 1969; Choi & Busch, 1970; Egawa et al., 1971; Choi & Busch, 1974; Saponara & Enger, 1974; Maden & Salim, 1974; Maden et al., 1975; Hashimoto et al., 1975; Hughes et al., 1976). Structural differences in polypurine sequences (Seeber et al., 1971; Seeber & Busch, 1971a,b) and polypyrimidine sequences (Nazar & Busch, 1973, 1974) were found between rRNA species and their precursors. Sequences up to 160 nucleotides in length of T_1 RNase derived oligonucleotides were isolated from different rRNA and pre-rRNA species and their primary nucleotide sequences have been determined (Inagaki & Busch, 1972a,b; Eladari & Galibert, 1975; Fuke & Busch, 1975; Fuke et al., 1976; Choi et al., 1976; Fuke & Busch, 1977a,b; Maden & Robertson, 1974; Nazar & Busch, 1975; Nazar et al., 1975; Kahn & Maden, 1976; Kanamaru et al., 1972, 1974; Maden & Kahn, 1977).

In the present report, the modified nucleotides were characterized in 18S rRNA of Novikoff hepatoma cells. Emphasis was placed on locating the modified nucleotides in T_1 RNase derived oligonucleotides which were fractionated by improved homochromatography (Choi et al., 1976; Maden & Kahn, 1977). There are 83 modification sites found in 66 different oligonucleotides. They include 2 $m^6{}_2A$, 1 m^6A, 1 m^7G, 1 m^1cap$^3\psi$, 7 Cm, 13 Am, 9 Gm, 11 Um, and 38 ψ. Regardless of the methods of total sequence of 18S rRNA either by RNA sequence method or by DNA sequence method, the structural determination of modified nucleotides is a prerequisite for further work of 18S rRNA.

Materials and Methods

Preparation of ^{32}P-Labeled 18S rRNA from Novikoff Hepatoma Cells. The harvested Novikoff hepatoma ascites cells were incubated in modified Eagle's medium with [^{32}P]-orthophosphate as described previously (Mauritzen et al., 1971). To prepare uncontaminated 18S rRNA, polysomes were isolated (Prestayko et al., 1974); the ribosomes were dissociated into subunits (Prestayko et al., 1974), and the 40S subunits were purified by sucrose density gradient centrifugation (Prestayko et al., 1974). ^{32}P-labeled 18S rRNA was extracted by the phenol–sodium dodecyl sulfate method (Steele et al., 1965) at room temperature and further purified by sucrose density gradient centrifugation. The specific activity of the isolated 18S rRNA was approximately 500 μCi/mg (Kanamaru et al., 1974).

Enzyme Digestion and Fractionation of Resultant Oligonucleotides. The techniques and conditions for enzyme digestion were essentially similar to those described by Brownlee (1972) and Barrel (1971). For complete digestion with U_2 RNase, 20 μL of the enzyme solution containing U_2 RNase

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[1] Abbreviations used: rDNA, ribosomal DNA; rRNA, ribosomal RNA; pre-rRNA, pre-ribosomal RNA; m^7G, 7-methylguanylate; m^6A, 6-methyladenylate; $m^6{}_2A$, 6-dimethyladenylate; m^1cap$^3\psi$, 1-methyl-3-γ-(α-carboxy-α-aminopropyl)pseudouridylate; Am, 2′-*O*-methyladenylate; Cm, 2′-*O*-methylcytidylate; Gm, 2′-*O*-methylguanylate; Um, 2′-*O*-methyluridylate; "m^7G", 4-amino-5-(*N*-methyl)formamidoisocytidylate.

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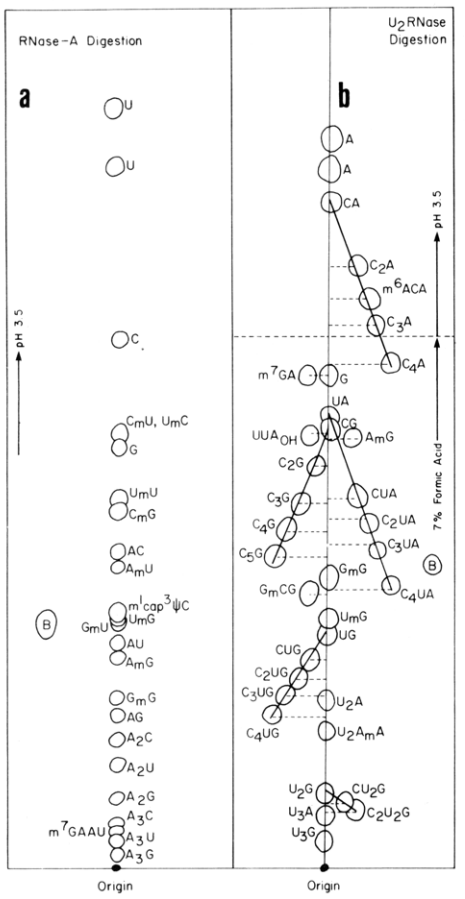


FIGURE 1: Electrophoretic mobilities of oligonucleotides on DEAE paper. (a) Mobilities at pH 3.5. The oligonucleotides were obtained by complete digestion with T_1 RNase and pancreatic RNase of 18S rRNA. B is the position of xylene cyanol FF. (b) Mobilities in 7% formic acid. Oligonucleotides were obtained by complete digestion with T_1 RNase and U_2 RNase of 18S rRNA. Mobilities of A, CA, C_2A, and C_3A are shown at pH 3.5 to represent two step electrophoresis. B is the position of xylene cyanol FF. The separation of a series of homologous sequences was presented in slanted manners to avoid crowded overlap of spots. Linear representation can be recognized by dotted lines drawn to midline.

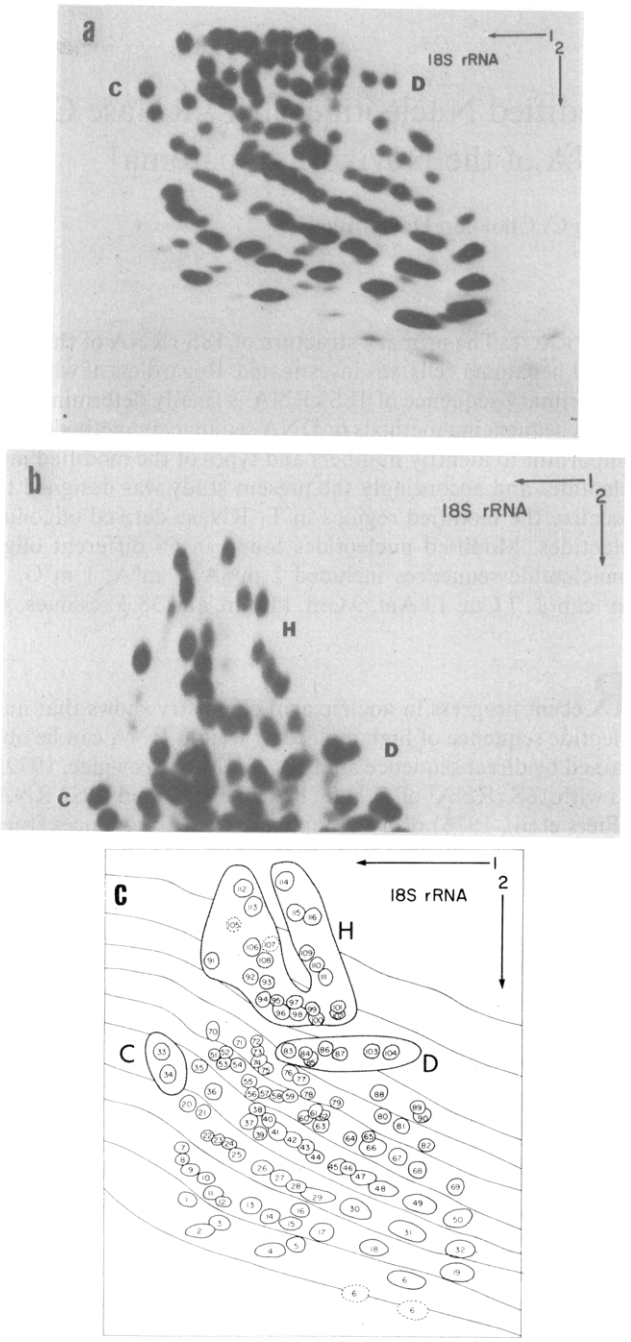


FIGURE 2: Homochromatographic fractionation of T_1 RNase derived oligonucleotides of 18S rRNA of Novikoff hepatoma cells. (a) Fractionation with C15 mixture. (b) Fractionation with C5 mixture. (c) Diagram of the fractionated T_1 RNase derived oligonucleotides of 18S rRNA based on the fractionations achieved with a combination of a and b. The characteristic regions H, C, and D are shaded and should be compared with a, b, and Figure 3.

(100 U/mL), T_1 RNase (500 U/mL), 0.05 M ammonium acetate, 2 mM EDTA at pH 4.5 was used, and the incubation period was 3–5 h at 37°C. Two-dimensional fractionation was performed by homochromatography fingerprinting for fractionation of oligonucleotides obtained by complete digestion of 18S rRNA (Busch et al., 1976). One-dimensional high voltage electrophoresis was performed on DEAE paper at pH 3.5 for the fractionation of pancreatic RNase digestion products of oligonucleotides recovered from homochromatography. For the fractionation of complete U_2 RNase digestion products of oligonucleotides recovered from homochromatography, one-dimensional high voltage electrophoresis was first performed at pH 3.5 and subsequently in 7% formic acid; or after electrophoresis in 7% formic acid, the portion of DEAE paper containing fast moving oligonucleotides was cut off, stitched to new DEAE paper, and subjected to electrophoresis at pH 3.5.

Figure 1 shows the different electrophoretic mobilities of various oligonucleotides on DEAE paper. To fractionate slow moving oligonucleotides on DEAE paper, the oligonucleotide spots were eluted and subjected to one-dimensional homochromatography. To further fractionate mixtures of oligonucleotides by thin-layer homochromatography, the oligonucleotides were eluted and subjected to stepwise salt gradient thin-layer chromatography (0.05, 0.1, 0.15, 0.2, and 0.25 M NaCl in 7 M urea, pH 3.5, with formic acid).

Recovery of Oligonucleotides from Papers and Thin Layers. Capillary techniques were employed for papers (Brownlee, 1972; Barrell, 1971) and Pasteur pipette techniques were employed for thin layers (Busch et al., 1976).

Determination of Nucleotide Composition and Modified Nucleotides. After alkaline hydrolysis or complete T_2 RNase digestion, high voltage electrophoresis was performed at pH 3.5 on Whatman 3 MM paper. For further fractionation for identification of modified nucleotides, chromatography was performed as a second dimensional fractionation using the

TABLE I: Relative Electrophoretic Mobility (R_u) and Chromatographic Migration (R_f) of Modified Nucleotides.[a]

Mononucleotides	R_u	R_f	Dinucleotides	R_u	R_f
Ap	0.44	0.50	AmpAp	0.57	0.27
Cp	0.26	0.59	AmpCp	0.55	0.32
Gp	0.81	0.45	AmpGp	0.80	0.23
Up	1.00	0.82	AmpUp	0.88	0.48
Amp	0.49	0.68	CmpAp	0.55	0.33
Cmp	0.30	0.73	CmpCp	0.37	0.37
Gmp	0.86	0.65	CmpGp	0.69	0.28
Ump	1.01	0.94	CmpUp	0.79	0.59
			GmpAp	0.81	0.24
$m^1cap^3\psi p$	1.04	0.85	GmpCp	0.70	0.30
m^6Ap	0.44	0.68	GmpGp	0.97	0.20
$m^6{}_2Ap$	0.43	0.73	GmpUp	1.11	0.41
m^7Gp	0.08	0.53	UmpAp	0.87	0.52
"m^7Gp"	0.87	0.59	UmpCp	0.78	0.59
ψp	0.95	0.65	UmpGp	1.10	0.51
			UmpUp	1.19	0.75

[a] Relative electrophoretic mobility (R_u) and chromatographic migration (R_f) were determined in a two-dimensional system. The first dimension was electrophoresis on Whatman 3 MM paper at pH 3.5 (5% acetic acid–ammonium hydroxide). The second dimension was paper chromatography using HCl–isopropyl alcohol–H_2O (16.5:65:19; Wyatt, 1955). Molar yields of the modified nucleotides (Appendix) were determined by analyses of T_1 RNase derived oligonucleotides, which were fractionated by homochromatography. (1) For identification of ^{32}P-labeled 2′-O-methylated mononucleotides and ^{32}P-labeled alkali-resistant dinucleotides, the standards were prepared from 1 g of rRNA from Novikoff hepatoma cells (Choi & Busch, 1970). The standards were characterized by an UV spectrum, paper chromatography (Choi & Busch, 1970), as well as mass spectrometric analyses of constituent nucleosides. (2) Standard ψp was obtained from P-L Biochemicals for identification of ^{32}P-labeled ψp. (3) A $m^1cap^3\psi p$ standard (Maden et al., 1975) was prepared from ^{32}P-labeled 17S rRNA of yeast (*Saccharomyces cerevisiae* strain S288C). The ^{32}P-labeled $m^1cap^3\psi pCp$ (Figure 1) was isolated after RNase A digestion. The R_u was identical with that of $m^1cap^3\psi p$ from yeast 17S rRNA after T_2 RNase digestion or after 0.3 N NaOH hydrolysis of 18S rRNA. (4) The assignments of $m^6{}_2Ap$ and m^6Ap were made on the basis of previous studies of Saneyoshi et al. (1969) and Brownless (1972) and by electrophoretic mobility on cellulose–acetate and DEAE-paper at pH 3.5. Their migration on cellulose–acetate is much slower than Ap and their migration on DEAE-paper is much faster than Ap (Adams & Cory, 1975). (5) The assignment of m^7Gp was based on the reports of Saneyoshi et al. (1969) and Brownlee (1972). Approximately 50% of m^7Gp was converted to "m^7Gp" when recovered after elution with 30% triethylaminecarbonate from DEAE-cellulose thin layer.

HCl–isopropyl alcohol–H_2O system (Wyatt, 1955). To determine the structure of alkali-resistant dinucleotides (NmpNp), the dinucleotides were treated with bacterial alkaline phosphatase to form the dinucleoside monophosphates. Then the dinucleoside monophosphates were either treated with snake venom phosphodiesterase for the determination of the 3′-terminal nucleoside 5′-phosphate or subjected to periodate oxidation followed by β elimination for the determination of the 5′-terminal nucleoside 3′-phosphate (Randerath et al., 1974). The modified nucleotides (Table I) of 18S rRNA were identified by their electrophoretic mobility and by chromatographic migration (Saponara & Enger, 1974; Maden & Salim, 1974; Maden et al., 1975; Hughes et al., 1976; Fellner, 1969; Saneyoshi et al., 1969).

Determination of Radioactivity. Radioactivity was detected by autoradiography. Quantitative analysis of isotope was determined with a Packard Tri-Carb liquid scintillation spectrometer Model 3375 (Kanamaru et al., 1974).

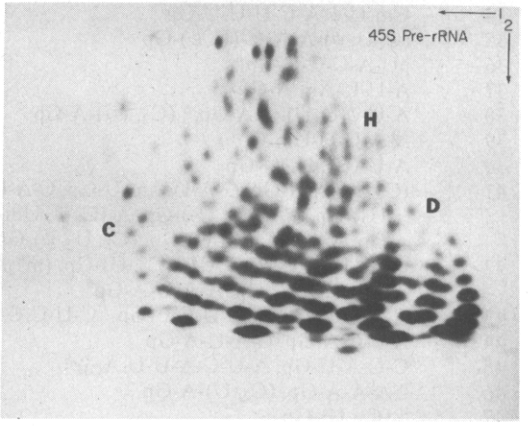


FIGURE 3: Homochromatographic fractionation of T_1 RNase derived oligonucleotides of 45S pre-rRNA (C5 mixture was used).

Results

Characteristic Pattern of Fractionated Oligonucleotides Obtained by Complete T_1 RNase Digestion of 18S rRNA. To obtain the most satisfactory fractionation of small and large T_1 RNase derived oligonucleotides, two different homochromatographic runs were made with C-15 and C-5 mixtures (Busch et al., 1976). Figure 2a shows a representative homochromatographic pattern of small T_1 RNase derived oligonucleotides (chain lengths = 1–10) obtained with the C-15 mixture. Figure 2b is a representative homochromatographic pattern of large T_1 RNase derived oligonucleotides (chain lengths > 10) obtained by prolonged homochromatography with the C-5 mixture. These two separate runs were very effective to define a consistent number of fractionated oligonucleotide spots.

Figure 2c shows a diagram based on the combination of these two separate fractionations performed in ten separate experiments. There are three clustered regions of oligonucleotides which provide a characteristic pattern of 18S rRNA of Novikoff hepatoma ascites cells. These were designated as H, D, and C.

Comparison of 18S rRNA with 45S Pre-RNA. To determine whether the characteristic pattern of 18S rRNA distinguishes oligonucleotides of 18S rRNA from those of other rRNA, homochromatography fingerprints of 45S rRNA were also made (Figure 3). As reported earlier (Choi et al., 1976),

TABLE II: Oligonucleotides in T_1 RNase Digests of 18S rRNA.[a]

Spot no.	Partial sequence	Spot no.	Partial sequence
1	ψ-U-Gm-Gp[j]	59	A-C-(C, U_2)-A-Gp
2	3 U-U-Gp, (U, ψ)-Gp	60	U-A-A-A-A-Gp
3	Gm-U-Gp[j]	61	A-A-A-C-U-Gp, A-U-A-A-C-Gp
4	19 U-Gp, Um-Gp	62	2 A-A-U-C-A-Gp, (C_3, U_2)-A-Gp, A-A-U-(C, U)-A-Gp
5	Gm-Gp		
6	120 Gp	63	A-U-A-C-C-Gp, (C_4, U_2)-Gp
7	2 (C, U_4)-Gp	64	(C, U)-A-C-C-Gp
8	A-U-U-U-Gp	65	A-C-A-A-Gp, A-A-A-C-Gp
9	U-A-U-U-Gp, (U, ψ)-A-U-Gp, U-U-U-A-Gp	66	U-C-C-C-C-Gp,[f] C-A-A-A-Gp, A-C-A-A-Gp
10	(C, U_3)-Gp	67	C-C-A-A-Gp, A-C-A-C-Gp, A-C-C-A-Gp
11	3 A-U-U-Gp, A-U-ψ-Gp, A-Um-U-Gp	68	3 A-C-C-C-Gp
12	U-A-ψ-Gp	69	C-C-C-C-Gp
13	3(C, U_2)-Gp, (C, U)-Um-Gp, (C, U, ψ)-Gp	70	U-U-A-ψ-U-A-A-A-Gp, [U-A, ψ-A,-(C_2, U_2)-A]-U-Gp
14	7 A-U-Gp		
15	5 U-A-Gp, U-Am-Gp	71	C-A-U-C-A-ψ-(U, ψ)-A-Gp
16	2 A-Gm-Gp	72	(C, U)-A-(Um-A, A)-Cm-A-U-Gp[j]
17	20 (C, U)-Gp	73	[A, (C, U)-A, (C, U_2)-A]-Gp
18	30 A-Gp, Am-Gp	74	(A, Um-A)-U-(C_2, U)-A-Gp
19	40 C-Gp, Cm-Gp	75	U-U-Am-A-A-A-A-Gp[j]
20	(C, Um-C, U_2, ψ)-Um-Gp	76	(C_2, $ψ_2$)-A-A-A-Gp, (C, U)-A-A-C-U-A-Gp
21	(C, U_4)-A-Gp	77	[C-A, (C, U)-A]-C-U-Gp
22	U-A-A-U-U-Gp	78	U-A-m^6A-C-A-A-Gp[j]
23	A-U-(C, U, ψ)-Gp	79	U-A-A-C-C-C-Gp
24	C-A-U-(U, ψ)-Gp	80	C-U-C-C-C-C-Gp,[j] C-C-C-U-C-C-Gp[f]
25	A-A-U-U-Gp, (C_2, U_3)-Gp	81	A-C-C-A-C-Gp
26	U-U-A-C-Gp, (C, U, U)-A-Gp	82	C-C-C-A-C-Gp
27	A-U-A-Gp, A-A-U-Gp, A-A-ψ-Gp, 2(C_2, U_2)-Gp	83	A-U-A-C-(C_2, U)-Gp
28	U-A-A-Gp	84	A-C-C-C-A-U-(C, U)-Gp
29	3 A-U-C-Gp, 3 A-C-U-Gp, 3(C, U)-A-Gp, 3 C-A-U-Gp	85	[C-C-A, (C_2, U)-A]-U-Gp, [(C, U)-A, (C, U)-A]-C-C-Gp
30	9 A-A-Gp, Am-A-Gp, 10 (C_2, U)-Gp, (C_2, ψ)-Gp	86	m^7G-A-A-U-(C_3, ψ)-A-Gp[j]
31	12 A-C-Gp, 10 C-A-Gp, A-Cm-Gp	87	U-A-C-A-C-A-C-C-Gp
32	20 C-C-Gp	88	C-C-C-C-U-C-C-Gp[f]
33	U-U-C-U-A-U-U-U-U-Gp[i]	89	A-C-C-C-C-C-Gp
34	Um-U-ψ-A-C-U-U-U-Gp[i]	90	C-C-A-C-C-C-Gp
35	(C, U, ψ)-A-A-U-(U, ψ)-Gp	91	U-U-U-U-C-A-U-U-A-A-U-C-A-A-Gp[i]
36	pU-A-C-C-U-Gp[h]	92	C-U-C-C-U-C-U-C-C-U-A-C-U-U-Gp[i]
37	A-U-U-Am-A-Gp[j]	93	C-U-C-Am-U-U-A-A-A-ψ-C-A-Gp[f,j]
38	A-U-ψ(or U)-A-A-Gp,[b] (C_2, U_3)-A-Gp	94	A-A-A-A-A-A-ψ-ψ-A-Gp
39	A-A-C-U-Um-Gp	95	A-A-A-C-U-U-A-A-A-Gp
40	A-U-(C_2, U, ψ)-Gp	96	[U, A-A, (C_3, U)]-A-Gm-C-Gp[j]
41	(C, U_2)-A-A-Gp, C-A-U-Am-U-Gp, C-A-(C, U_2)-Gp, 3 A-(C_2, U_2)-Gp, A-(C, U, Um-C)-Gp, A-(C, U, Cm-U)-Gp, (C_3, U_2, ψ)-Gp	97	A-A-C-C-C-C-A-U-(C, U)-Gp
		98	(C, A-A-C, A-A-U)-A-Gm-Gp[j]
		99	(A-ψ, A-A-A-C, C, A-C)-Gp
42	C-A-U-(C, U)-Gp, A-U-(C_2, U)-Gp, (m^6_2A-m^6_2A-C, U)-Gp, A-A-U-A-Gp	100	(C_2, U)-A-(C_2, U)-A-C-Gp, [(C_3, U)-A, (C, U)-A]-C-Gp
43	A-A-C-U-Gp, U-C-U-C-C-Gp,[f] C-U-C-C-U-Gp[f]	101	A-A-A-C-C-A-A-A-Gp
44	A-ψ-C-A-Gp, C-A-U-A-Gp	102	A-A-C-(m^1cap^{3}ψ-C, A-C)-A-C-G
45	C-C-A-U-Gp, A-U-C-A-U-U-A_{OH}[h]	103	C-C-C-C-C-U-C-C-C-Gp[i]
46	2 A-A-A-Gp, (C_2, U)-A-Gp	104	C-A-C-C-A-C-C-A-Gp[i]
47	5 (C_3, U)-Gp	105	0.1 U-C-C-A-C-(U, U, ψ)-A-A-Am-U-C-C-U-U-Up[i]
48	2 A-Am-C-Gp, 2 A-A-C-Gp, 2 A-C-A-Gp, 2 C-A-A-Gp		
		106	C-A-A-U-U-A-U-ψ-C-C-C-C-A-U-Gp[i]
49	2 A-C-C-Gp, 3 C-A-C-Gp, 2 C-C-A-Gp	107	0.2 U-C-C-C-C-C-A-A-C-U-Um-C-U-U-A-Gp[c,i]
50	10 C-C-C-Gp, C-Cm-C-Gp,[g] Cm-C-C-Gp[g]		
51	A-A-A-U-(C, U_2, ψ)-Gp	108	C-C-C-U-A-U-C-A-A-C-U-U-U-C-Gp[i]
52	U-A-Am-U-(C, U_2)-A-Gp,[j] (U, ψ)-A-A-U-(C_2, U)-Gp, C-C-U-U-U-U-C-U-C-Gp[f]	109	C-U-A-C-C-A-C-A-U-Cm-C-A-A-Gp[i]
		110	A-C-C-C-C-C-C-U-U-C-C-C-Gp[i]
		111	A-A-A-C-C-U-A-C-C-C-Gp[i]
53	A-C-U-U-C-U-C-U-Gp[f]	112	U-C-C-A-C-(U, U, Ψ-A-A-Am-U)-C-C-U-U-U-A-A-C-Gp[i]
54	A-A-U-A-A-ψ-Gm-Gp[j]		
55	(Am-U, C, C)-A-U-U-Gp		
56	A-A-ψ-A-A-ψ-Gp	113	U-C-C-C-C-C-A-A-C-U-Um-C-U-U-A-Gm-A-Gp[c,i] (see spot 107)
57	A-U-A-A-C-U-Gp		
58	(C, U, ψ)-A-A-A-Gp, [U-A, (C, U)-A]-A-Gp	114	A-A-A-A-A-U-A-A-C-A-A-U-A-C-A-Gp[i]
		115	C-Am-A-A-U-U-A-C-C-C-A-C-U-C-C-C-Gp[i]
		116	A-U-C-A-A-A-A-C-C-A-A-A-C-C-C-Gp[i]

[a] Molar yields of oligonucleotides were calculated by two criteria: (1) yield of isotope in Gp and modified nucleotides; and (2) comparison of relative yields among different oligonucleotides in a given spot. The references cited show origins of some analyses of footnotes *e–h*. [b] Molar yield of ψ is 0.5. [c] Position of ψ is not determined in C-U-Um-C-U-U-Ap. [d] Spots were separated and their sequences were determined individually (spots 45, 95–99, 101, 102). [e] Spots were not separated; the sequences were deduced from the fragments shown in the Appendix (spots 9, 11, 25–27, 29–31, 38, 41, 44, 46, 48, 49, 61–67, 70, 76, 80). [f] Nazar & Busch, 1973. [g] Maden & Salim, 1974. [h] Eladari & Galibert, 1975. [i] Fuke & Busch, 1977. [j] Maden & Khan, 1977.

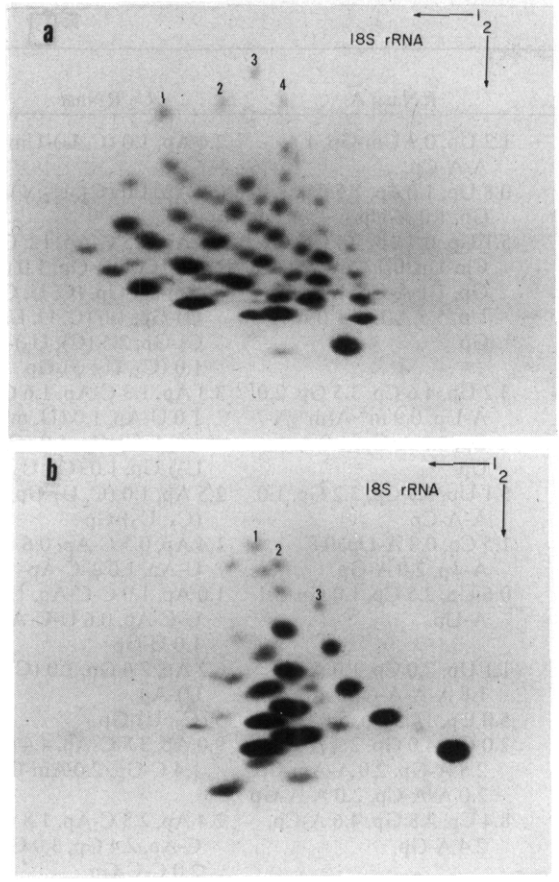


FIGURE 4: Homochromatographic fractionation of pyrimidine sequences and A-repeating sequences. (a) Fractionation of oligonucleotides obtained by combined digestion of 18S rRNA with T_1 RNase and U_2 RNase (C15 mixture was used). (1) (C_4, U_5)-Gp; (2) (C_6, U_4)-Ap; (3) (C_9, U_2)-Gp; (4) (C_8, U)-Gp. (b) Fractionation of oligonucleotides obtained by combined digestion of 18S rRNA with T_1 RNase and RNase A (C15 mixture was used). (1) A-A-A-A-A-A-ψp; (2) Am-A-A-A-A-Gp; (3) A-A-A-A-Cp.

in 45S pre-RNA the marker oligonucleotides of 18S rRNA (C, D, and H) are clearly distinguishable from other oligonucleotides.

Examination of Pyrimidine and A-Repeating Sequences. Prior to characterization of T_1 RNase oligonucleotides shown in Figures 2a and 2b, similar homochromatography fingerprints were also made for pyrimidine and A-repeating sequences. Figure 4a shows the pattern for the U_2 digest of the T_1 RNase oligonucleotides of 18S rRNA. Spots noted by numbers are (1) (C_4, U_5)-Gp in spot 52; (2) (C_6, U_4)-Ap in spot 92; (3) (C_9, U_2)-Gp in spot 110; and (4) (C_8, U)-Gp in spot 103.

Migration in the first electrophoretic dimension reflects the net negative charge per mass at pH 3.5; Up > Gp > Ap > Cp. Migration in the second dimension, DEAE-cellulose thin layers, reflects the net negative charge (ionic bonding) and hydrophilic and hydrophobic bonding of nucleotides, i.e., Up > Cp > Ap > Gp (Ling, 1972).

Figure 4b shows the types of A-containing oligomers derived by pancreatic RNase digestion of the T_1 RNase fragments. The corresponding sequences were found in oligonucleotides shown in Figure 2c. Among the A-containing oligomers in Figure 4b are the numbered sequences which were found in oligonucleotides in Figure 2c: (1) A-A-A-A-A-A-ψp in spot 94; (2) Am-A-A-A-A-Gp in spot 75; and (3) A-A-A-A-Cp in spot 116.

Characterization of T_1 RNase Oligonucleotides of 18S rRNA. Table II lists the complete and partial sequences of oligonucleotides of 18S rRNA. These findings were based on the two different types of analyses. The first is an analysis of the nucleotide composition of spots by two-dimensional fractionation (electrophoresis on Whatman 3 MM paper at pH 3.5 and paper chromatography by the system of HCl–isopropyl alcohol–H_2O). The second is the analysis of secondary digestion products (RNase-A or U_2 RNase) by electrophoresis on DEAE paper or by homochromatography. The mobility shifts of oligonucleotides were also used for analyses of composition and sequences (see Appendix). The mobility shifts noted in homochromatography (Figure 2c) were very useful in characterization of T_1 RNase derived oligonucleotides of 18S rRNA.

TABLE III: 2′-O-Methylated Nucleotides.

Modified nucleotide	Form of alkali stable dinucleotide	Found in spot no.
Amp	AmpAp	30, 37, 75, 115
	AmpCp	48 (2 mol)
	AmpGp	15, 18
	AmpUp	41, 52, 55, 93, 112
Cmp	CmpAp	72
	CmpCp	50 (2 mol), 109
	CmpGp	19, 31
	CmpUp	41
Gmp	GmpAp	113
	GmpCp	96
	GmpGp	1, 5, 16 (2 mol), 54, 98
	GmpUp	3
Ump	UmpAp	72, 74
	UmpCp	20, 41, 113
	UmpGp	4, 13, 20, 39
	UmpUp	11, 34

Characterization of Modified Nucleotides. Prior to the determination of modified nucleotides in the secondary digestion products, it was necessary to locate the modified nucleotides in the fractionated spots.

After alkaline hydrolysis or T_2 RNase digestion, all the oligonucleotide spots were examined by two-dimensional fractionation (electrophoresis and chromatography) for detection of alkali-resistant dinucleotides, base methylated nucleotides, and pseudouridylate. Subsequently, alkali resistant dinucleotides were converted to dinucleoside monophosphates with alkaline phosphatase and were subjected to β elimination to form 2′-*O*-methyl nucleoside 3′-phosphates or were treated with snake venom phosphodiesterase to form nucleoside 5′-phosphates. These mononucleotides were characterized by electrophoresis and chromatography. Analysis of alkali resistant dinucleotides showed no base modified nucleotides were present.

1. *2′-O-Methylated Nucleotides.* Table III shows 40 sites of 2′-O-methylation found in 18S rRNA. Most of them exist in T_1 RNase derived oligonucleotides with a relationship of 1 mol per oligonucleotide except spots 20, 72, and 113. The 2′-O-methylated nucleotides were identified as alkali-resistant dinucleotides. There are 16 kinds of alkali-resistant dinucleotides in 18S rRNA.

2. *Pseudouridylate.* There are 38 sites of pseudouridylate formation as a result of rearrangement of the uracil base. Examination of this modified nucleotide in T_1 RNase derived oligonucleotides showed that the distribution is mostly related

TABLE IV: Supplementary Results from Enzymatic Analyses of the Spots.

Spot no.	RNase A	U_2 RNase
1	1.1 Up, 1.0 ψp, 1.0 Gm-Gp	(U, ψ)-Gm-Gp
2	7.2 Up, 0.8 ψp, 4.0 Gp	3.0 U-U-Gp, 1.0 (U, ψ)-Gp
3	1.0 Gp, 1.0 Gm-Up	Gm-U-Gp
4	19.0 Up, 18.0 Gp, 1.0 Um-Gp	19.0 U-Gp, 1.0 Um-Gp
5	0.5 Gm-Gp	0.5 Gm-Gp
6	120 Gp	120 Gp
7	2.0 Cp, 7.4 Up, 2.0 Gp	2 (C, U_4)-Gp
8	2.4 Up, 1.0 Gp, 0.8 A-Up	1.0 Ap, 1.0 U-U-U-Gp
9	5.6 Up, 0.8 ψp, 1.8 Gp, 2.0 A-Up, 0.9 A-Gp	1.3 Gp, 0.9 U-Ap, 0.7 U-Gp, 0.8 (U, ψ)-Ap, 1.0 U-U-Gp, 1.0 U-U-U-Ap
10	2.7 Up, 0.9 Cp, 1.0 Gp	(C, U_3)-Gp
11	3.2 Up, 0.7 ψp, 4.6 Gp, 4.4 A-Up, 1.2 A-Um-Up	5.1 Ap, 1.0 (U, ψ)-Gp, 1.0 Um-U-Gp, 3.0 U-U-Gp
12	1.2 Up, 1.0 Gp, 1.3 A-ψp	1.0 U-Ap, 1.2 ψ-Gp
13	7.2 Up, 1.0 ψp, 4.8 Cp, 4.0 Gp, 0.8 Um-Gp	3.0 (C, U_2)-Gp, 1.0 (C, U)-Um-Gp, 1.2 (C, U, ψ)-Gp
14	7.0 Gp, 8.4 A-Up	7.0 Ap, 7.0 U-Gp
15	5.5 Up, 1.0 Am-Gp, 5.0 A-Gp	5.2 Gp, 4.2 U-Ap, 1.0 U-Am-Gp
16	1.5 A-Gm-Gp	1.2 Ap, 1.5 Gm-Gp
17	18.0 Up, 20.0 Cp, 20.0 Gp	20 (C, U)-Gp
18	1.0 Am-Gp, 30.3 A-Gp	31.1 Ap, 30.0 Gp, 1.0 Am-Gp
19	39.6 Cp, 38.0 Gp, 1.0 Cm-Gp	1.0 Cm-Gp, 39.7 C-Gp
20	2.4 Up, 1.0 ψp, 1.2 Cp, 1.2 Um-Cp, 1.0 Um-Gp	(C, U_2, ψ, Um-C)-Um-Gp
21	3.8 Up, 1.1 Cp, 1.0 A-Gp	1.0 Gp, 1.0 (C, U_4)-Ap
22	2.2 Up, 1.0 Gp, 1.0 A-A-Up	1.0 Ap, 0.7 U-Ap, 1.0 U-U-Gp
23	1.2 Up, 1.0 ψp, 1.2 Cp, 1.0 Gp	1.1 Ap, 1.0 (C, U_2, ψ)-Gp
24	1.2 Up, 1.0 ψp, 0.9 Cp, 1.0 Gp, 1.1 A-Up	0.6 C-Ap, 1.0 (U_2, ψ)-Gp
25	3.5 Up, 1.4 Cp, 2.0 Gp, 0.9 A-A-Up	2.8 Ap, 1.3 U-U-Gp, 0.6 (C_2, U_3)-G
26	3.6 Up, 1.1 Cp, 1.1 Gp, 1.0 A-Cp 1.0 A-Gp	1.0 Gp, 0.9 C-Gp, 1.0 U-U-Ap, 1.0 (C, U_2)-Ap
27	3.7 Up, 3.6 Cp, 3.9 Gp, 1.0 A-Up, 1.0 A-Gp, 1.4 A-A-Up, 1.0 A-A-ψp	5.2 Ap, 1.3 Gp, 1.0 U-Ap, 1.3 U-Gp, 1.3 ψ-Gp, 2.0 (C_2, U_2)-Gp
28	1.4 Up, 1.0 A-A-Gp	1.0 Ap, 1.4 Gp, 1.0 U-Ap
29	6.6 Up, 8.4 Cp, 8.7 Gp, 3.0 A-Cp, 6.2 A-Up, 3.0 A-Gp	6.6 Ap, 3.0 C-Ap, 3.3 Gp, 3.0 (C, U)-Ap, 3.0 U-Gp, 6.1 (C, U)-Gp
30	8.8 Up, 1.0 ψp, 22.0 Cp, 10.0 Gp, 1.0 Am-A-Gp, 9.0 A-A-Gp	16.0 Ap, 1.0 Am-Ap, 10.0 Gp, 10.0 (C_2, U)-Gp, 1.0 (C_2, ψ)-Gp
31	10.3 Cp, 10.2 Gp, 11.0 A-Cp, 10.0 A-Gp, 1.0 A-Cm-Gp	10.1 Ap, 10.2 C-Ap, 11.0 Gp, 12.0 C-Gp, 1.0 Cm-Gp
32	41.0 Cp, 20.0 Gp	20 C-C-Gp
33	5.9 Up, 1.3 Cp, 1.0 Gp, 0.5 A-Up	1.0 (C, U_3)-Ap, 1.0 U-U-U-U-Gp
34	3.3 Up, 0.8 ψp, 0.8 Gp, 1.0 Um-Up, 0.9 A-Cp	1.0 (Um-U, ψ)-Ap, 1.0 (C, U_3)-Gp
35	2.4 Up, 2.0 ψp, 1.0 Cp, 0.8 Gp, 1.1 A-A-Up	1.0 Ap, 0.8 (C, U, ψ)-Ap, 1.0 (U_2, ψ)-Gp
36	1.4 Up, 1.2 Cp, 1.0 Gp, 0.8 A-Cp, 0.8 pUp	1.0 pU-Ap, 1.0 (C_2, U)-Gp
37	1.2 Up, 1.0 A-Up, 0.8 Am-A-Gp	0.8 Ap, 1.0 Gp, 1.0 U-U-Am-Ap
38	2.8 Up, 0.4 ψp, 2.0 Cp, 1.5 A-Up, 0.7 A-Gp, 1.0 A-A-Gp	1.8 Ap, 2.3 Gp, 1.4 ($U_{1.5}$, $\psi_{0.5}$)-Ap, 1.0 (C_2, U_3)-Ap
39	1.2 Up, 0.9 Um-Gp, 1.4 A-A-Cp	2.4 Ap, 1.0 (C, U)-Um-Gp
40	0.8 Up, 1.0 ψp, 1.5 Cp, 1.1 Gp, 1.0 A-Up	0.9 Ap, 1.0 (C_2, U_2, ψ)-Gp
41	5.1 Up, 0.7 ψp, 7.4 Cp, 0.7 Cm-Up, 0.7 Um-Cp, 7.8 Gp, 1.1 A-Cp, 1.2 Am-Up, 5.5 A-Up, 1.0 A-A-Gp	6.6 Ap, 1.5 C-Ap, 1.5 Gp, 1.0 (C, U_2)-Gp, 1.0 (U, Am-U)-Gp, (C, U, Cm-U)-Gp, 1.0 (C, U, Um-C)-Gp, 2.5 (C_2, U_2)-Gp, 1.0 (C_3, U_2, ψ)-Gp
42	3.2 Up, 4.6 Cp, 3.5 Gp, 2.0 A-Up, 0.9 m^6_2A-m^6_2A-Cp, 0.9 A-Gp, 1.0 A-A-Up	3.3 Ap, 1.3 C-Ap, 1.6 Gp, 1.0 U-Ap, 1.0 (U, m^6_2A-m^6_2A-C)-Gp, 1.0 (C, U_2)-Gp, 1.0 (C_2, U_2)-Gp
43	5.1 Up, 5.9 Cp, 3.2 Gp, 1.0 A-A-Cp	2.5 Ap, 1.0 (C, U)-Gp, 2.0 (C_3, U_2)-Gp
44	1.5 Cp, 0.8 A-Up, 0.8 A-ψp, 2.0 A-Gp	1.4 Ap, 0.5 C-Ap, 0.6 U-Ap, 1.0 ψ-C-Ap
45	0.6 Up, 2.5 Cp, 1.0 Gp, 3.1 A-Up	1.0 Ap, 1.0 C-C-Ap, 1.0 U-C-Ap, 0.6 U-U-A_{OH}, 1.0 U-Gp
46	1.1 Up, 2.0 Cp, 1.0 A-Gp, 1.8 A-A-A-Gp	6.2 Ap, 2.6 Gp, 1.0 (C_2, U)-Ap
47	5.0 Up, 14.5 Cp, 5.0 Gp	5 (C_3, U)-Gp
48	2.0 Cp, 4.0 Gp, 2.4 A-Cp, 2.8 A-Gp, 2.0 A-Am-Cp, 2.0 A-A-Cp, 2.0 A-A-Gp	9.0 Ap, 3.8 C-Ap, 4.4 Gp, 1.4 C-Gp, 2.0 Am-C-Gp
49	8.4 Cp, 4.8 Gp, 4.6 A-Cp, 2.4 A-Gp	2.4 Ap, 2.8 C-Ap, 1.8 C-C-Ap, 2.4 Gp, 3.0 C-Gp, 2.0 C-C-Gp
50	32.0 Cp, 2.0 Cm-Cp, 11.8 Gp	9.6 C-C-C-Gp, 2.0 (Cm-C, C)-Gp
51	2.1 Up, 0.7 ψp, 1.4 Cp, 0.7 Gp, 1.0 A-A-A-Up	2.7 Ap, 1.0 (C, U_3, ψ)-Gp
52	9.0 Up, 1.0 ψp, 6.0 Cp, 2.0 Gp, 1.2 A-Gp, 1.2 A-Am-Up, 1.0 A-A-Up	1.5 Ap, 1.4 Gp, 1.4 U-Ap, 1.2 (U, ψ)-Ap, 0.8 (C_2, U_2)-Gp, 1.0 (C, U_2, Am-U)-Ap, 1.0 (C_4, U_5)-Gp
53	4.4 Up, 2.1 Cp, 1.0 Gp, 0.5 A-Cp	0.8 Ap, 1.0 (C_3, U_4)-Gp
54	0.8 Gm-Gp, 1.0 A-A-Up, 0.8 A-A-ψp	2.9 Ap, 0.7 U-Ap, 0.6 ψ-Gm-Gp
55	1.1 Up, 2.0 Cp, 1.3 Gp, 1.0 Am-Up, 1.4 A-Up	1.0 (C_2, Am-U)-Ap, 1.0 U-U-Gp
56	1.0 Gp, 2.1 A-A-ψp	3.1 Ap, 1.0 ψ-Ap, 1.0 ψ-Gp
57	1.1 Up, 1.0 Gp, 0.9 A-Up, 1.1 A-A-Cp	2.2 Ap, 1.0 U-Ap, 1.0 C-U-Gp
58	1.8 Up, 0.7 ψp, 2.0 Cp, 1.1 A-Up, 1.0 A-A-Gp, 1.4 A-A-A-Gp	3.2 Ap, 1.5 Gp, 0.6 U-Ap, 0.6 (C, U)-Ap, 1.0 (C, U, ψ)-Ap
59	2.3 Up, 1.1 Cp, 0.9 A-Cp, 1.0 A-Gp	1.2 Ap, 0.8 Gp, 1.2 (C_2, U_2)-Ap
60	0.7 Up, 0.9 A-A-A-A-Gp	3.1 Ap, 1.0 Gp, 0.4 U-Ap
61	0.7 Up, 2.0 Gp, 1.4 A-Up, 1.4 A-A-Cp, 1.2 A-A-A-Cp	4.8 Ap, 0.6 U-Ap, 0.6 C-Gp, 1.0 (C, U)-Gp
62	3.2 Up, 6.0 Cp, 4.0 A-Gp, 2.8 A-A-Up	5.9 Ap, 3.9 Gp, 2.1 (C, U)-Ap, 0.8 (C, U_2)-Ap, 1.0 (C_3, U_2)-Ap
63	2.2 Up, 5.5 Cp, 1.8 Gp, 1.0 A-Cp, 1.2 A-Up	1.4 Ap, 1.0 U-Ap, 1.0 C-C-Gp, 1.0 (C_4, U_2)-G
64	1.1 Up, 1.2 Cp, 1.0 Gp, 1.0 A-Cp	1.1 C-C-Gp, 1.0 (C, U)-Ap
65	1.0 Gp, 0.9 A-Cp, 1.0 A-A-Gp, 0.6 A-A-A-Cp	4.9 Ap, 1.1 C-Ap, 1.4 Gp, 1.0 C-Gp
66	1.4 Up, 5.5 Cp, 1.2 Gp, 1.0 A-A-A-Gp	2.3 Ap, 0.9 C-Ap, 1.0 Gp, 1.0 (C_4, U)-Gp
67	3.7 Cp, 1.3 Gp, 3.4 A-Cp, 1.2 A-Gp 1.0 A-A-Gp	2.2 Ap, 0.8 C-Ap, 1.0 C-C-Ap, 1.3 Gp, 0.8 C-Gp
68	7.8 Cp, 3.0 Gp, 3.0 A-Cp	3.0 Ap, 3.3 C-C-C-Gp
69	4.2 Cp, 1.0 Gp	C-C-C-C-Gp

TABLE IV (*Continued*)

Spot no.	RNase A	U_2 RNase	Spot no.	RNase A	U_2 RNase
70	4.9 Up, 2.1 Cp, 1.0 Gp, 2.0 A-ψp, 2.0 A-Up, 1.0 A-A-A-Gp	1.5 Ap, 1.2 Gp, 0.8 U-Ap, 0.8 ψ-Ap, 1.0 U-Gp, 0.8 (U, ψ)-Ap, 0.8 U-U-Ap, 1.2 (C_2, U_2)-Ap	96	1.1 Up, 3.1 Cp, 1.0 Gp, 1.1 A-Gm-Cp, 1.0 A-A-Up	1.5 Ap, 0.9 U-Ap, 0.9 (C_3, U)-Ap, 1.0 Gm-C-Gp
			97	0.8 Up, 4.0 Cp, 1.2 A-Up, 0.9 A-A-Cp	2.0 Ap, 1.2 C-C-C-C-Ap, 1.0 (C, U_2)-Gp
71	1.2 Up, 1.0 ψp, 2.0 Cp, 0.9 A-Up, 0.9 A-ψp, 1.0 A-Gp	1.0 C-Ap, 0.8 Gp, 1.0 (C, U)-Ap, 1.2 (U, $ψ_2$)-Ap	98	0.6 Cp, 0.9 A-A-Cp, 1.0 A-A-Up, 1.0 A-Gm-Gp	2.0 Ap, 1.6 C-Ap, 1.0 U-Ap, 1.0 Gm-Gp
			99	1.5 Cp, 1.0 Gp, 1.1 A-Cp, 1.1 A-ψp, 0.9 A-A-A-Cp	3.9 Ap, 1.0 C-C-Ap, 1.0 ψ-Ap, 1.0 C-Gp
72	1.3 Up, 1.3 Cp, 0.8 Gp, 0.8 (Um-A, Cm-A)-A-A-Up	0.8 Ap, 0.7 Cm-Ap, 0.6 Um-Ap, 1.0 (C, U)-Ap, 0.9 U-Gp	100	4.2 Up, 7.1 Cp, 2.0 Gp, 4.6 A-Cp	2.0 C-Gp, 0.9 (C, U)-Ap, 1.9 (C_2, U)-Ap, 1.0 (C_3, U)-Ap
73	2.0 Up, 1.6 Cp, 0.9 A-Cp, 1.0 A-Up, 1.0 A-Gp	1.6 Ap, 1.5 Gp, 0.9 (C, U)-Ap, 1.0 (C, U_2)-Ap	101	1.0 Cp, 1.0 A-A-A-Cp, 1.0 A-A-A-Gp	4.4 Ap, 0.7 C-C-Ap, 1.0 Gp
74	1.6 Up, 1.9 Cp, 1.0 A-Gp, 1.0 A-Um-A-Up	1.8 Ap, 0.8 Gp, 0.8 Um-Ap, 1.0 (C_2, U_2)-Ap	102	1.0 Gp, 2.1 A-Cp, 1.0 $m^1cap^3ψ$-Cp, 1.0 A-A-Cp	1.9 Ap, 1.0 C-Ap, 1.0 C-Gp, 0.6 ($m^1cap^3ψ$-C, C)-Ap
75	2.4 Up, 1.3 Am-A-A-A-A-Gp	2.4 Ap, 1.0 Gp, 1.0 U-U-Am-Ap	103	1.1 Up, 9.1 Cp, 1.0 Gp	(C_8, U)-Gp
76	2.8 Up, 2.0 ψp, 2.6 Cp, 1.0 A-Gp, 0.8 A-A-Cp, 0.6 A-A-A-Gp	3.6 Ap, 2.0 Gp, 2.2 (C, U)-Ap, 1.0 (C_2, $ψ_2$)-Ap	104	3.0 Cp, 1.7 A-Cp, 1.0 A-Gp	1.0 C-Ap, 2.0 C-C-Ap, 1.1 Gp
77	1.9 Up, 2.3 Cp, 1.0 Gp, 2.1 A-Cp	0.8 C-Ap, 1.0 (C, U)-Ap, 1.0 (C, U)-Gp	105	5.5 Up, 1.1 ψp, 4.5 Cp, 1.1 A-Cp, 1.0 A-A-Am-Up	0.8 Ap, 0.8 (C_2, U)-Ap, 1.3 (C, U_2, ψ)-Ap, 1.0 (C_2, U_3, Am-U)
78	1.4 Up, 1.0 A-m^6A-Cp, 1.0 A-A-Gp	1.4 Ap, 1.0 m^6A-C-Ap, 1.4 Gp, 1.0 U-Ap	106	0.9 Up, 0.8 ψp, 5.2 Cp, 1.0 Gp, 1.9 A-Up, 1.0 A-A-Up	1.4 Ap, 0.7 C-Ap, 1.0 U-Gp, 1.0 U-U-Ap, 0.8 U-(C_4, ψ)-Ap
79	1.0 Up, 1.9 Cp, 1.0 Gp, 0.8 A-A-Cp	1.2 Ap, 1.0 U-Ap, 1.0 C-C-C-Gp	107	4.0 Up, 4.8 Cp, 1.0 Um-Cp, 1.0 A-Gp, 1.0 A-A-Cp	1.0 Ap, 1.0 Gp, 1.1 (C_5, U)-Ap, 1.0 (C, U_2, ψ, Um-C)-Ap
80	2.4 Up, 9.4 Cp, 2.0 Gp	1.6 (C_5, U)-Gp			
81	1.2 Cp, 0.8 Gp, 1.5 A-Cp	1.1 Ap, 0.8 C-C-Ap, 1.0 C-Gp			
82	3.3 Cp, 1.0 Gp, 0.9 A-Cp	0.7 C-C-C-Ap, 1.0 C-Gp	108	4.3 Up, 5.3 Cp, 1.0 Gp, 1.4 A-Up, 1.0 A-A-Cp	1.0 Ap, 1.0 (C, U)-Ap, 0.8 (C_3, U)-Ap, 1.0 (C_2, U_3)-Gp
83	1.3 Up, 2.2 Cp, 1.0 Gp, 1.2 A-Cp 1.6 A-Up	1.6 Ap, 1.0 U-Ap, 1.1 (C_3, U)-G			
84	1.3 Up, 3.4 Cp, 1.0 Gp, 1.0 A-Cp, 1.0 A-Up	1.0 Ap, 1.3 C-C-C-Ap, 1.6 (C, U_2)-Gp	109	0.9 Up, 2.0 Cp, 0.9 Cm-Cp, 1.8 A-Cp, 1.0 A-Up, 1.0 A-A-Gp	1.7 Ap, 1.1 C-Ap, 1.1 C-C-Ap, 1.4 Gp, 1.0 (C, U)-Ap, 1.0 (U, Cm-C)-Ap
85	2.8 Up, 6.4 Cp, 2.0 Gp, 3.0 A-Cp, 1.4 A-Up	0.8 C-C-Ap, 1.0 C-C-Gp, 1.8 (C, U)-Ap, 0.8 (C_2, U)-Ap, 1.0 U-Gp	110	1.9 Up, 6.9 Cp, 1.0 Gp, 1.0 A-Cp	1.4 Ap, 1.0 (C_9, U_2)-Gp
86	1.0ψp, 2.9 Cp, 1.0 A-Gp, 0.8 m^7G-A-A-Up	1.4 Ap, 0.7 Gp, 0.7 m^7G-Ap, 1.0 U-(C_3, ψ)-Ap	111	1.1 Up, 3.6 Cp, 1.0 Gp, 1.1 A-Cp, 1.2 A-A-A-Cp	3.6 Ap, 1.0 C-C-C-Gp, 0.9 (C_2, U)-Ap
87	1.0 Up, 1.4 Cp, 1.0 Gp, 3.0 A-Cp	2.0 C-Ap, 1.0 U-Ap, 1.0 C-C-Gp	112	6.2 Up, 1.2 ψp, 4.1 Cp, 1.0 Gp, 1.1 A-Cp, 1.1 A-A-Cp, 1.0 A-A-Am-Up	1.6 Ap, 0.9 C-Gp, 0.7 (C_2, U)-Ap, 1.1 (C, U_2, ψ)-Ap, 1.0 (C_2, U_3, Am-U)-Ap
88	1.2 Up, 7.0 Cp, 1.0 Gp	(C_6, U)-Gp			
89	4.5 Cp, 1.0 Gp, 0.8 A-Cp	1.0 Ap, 1.0 C-C-C-C-C-Gp			
90	4.7 Cp, 1.0 Gp, 1.0 A-Cp	1.0 C-C-Ap, 1.0 C-C-C-Gp	113	3.2 Up, 1.0 ψp, 4.7 Cp, 0.7 Um-Cp, 1.0 A-A-Cp, 1.0 A-Gm-A-Gp	1.3 Ap, 1.3 Gp, 0.9 Gm-Ap, 0.7 (C_5, U)-Ap, 1.0 (C, U_2, ψ, Um-C)-Ap
91	4.9 Up, 1.7 Cp, 1.1 A-Up, 1.0 A-A-Up, 0.9 A-A-Gp	2.0 Ap, 1.0 Gp, 0.9 (C, U)-Ap, 1.1 U-U-Ap, 1.2 (C, U_4)-Ap	114	1.0 A-Cp, 1.0 A-Gp, 1.0 A-A-Cp, 1.2 A-A-Up, 1.0 A-A-A-A-A-Up	7.6 Ap, 2.0 C-Ap, 1.4 Gp, 2.0 U-Ap
92	5.2 Up, 5.6 Cp, 0.8 Gp, 1.0 A-Cp	1.0 (C, U_2)-Gp, 1.0 (C_6, U_4)-Ap			
93	1.4 Up, 2.3 Cp, 0.8 Am-Up, 1.0 A-Gp, 0.8 A-A-A-ψp	2.2 Ap, 1.2 Gp, 0.9 (C, ψ)-Ap, 1.0 (C_2, U_2, Am-U)-Ap	115	2.1 Up, 6.3 Cp, 1.0 Gp, 2.2 A-Cp, 1.3 A-Am-A-Up	1.3 Ap, 0.9 C-Am-Ap, 0.8 C-C-C-Ap, 1.0 U-U-Ap, 1.1 (C_4, U)-Gp
94	1.1 ψp, 1.0 A-Gp, 1.0 A-A-A-A-A-A-ψp	6.4 Ap, 1.3 Gp, 1.0 ψ-ψ-Ap	116	4.1 Cp, 1.0 Gp, 1.2 A-Up, 1.2 A-A-Cp 1.1 A-A-A-Cp	5.6 Ap, 1.0 C-C-Ap, 1.0 (C, U)-Ap, 1.0 C-C-C-Gp
95	2.2 Up, 1.0 A-A-A-Cp, 1.0 A-A-A-Gp	4.5 Ap, 1.0 Gp, 1.0 (C, U_2)-Ap			

to 1 mol of ψ per one T_1 RNase derived oligonucleotide and maximal occurrence in one oligonucleotide is two. Twenty-seven moles of pseudouridylate was found in a relationship of 1 mol of ψ per T_1 RNase oligonucleotide in spots 1, 2, 9, 11, 12, 13, 20, 23, 24, 27, 30, 34, 40, 41, 44, 51, 52, 54, 58, 70 (2 mol), 86, 93, 99, 106, 112, and 113. Ten moles was detected in a relationship of 2 mol per oligonucleotide in spots 35, 56, 71, 76, and 94. Spot 38 was in submolar amounts.

3. *Base Methylated Nucleotides.* There were five major sites of base methylation. The occurrence of base methylated nucleotides is lowest among modified nucleotides.

(a) *6-Methyl Adenylate.* Spot 78 contained a single oligonucleotide; nucleotide composition was (A_3, m^6A, C, U)-G. RNase A digestion had 1 Up, 1 A-m^6A-Cp, and 1 A-A-Gp. U_2 RNase digestion for longer than 5 h showed 2 Ap, 1 m^6A-C-Ap, 1 Gp, and 1 U-Ap. It is interesting to observe that m^6A-C-Ap is resistant to U_2 RNase. The sequence was deduced to be U-A-m^6A-C-A-A-Cp.

(b) *6-Dimethyl Adenylate.* Spot 42 consisted of a mixture of oligonucleotides which contained 2 mol of $m^6{}_2$A. RNase A digestion produced $m^6{}_2$A-$m^6{}_2$A-Cp. U_2 RNase digestion for more than 5 h produced a U_2 RNase resistant oligonucleotide. This resistant oligonucleotide had a somewhat faster electrophoretic mobility than U-U-Gp on DEAE paper in 7% formic

acid. Its nucleotide composition was (2 m^6_2A, 1–2 Cp, Up)-Gp. One-dimensional homochromatography showed a similar mobility to (C_3, U_2)-Gp. From this evidence, the partial sequence was deduced to be (m^6_2A-m^6_2A-C, U)-Gp. The sequence (m^6_2A-m^6_2A-C-U-Gp) was found earlier in 18S rRNA of HeLa cells (Maden & Salim, 1974). However, it is an interesting peculiarity that the sequence A-A-C-U-Gp in spot 43 had a slower electrophoretic mobility than (m^6_2A-m^6_2A-C, U)-Gp despite the fact that m^6_2Ap had a slower electrophoretic mobility than Ap (Adams & Cory, 1975).

(c) *7-Methyl Guanylate.* Spot 86 contained a single oligonucleotide with 1 mol of m^7G and its degradation product "m^7G" had a similar electrophoretic and chromatographic mobility to Um-Ap which could readily be differentiated by alkaline phosphatase treatment. Its nucleotide composition was (m^7G, 3A, 3C, U, ψ)-Gp. RNase A digestion produced 1 ψp, 3 Cp, 1 m^7G-A-A-Up, and 1 A-Gp. Prolonged U_2 RNase digestion produced 1 Ap, m^7G-Ap, 1 Gp, and 1 U-(C_3, ψ)-Gp. The partial sequence deduced was m^7G-A-A-U-(C_3, ψ)-A-Gp.

(d) *1-Methyl-3-γ-(α-carboxy-α-aminopropyl)pseudouridylate.* Spot 102 was readily purified by salt gradient thin-layer chromatography at pH 3.5 in the presence of 7 M urea and was found to contain 1 mol of the hypermodified nucleotide. This hypermodified nucleotide was first described for 18S rRNA of Chinese hamster cells and was characterized by the method of chemical degradation (Saponara & Enger, 1974). Subsequently, the nucleotide sequence of T_1 RNase derived oligonucleotide was determined in 17S rRNA of yeast (Maden et al., 1975). RNase A digestion produced 1 Gp, 2 A-Cp, RNase A resistant fragment 1 $m^1cap^3\psi$-Cp, and 1 A-A-Cp. U_2 RNase digestion produced 2 Ap, 1 C-Ap, 1 C-Gp, and 1 ($m^1cap^3\psi$-C, C)-Ap. The partial sequence obtained is A-A-C-($m^1cap^3\psi$-C, A-C)-A-C-Gp which has a remarkable similarity to A-A-C-$m^1cap^3\psi$-C-A-C-A-C-Gp found in 17S rRNA of yeast (Maden et al., 1975). This structural similarity indicates clearly its well-conserved sequence through evolution.

Number of Modifications of Oligonucleotides. Examination of yields of oligonucleotides and yields of modified nucleotides present in oligonucleotides made it possible to define the number of modification sites which included sites for full modification and sites for undermodification. As described above, a total of 83 modification sites were found which were classified into 40 sites for 2′-O-methylation, 38 sites for pseudouridylation, and 5 sites for base methylation. Most of the modification sites were found fully modified except spots 5, 16, 38, and 107.

Spots 5 (Gm-Gp) and 16 (A-Gm-Gp) had a consistently low yield, 0.4–0.6 mol for Gm-Gp and (1.2–1.5 mol for A-Gm-Gp. Spot 107 had a 0.2 molar yield which is derived from spot 113 containing two modification sites (Um-Cp and Gm-Ap). It was readily found that the relationships of these two spots 107 and 113 were associated with one undermodification site (Gm-Ap) (Fuke & Busch, 1977a,b). It is interesting to observe that these partial 2′-O-methylations occur at G. Among 38 sites for pseudouridylation in 18S rRNA, spot 38 showed underpseudouridylation.

Oligonucleotides Produced by Specific Breakdown of 18S rRNA. Spot 105 contains an oligonucleotide which is derived from spot 112. The sequence is similar but lacks A-A-C-Gp. The oligonucleotide is present consistently. The molar yield is less than 0.1 mol. However, when 18S rRNA was prepared from the 40S ribosomal subunit stored for 1 week at −20 °C, this oligonucleotide appeared. Thus the oligonucleotide in spot 105 may offer a site for specific cleavage of 18S rRNA.

Useful Markers for 18S rRNA Sequence. Spots 33–34, 83–87, and 91–116 are clustered regions of spots which are useful for identification of 18S rRNA by homochromatography fingerprinting.

These oligonucleotides are readily recognized in 45S pre-RNA (Choi et al., 1976). These are characterized by various modified nucleotides.

It was previously shown that these oligonucleotides reflect evolutionary trends (Fuke et al., 1976). It was also shown that these oligonucleotides are useful for evaluation of fidelity of in vitro transcriptional system (Ballal et al., 1977).

Discussion

The present report is an extension of earlier studies from this laboratory (Fuke & Busch, 1975; Fuke et al., 1976) which are designed to complete the nucleotide sequence of 18S rRNA. The aim of this work was to establish locations of modified nucleotides and to obtain fundamental information on the primary structure of 18S rRNA. This work can now be extended to 18S rRNA or rDNA complementary to 18S rRNA.

Based on the improved method of homochromatography and the partial characterization of all the oligonucleotides studied, some important characteristics of 18S rRNA were observed.

1. The homochromatographic definition of characteristic pattern (the oligonucleotides at the regions of C, D and H) of 18S rRNA indicates some oligonucleotides are specific markers for 18S rRNA (Fuke & Busch, 1975).

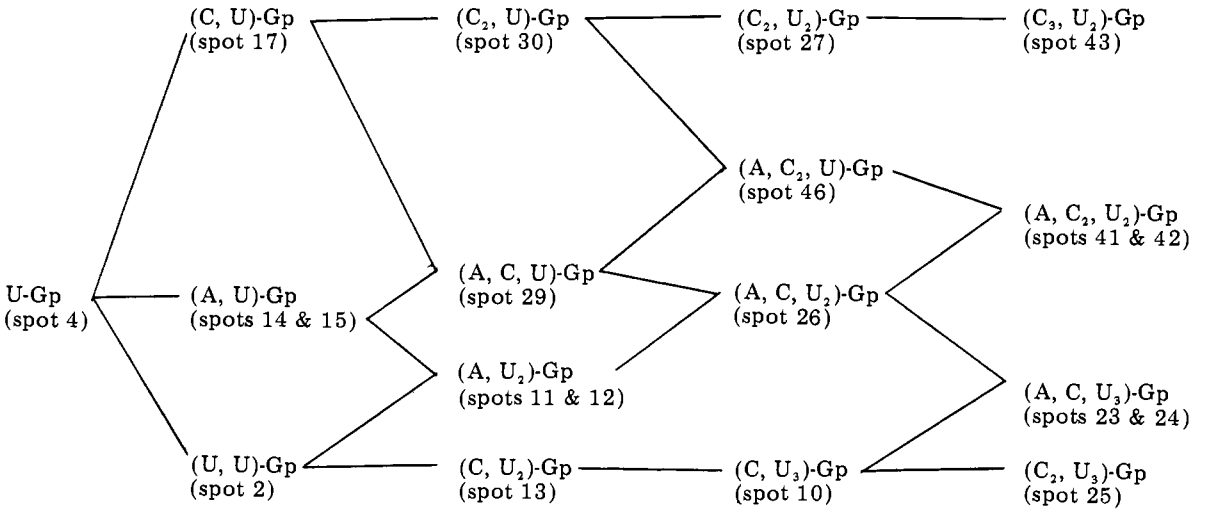
2. The partial characterization of types of oligonucleotides shows the location of modified nucleotides which are valuable for definition of posttranscriptional modification of rRNA synthesis.

3. The examination of oligonucleotides indicates that there are 176 types of oligonucleotides among which 66 types of oligonucleotides contain 83 modified nucleotides. The examination is based solely on the qualitative examination of oligonucleotides.

The localization of various modified nucleotides in 18S rRNA of Novikoff hepatoma provides clear evidence for nonrandom modification of rRNA. Structural homologies have been found in *E. coli* 16S rRNA and yeast 17S rRNA. The sequence (m^6_2A-m^6_2A-C, U)-Gp of 18S rRNA is homologous to the sequence m^6_2A-m^6_2A-C-C-U-Gp of *E. coli* 16S rRNA (Fellner, 1969), and m^6_2A-m^6_2A-C-U-C-Gp of yeast 17S rRNA (de Jonge et al., 1977). The sequence A-A-C-($m^1cap^3\psi$-C, A-C)-A-C-Gp of 18S rRNA was not found in *E. coli* 16S rRNA but is like the sequence A-A-C-$m^1cap^3\psi$-C-A-C-A-C-Gp of 17S rRNA of yeast (Maden et al., 1975). These structural homologies suggest that evolution of prokaryotic 16S rRNA to mammalian 18S rRNA involves mechanisms in which some prokaryotic and lower eukaryotic sequences are conserved. These homologous sequences may be required for protein synthesis or binding of very important ribosomal proteins.

The functional role of modified nucleotides in rRNAs has not been clarified. However, there is clear evidence for a role of modified nucleotides in tRNAs. The sequence of the G-T-ψ-C loop is specifically recognized by 50S ribosomal subunit (Richter et al., 1973). Methylation of phenylalanine tRNA, especially N^2-methylguanylate, gives rise to a modulation site of aminoacylation kinetics (Roe et al., 1973). Accordingly, modified nucleotides in tRNAs are apparently recognition sites for proteins. From these observations, it is reasonable that the modified nucleotides in rRNA are involved in recognition of interacting ribosomal proteins. Undermethylation of 45S

SCHEME I.



pre-rRNA is associated with low efficiency of processing and maturation of rRNAs (Hadjiolov & Nikolaev, 1976).

Although the detailed mechanism of modification and the recognition sites for modification are not known, there are some recognizable trends for the formation of modified nucleotides. For example, Table I shows that all four nucleotides can be 2′-O-methylated (7 Cm, 13 Am, 9 Gm, and 11 Um). It is interesting that no special trend was noted for nearest neighbors to base-substituted nucleotides. RNase cleavage was noted in spots 42, 78, 86, and 102. The 7 substitution of G is known to confer resistance to T_1 and U_2 RNase (Uchida & Egami, 1967). The 3 substitution of pyrimidines (Szer & Shugar, 1961) and 1 substitution of A (Uchida et al., 1970) make these modified nucleotides resistant to endonucleases. The N-6 substitution of A produced resistance to U_2 RNase. Apparently U_2 RNase activity is altered by the presence of substituted N^6 or N^7 in purine nucleotides.

The partial cleavage of spot 112 (spot 105) suggests that this oligonucleotide is partially cleaved in the 40S subunit. Oligonucleotide 105 varied in yield up to 10%. The consistent presence of spot 105 suggests that oligonucleotide 112 is localized in an endonuclease accessible position in the 40S subunit.

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Appendix

Two representative pyrimidine sequences are shown as tracts. One is (Cn, U)-Gp (n = 0–8) which is located in spots 4, 17, 30, 47, 66, 80, 88, and 103 except that (C_7, U)-Gp is absent. The other is (Cn, U_2)-Gp (n = 0–4) which is located in spots 2, 13, 27, 43, and 63 (see Table IV).

An example of progression from U-Gp is shown in Scheme I.

Spots 41 and 42 contained a number of oligonucleotides which were not fractionated. However, when a series of spots 37–50 was compared in terms of nucleotide composition, clear relationships were found which were valuable for deriving sequences. One was (C_3)-Gp (spot 50), (C_3, U)-Gp (spot 47), (C_3, U_2)-Gp (spot 43), (C_3, U_2, ψ)-Gp (spot 41). The second was (A_3)-Gp (spot 46), (A_3, U)-Gp (spot 42), and (A_3, U, ψ)-Gp (spot 38) and (A_2, Am, U_2)-Gp (spot 37). The third was (A_2, C, U_2)-Gp, which exists as modified structural isomers (spots 39 and 41), and (A, C_2, U_2)-Gp, which exists as modified structural isomers (spots 41 and 42). The sequence (U, Am-U)-Gp in spot 41 was suggested to be C-A-U-Am-U-Gp on the basis of those relationships.

Several possible structures existed for spot 70 which contained two sequences which are not structural isomers. RNase A digestion produced 5 Up, 2 Cp, 1 Gp, 2 A-ψp, 2 A-Up, and 1 A-A-A-Gp. U_2 RNase digestion produced 2 Ap, 1 Gp, 1 U-Ap, 1 ψ-Ap, 1 U-Gp, 1 (U, ψ)-Ap, 1 U-U-Ap, and 1 (C_2, U_2)-Ap. The results indicate that two sequences terminated in -A-U-Gp and -A-A-A-Gp. Spot 71 has the composition (C_2, A_3, U_2, ψ_2)-Gp. From the mobility shift, the best fit for partial sequences of spot 70 was U-U-A-ψ-U-A-A-A-Gp and [U-A, ψ-A, (C_2, U_2)A]-U-Gp.

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