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Evolutionary trends in 18S ribosomal RNA nucleotide sequences of rat, mouse, hamster and man.

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ABSTRACT

The large T1 ribonuclease fragments of 18S ribosomal RNA from four mammalian species, rat, mouse, hamster and man, were compared by two-dimensional homochromatography fingerprinting. The nucleotide sequences of the large T1 ribonuclease fragments, polypyrimidines and polypurines which were different among the four mammalian species were determined and compared. The method used for determining nucleotide sequences utilizes ^{32}P-labeling of oligonucleotides at their 5'-termini by polynucleotide kinase, partial digestion by ribonucleases and analysis of labeled spots by homochromatography-fingerprinting. Several examples of point mutations were detected. It was of interest that the 18S rRNA of Chinese hamster has more oligonucleotide sequences in common with those of man than rat or mouse.

INTRODUCTION

Much information has been accumulated regarding evolutionary trends of amino acid sequences of proteins (1), but there is little information about evolutionary trends of nucleic acid base sequences. In a comparative study on the primary structures of 16S ribosomal RNAs from 27 prokaryotes, Woese et al (2) localized several major conserved regions. The 3'-terminal sequence of 16S rRNA was studied extensively (3,4). Eukaryotic 5S ribosomal RNA nucleotide sequences were compared (5,6) and no differences were found in the sequence of 5S rRNA from several mammals, i.e., rat, mouse, rabbit and man (7,8). The large

oligonucleotide sequences of 5.8S rRNA from HeLa and Novikoff rat ascites hepatoma were found to be the same except for minor differences at their termini, which may reflect processing differences (9). The 3'-terminal sequence, ---GAUCAUUA$_{OH}$ (10-12) and the 5'-terminal sequence, pUACCUG--- (12,13) of 18S rRNA from several eukaryotes were found to be conserved. Many methylated nucleotide sequences were also found conserved (14-20). Previously we reported a difference in the nucleotide sequences of 18S rRNAs from human HeLa cells and rat Novikoff ascites hepatoma cells (21). In this study, T1 RNase digests of 18S and 28S rRNAs were analyzed from four mammalian species, i.e., rat, mouse, Chinese hamster and man. Large polypyrimidine and polypurine fragments were also compared for the 18S rRNAs of these four species.

MATERIALS AND METHODS

^{32}P-labeled ribosomal RNA. Novikoff rat ascites hepatoma and HeLa cells were grown as described earlier (21). The mouse L1210 cell line was obtained from Southern Research Institute, Birmingham, Alabama. The Chinese hamster ovary cell line was originally obtained from Dr. T. T. Puck's laboratory. Karyotype analysis by Dr. Samuel A. Ramirez confirmed the origins of the four cell lines. The cells were labeled with ^{32}P in a low phosphate medium (10^{-4} M) for 24 hours at a concentration of 20 μCi of ^{32}P/ml and washed twice with 50 mM-Tris (pH 7.5), 50 mM-KCl and 5 mM-$MgCl_2$. The cells were broekn by a Tissumizer® (model SDT, Tekmar Co., Cincinnati, Ohio) for 3 minutes at top speed. Cell debris

and nuclei were removed by centrifugation at 15,000 rpm for 15 minutes (Sorvall SS1 rotor). The supernatant was layered over 0.5 volumes of 1 M-sucrose and centrifuged at 35,000 rpm for 18 hours (Beckman Type 35 rotor). Ribosomal subunits were prepared by homogenization of the polysome pellet in 8 mM-EDTA and 5 mM-Tris (pH 7.5), and 40S and 60S subunits were separated by centrifugation through a 5% to 50% linear sucrose gradient in 10 mM-Tris (pH 7.5) at 26,000 rpm for 18 hours (Beckman SW27 rotor). 18S and 28S rRNAs were extracted from 40S and 60S ribosomal subunits, respectively with phenol equilibrated with 0.1 M-NaCl, 0.1 M-Tris (pH 7.5) and 0.01 M-EDTA.

Digestion. Three kinds of digestion were performed. A reaction mixture (100 μl) containing 100 μg of RNA and 10 units of T1 RNase (Sankyo, Tokyo) in 10 mM-Tris (pH 7.5) and 1 mM-EDTA was incubated at 37^{o}C for 2 hours to get T1 RNase fragments. To get polypyrimidine fragments, combined U2 RNase and T1 RNase digestion was carried out. 10 units of U2 RNase (Sankyo) was added to the above reaction mixture and the mixture was kept at 37^{o}C for 2 hours. Polypurine fragments were produced by pancreatic RNase. 100 μl of reaction mixture containing 100 μg of RNA and 10 units of pancreatic RNase (Worthington) in the above buffer were incubated at 37^{o}C for 2 hours. The reaction mixtures were dried in air and dissolved in 10 μl of water for application onto cellulose-acetate strips.

Homochromatography fingerprinting. A cellulose-acetate strip of 5 cm x 36 cm was extended with Whatman 3 MM paper and soaked with 5% acetic acid and 0.5% pyridine in 7

M-urea. Electrophoresis in the first dimension was performed for 1 hour at 2 kV. The oligonucleotides were transferred to a DEAE-cellulose plate (20 cm x 20 cm) by blotting. The ratio of DEAE-cellulose to cellulose was 1:7.5 (Analtech, Newark, Del.). A flap of 20 cm x 15 cm Whatman 3 MM paper was placed at the top of the plate and fastened with paper clips. The homomixture was prepared by keeping 4% torula yeast RNA (Sigma) solution in 7 M-urea at 90°C for 5 days for separation of T1 RNase fragments and for 10 days for separation of polypyrimidines and polypurines. Homochromatography was carried out for 18 hours at 60°C. The plate was dried and exposed onto an X-ray film. Portions of the thin layer which contain large fragments were scraped from the plate and the radioactivity was determined.

Base composition analysis. The oligonucleotides were eluted from DEAE-cellulose and their nucleotide composition was determined. Small columns (0.5 cm x 10 cm) were prepared with glass-wool stoppers at the bottom and the DEAE-cellulose containing the fragment was suspended in water and poured into the column. The column was then washed with water and ethanol to remove urea. The oligonucleotides were eluted from the column with 0.5 ml of 30% triethylamine (adjusted to pH 10 with CO_2). The triethylamine and water were air-evaporated and oligonucleotides were digested with 5 units of T2 RNase (Sankyo) for 1 hour at 37°C in 10 μl of 10 mM-sodium acetate and 1 mM-EDTA (pH 4.5). The nucleotides were separated by electrophoresis on Whatman 3 MM paper at pH 3.5 (5% acetic acid and adjusted to pH 3.5 with ammonium hydroxide). The nucleotides were detected by auto-

radiography, cut out, and their radioactivity was determined.

Determination of nucleotide sequences. Non-radioactive polypyrimidine and polypurine fragments of 18S rRNA were labeled with $[\gamma\text{-}^{32}P]$ ATP (specific activity about 100 Ci/mmole, ICN Pharmaceuticals, Inc., Calif) and polynucleotide kinase prepared from the RNase free strain of E. coli Q13 infected by bacteriophage T4 am N82 (22,23). After fingerprinting, the fragments were located by autoradiography and the DEAE-cellulose containing the fragments was scraped from the plate and suspended in 1 ml of water. Labeled fragments which gave more than 5,000 cpm (Cerenkov counts) were further analyzed by homochromatography after partial digestion. The polypyrimidine oligonucleotides were partially digested with pancreatic RNase and the polypurine oligonucleotides were partially digested with U2 RNase at a concentration of 5 units/ml in water (10 μl) for 1 hour at 37°C. After homochromatography, the plates were placed on X-ray films for several days. Nucleotide sequences were determined by following the movements of spots.

RESULTS

T1 RNase digests of 18S rRNA. The fingerprint patterns of the 18S rRNAs of the various species studied were quite similar; 18 numbered large fragments were found at corresponding positions (Fig. 1). The radioactivity in the numbered spots was determined and the molar yield was calculated (Table 1). Fragments were eluted from DEAE-cellulose and their base compositions were determined (Table 1). The molar yield was approximately one for each numbered fragment

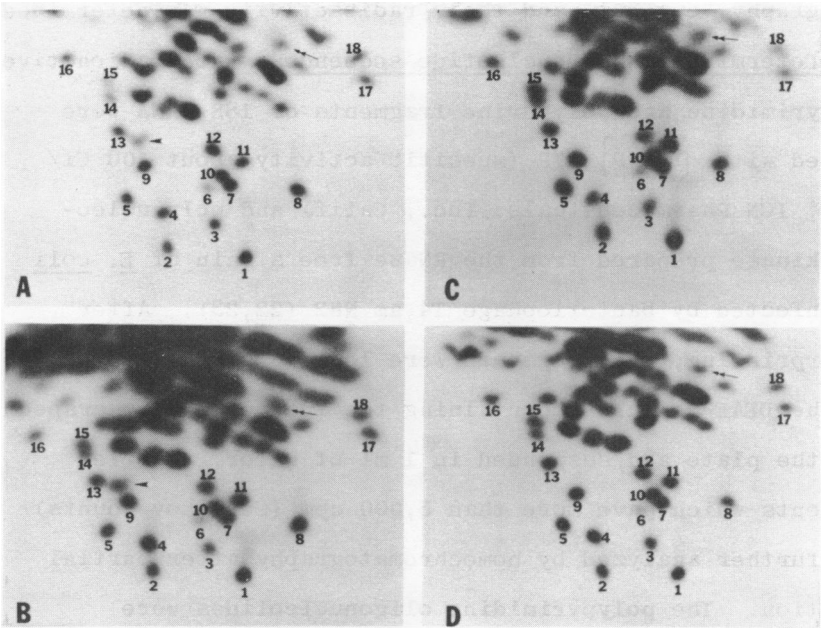

Figure 1. Homochromatography fingerprints of T1 RNase digest of 18S rRNAs from four species; (A) rat, (B) mouse, (C) Chinese hamster, and (D) man. In all homochromatography fingerprintings, the first dimension was run from left to right and the second dimension was run from bottom to top.

except fragments 3 and 6. Fragment 3 was found in less than one molar yield in all four 18S rRNAs and fragment 6 was found at approximately 10% molar yield in all four 18S rRNAs. Fragments 3 and 6 have the sequences of UCCCCCAAC (UmC,U_3)AGmAG and UCCCCCAAC(UmC,U_3)AG, respectively. Fragment 6 is a part of fragment 3 and GmA in 18S rRNA is undermethylated as was found in 5.8S rRNA (9). Khan and Maden (20) also reported AGmAG in a fractional molar yield in HeLa and chick 18S rRNAs. The spot for oligonucleotide ACCCCCCUUCCCG which is present in rat 18S rRNA (shown by a single arrow in Fig. 1A) and absent in human 18S rRNA (Fig. 1D) (21) was found in mouse 18S rRNA (Fig. 1B), but

Table 1: Nucleotide composition and molar yield of large T1 RNase fragments of the four 18S rRNAs. The ^{32}P was determined for the spots shown in Fig. 1. The ratio of the radioactivity in each spot to the radioactivity in spot 1 was calculated and it was normalized by the number of nucleotides in each fragment. Experiments were performed three times (rat and man) and one time (mouse and hamster) each. XmY in fragment 14 is an unidentified methylated dinucleotide.

Fragment	Nucleotide composition	Number of nucleotides in fragment	Molar Yield			
			Rat	Mouse	Hamster	Man
1	$AmUU_7C_6A_5G$	21	1.00	1.00	1.00	1.00
2	$U_2C_2A_{11}G$	16	1.20	1.17	1.38	1.30
3	$GmAUmCU_4C_6A_3G$	18	0.86	0.71	0.67	0.80
4	$AmAU_3C_6A_4G$	16	1.37	1.18	1.26	1.43
5	UC_6A_7G	15	1.14	1.27	1.27	1.29
6	$UmCU_4C_6A_3G$	16	0.12	0.13	0.13	0.07
7	$U_4C_5A_4G$	14	1.08	1.09	1.08	1.01
8	U_6CA_5G	13	1.30	1.37	1.25	1.33
9	$CmCU_2C_4A_5G$	14	1.30	1.33	1.15	1.33
10	$U_4C_5A_3G$	13	1.12	1.14	1.03	1.13
11	U_6C_7AG	15	1.07	1.19	1.13	1.10
12	$AmUU_3C_2A_4G$	12	1.14	1.05	1.29	1.35
13	UC_6A_4G	12	1.39	1.44	1.29	1.12
14	$XmYC_4A_4G$	11	1.30	1.38	1.34	1.22
15	C_2A_6G	9	1.47	1.34	1.28	1.23
16	C_5A_3G	9	1.38	1.39	1.27	1.27
17	U_7CAG	10	1.18	1.26	1.21	1.18
18	$UmUU_4CAG$	9	1.39	1.22	1.37	1.39

not in Chinese hamster 18S rRNA (Fig. 1C). The spots indicated by double arrows show the position reported by Eladari and Galibert (12) for fragment pUACCUG, the 5'-terminal oligonucleotide. The sequence of the fragment was determined for mouse L5178Y cells. The same sequence was found in rat Novikoff ascites hepatoma cells (Williams, R. and Nazar, R. N., personal communication). The double arrowed spot was found at the same position in the four homochromatography fingerprints indicating this 5'-terminal oligo-

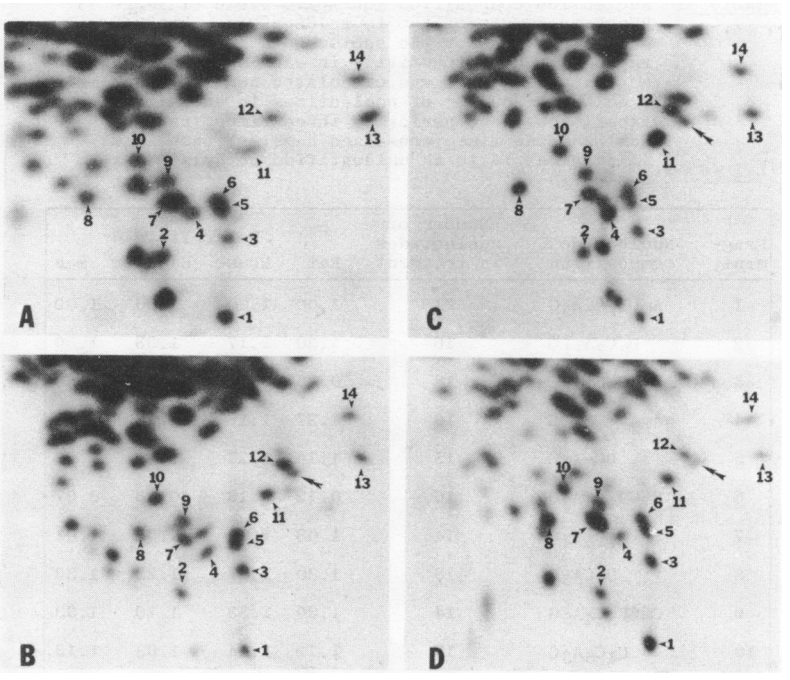

Figure 2. Homochromatography fingerprints of T1 RNase digest of 28S rRNAs from four species; (A) rat, (B) mouse, (C) Chinese hamster and (D) man.

nucleotide sequence is conserved among the four mammals.

T1 RNase digests of 28S rRNA. Figure 2 shows homochromatography fingerprints of T1 RNase digests of 28S rRNAs from the four species. Only one of the large RNase T1 fragments of 18S rRNA was found different among at least 19 fragments shown in Figure 1. Although more changes were found in 28S rRNA, 14 spots were found at corresponding positions in all four fingerprints. Of the large RNase T1 fragments of 28S rRNA, at least 7 spots were not conserved in the rat, 6 spots in the mouse, 6 spots in the hamster and 8 spots in human 28S rRNA. Fragment 1 is the largest T1 RNase fragment in rat 28S rRNA; its length, 28 nucleotides, and nu-

cleotide sequence were reported earlier (24). The spot indicated by the double arrows was found in mouse, Chinese hamster and human, but not in rat 28S rRNA.

Polypyrimidines and polypurines of 18S rRNA. For studies on the polypyrimidine fragments, combined U2 RNase and T1 RNase digestion was carried out on 18S rRNA. More differences were found in the homochromatography fingerprints of large polypyrimidine fragments (Fig. 3) than in the fingerprints of large T1 RNase fragments (Fig. 1). The large fragment spots which appear at corresponding positions in the four fingerprints (Fig. 3) are numbered and the spots which are not common to all four species are indicated by arrows and letters. The oligonucleotides were eluted from the plates and their content of ^{32}P and nucleotide compositions were determined (diagram in Fig. 3). All spots were present in approximately one molar yield except spot 5 which was present in two molar yield in all four 18S rRNAs.

The T1 RNase fragment ACCCCCCUUCCCG, AC_9U_2G (single arrowhead, Fig. 1) which is present in rat and mouse 18S rRNAs but not in Chinese hamster and human 18S rRNAs contains a large polypyrimidine fragment C_9U_2G (21). This fragment C_9U_2G was found in rat 18S rRNA (spot a, Fig. 3A) and in mouse 18S rRNA (spot d, Fig. 3B), but not in Chinese hamster 18S rRNA (Fig. 3C) or in human 18S rRNA (Fig. 3D). However, both Chinese hamster and human 18S rRNAs contained a fragment C_6U_2G (spots h and k, Fig. 3). Fragment CCCCCCG, C_6G, (spots f, i and l, Fig. 3) was found in mouse, Chinese hamster and human 18S rRNA but not in rat 18S rRNA. In

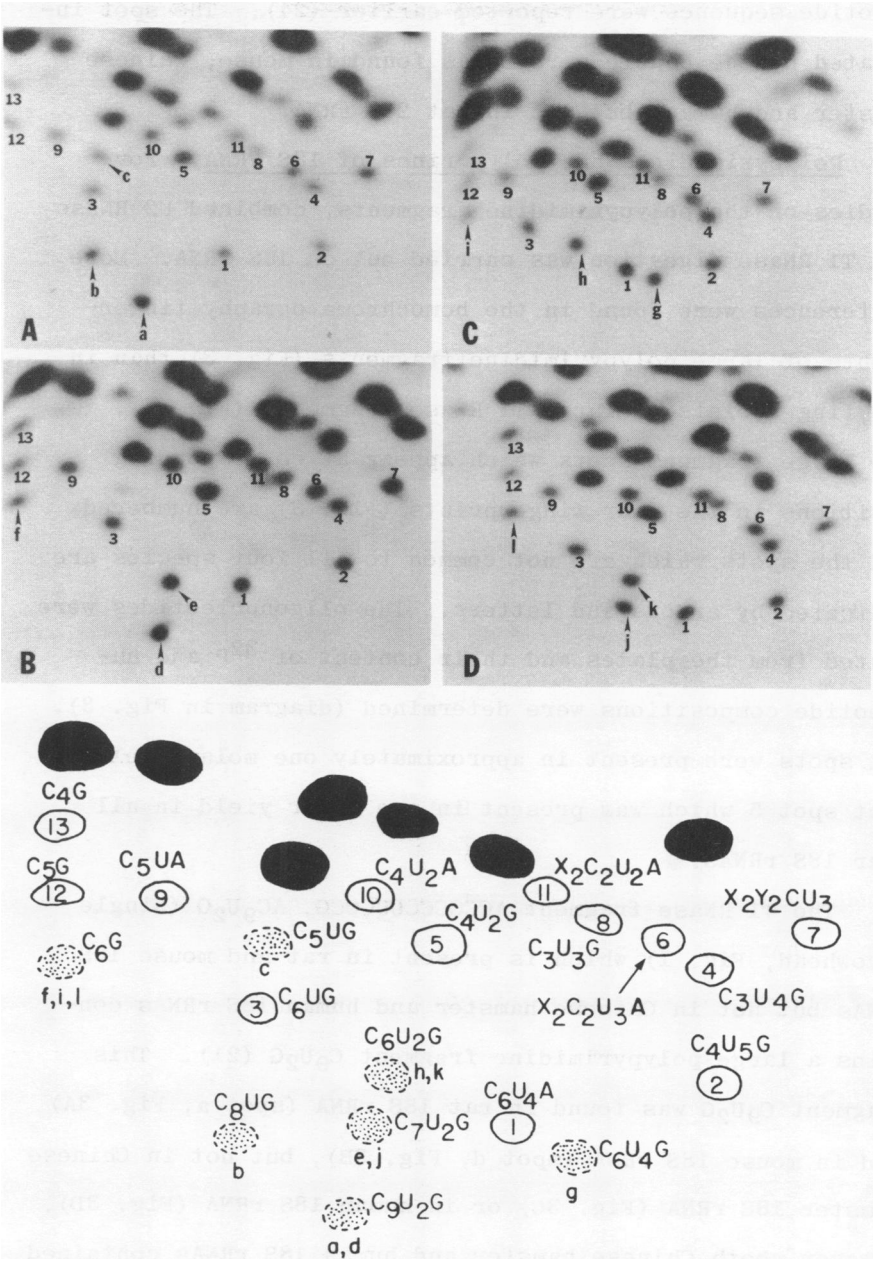

Figure 3. Homochromatography fingerprints and a diagram of polypyrimidine fragments of 18S rRNAs from four species; (A) rat, (B) mouse, (C) Chinese hamster and (D) man. Dotted spots appeared differently among the four species. Their nucleotide sequences are shown in Table 1.

its place, rat contained a fragment $(C_5U)G$ (spot c, Fig. 3). This suggests a single point mutation A ⟷ G occurred in rDNA that resulted in the U ⟷ C change in 18S rRNA between rat and other three species. Rat 18S rRNA contains the oligonucleotide C_8UG and mouse and human 18S rRNA contain oligonucleotide C_7U_2G. This suggests the possibility of another gene mutation resulting in a C ⟷ U change in 18S rRNA between rat, mouse and man. Chinese hamster 18S rRNA contains neither oligonucleotide C_8UG nor oligonucleotide C_7U_2G but contains oligonucleotide C_6U_4G, which is one nucleotide larger.

Figure 4 shows homochromatography fingerprints and a diagram of the 18S rRNA polypurine fragments produced by pancreatic RNase. The molar yield of spot 5 was one for rat or mouse but two for Chinese hamster (indicated also by c in Fig. 4C) and man (indicated by e in Fig. 4D). Fragments d and g had the nucleotide compositions G_8C and G_6C; their nucleotide sequences are GGGGGGGGC and GGGGGGC. Spots a, b, d and f were found to be marker spots for rat, mouse, Chinese hamster and human 18S rRNA, respectively.

Nucleotide sequence determination. Nucleotide sequences were determined for the polypyrimidine and polypurine fragments which differed in the four 18S rRNAs. The method employed for sequence-analysis of the largest polypyrimidine oligonucleotide CCCCCCUUCCCG is demonstrated in Fig. 5 and shown diagrammatically in Fig. 6. The smallest fragment, the top spot, migrated at the position of pCp in the first dimension (pH 3.5 cellulose-acetate electrophoresis). The 5'-terminal nucleotide of the fragment was confirmed to

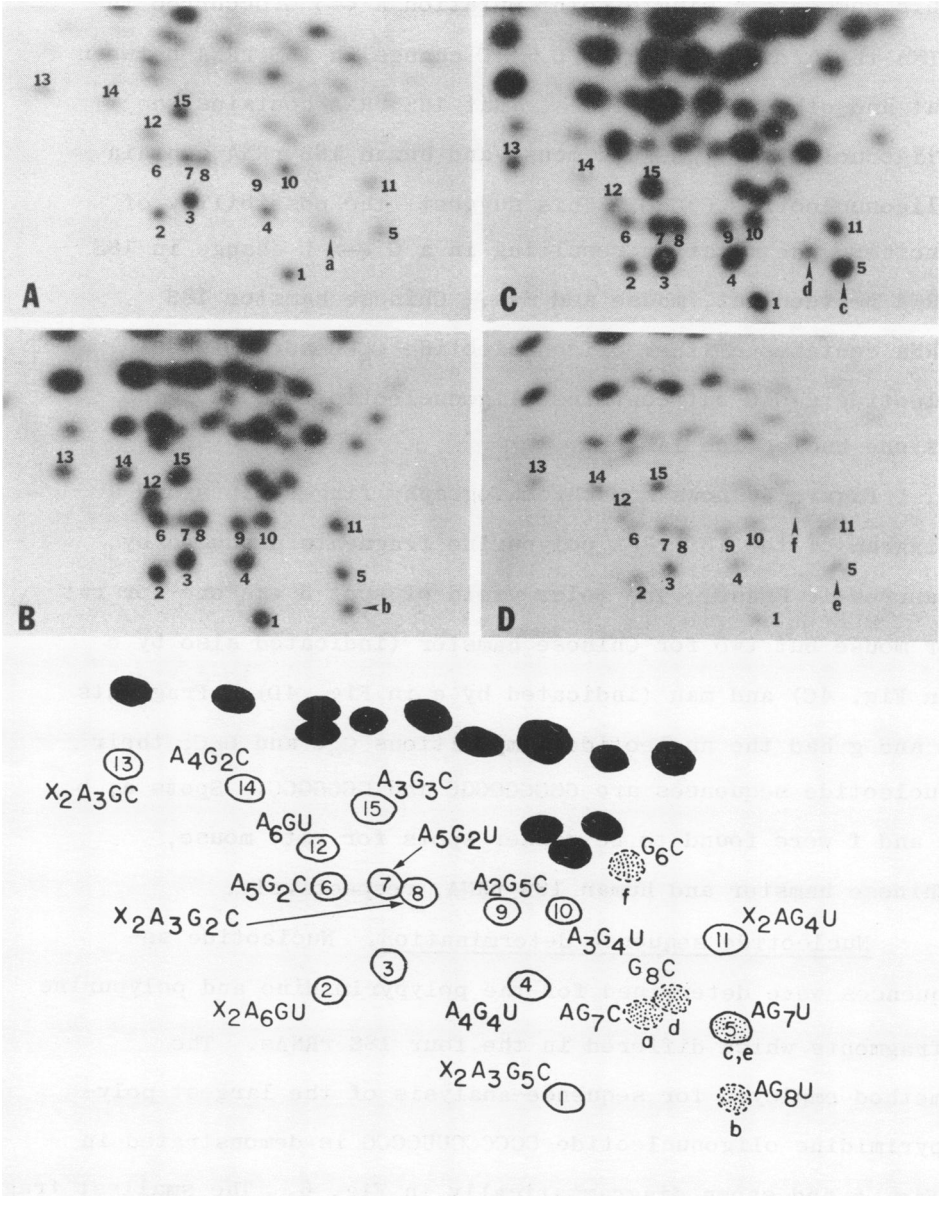

Figure 4. Homochromatography fingerprints and a diagram of polypurine fragments of 18S rRNA from four species; (A) rat, (B) mouse, (C) Chinese hamster and (D) man. Spot 3 appeared in two molar yield in all four rRNAs. It contains two fragments and one has a T2 RNase resistant methylated dinucleotide.

be pCp independently by pH 3.5 paper electrophoresis after complete digestion of the ^{32}P-labeled fragment by T2 RNase. When one more cytydyl residue is added, the spot moves left and down and one more uridylyl residue is added, the spot moves right and down. The 3'-terminal nucleotide was known to be G from nucleotide compositional analysis and the enzyme specificity. By following the spot positions, the nucleotide sequence of the fragment was determined to be CCCCCCUUCCCG. The obtained result agrees with the sequence determined by spleen phosphodiesterase partial digestion (25). The oligonucleotide sequences and their possible mutational relationships are summarized in Table 2.

Mouse polypyrimidine fragment d, C_9U_2G, had the same sequence as rat fragment a; CCCCCCUUCCCG. The nucleotide sequence, CCCCCUUCG, of the fragments h and k, C_6U_2G, of Chinese hamster and human 18S rRNA was the same. If the change between the two fragments C_9U_2G and C_6U_2G were caused by mutation, at least two point mutations occurred (Table 2). An example of the result of a point mutation is shown between rat fragment b, CCCCCUCCCG and mouse fragment e, CUCCCUCCCG. Fragment j, CCCCUCUCCG (C_7U_2G), from human 18S rRNA has the same nucleotide composition to mouse fragment e, CUCCCUCCCG (C_7U_2G), but their sequences are different. Four nucleotides differ in fragments j and e and three nucleotides differ in fragments j and b. The nucleotide sequence CUUCCUUCCCG was obtained for fragment g, C_6U_4G, of Chinese hamster.

The nucleotide sequences of polypurine fragments were determined by two methods. In one, the method employed was the same as for polypyrimidine fragments (Fig. 5) except for

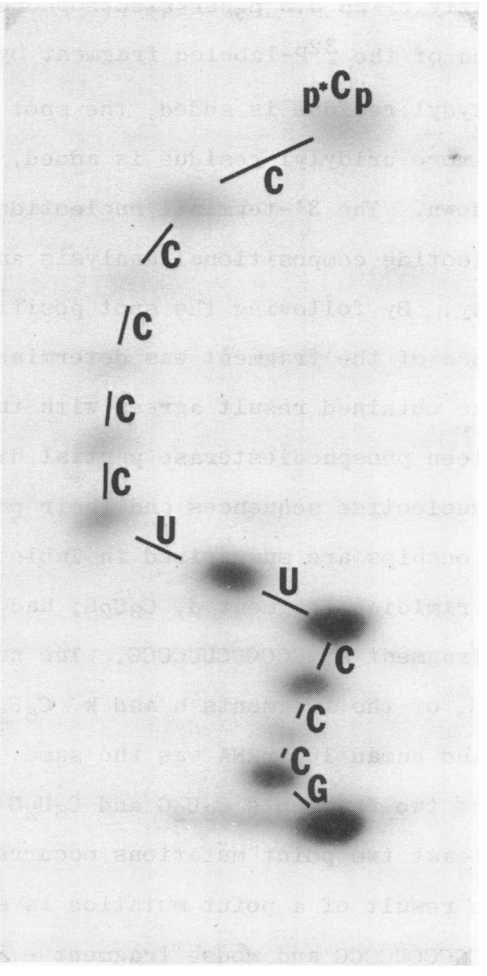

Figure 5. Determination of the nucleotide sequence of polypyrimidine oligonucleotide CCCCCCUUCCCG.

partial U2 RNase digestion of the polypurine fragments. The other method employed complete T1 RNase digestion followed by DEAE-paper electrophoresis at pH 1.9. Fragment a, AG_7C, was cleaved to AC and guanylyl residues and fragment b, AG_8U, was cleaved to AU and guanylyl residues and their sequences were determined to be GGGGGGGAC and GGGGGGGGAU, respectively.

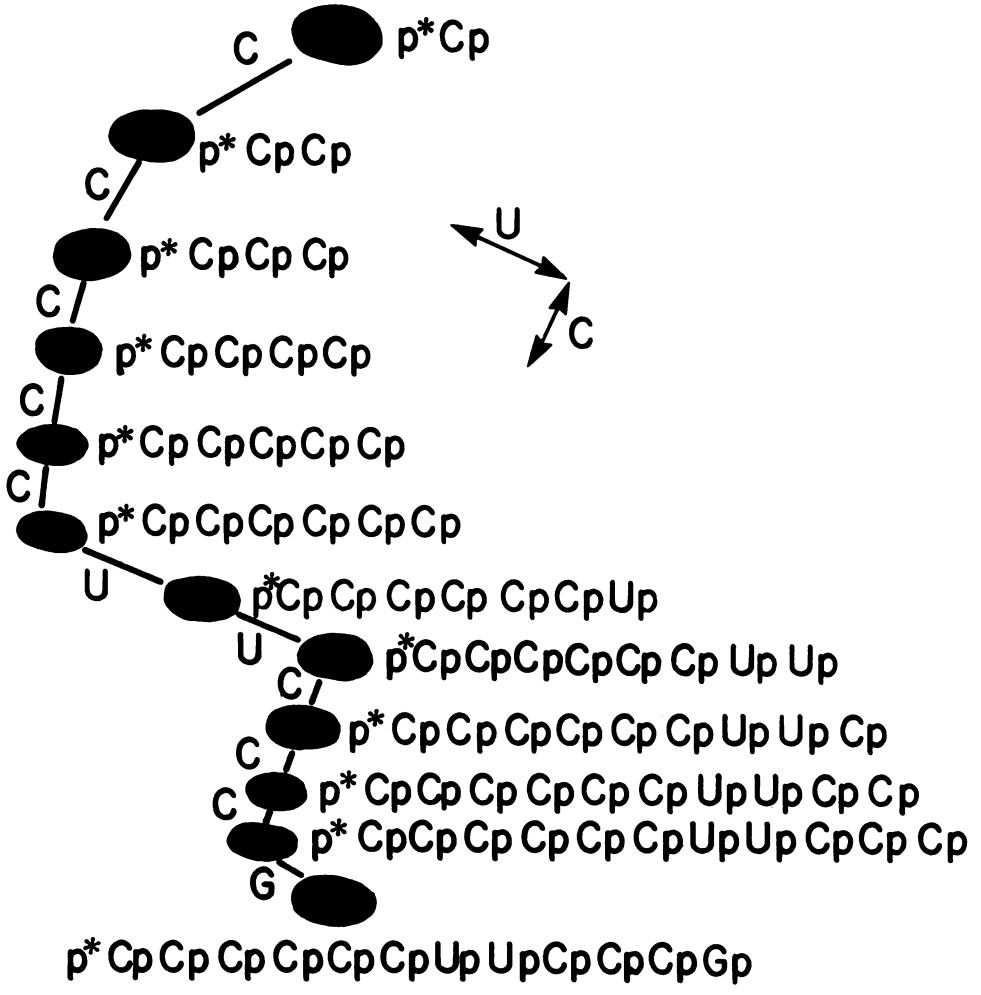

Figure 6. A diagram for Figure 5.

Fragments c and e comigrated with fragment 5 in the fingerprints of Chinese hamster and human 18S rRNAs. Complete T1 RNase digestion of the mixture of fragments 5 and c, or fragments 5 and e produced U, G, AU and AG (Fig. 7). The ratio of the radioactivity of AU to AG was 1:1. Fragment 5 in rat or mouse gave three spots, U, G and AG, after complete T1 RNase digestion. Therefore, spots G and AU belong to frag-

Table 2: Possible mutational relationship of oligonucleotide sequences which differ among four 18S rRNA species. Polypyrimidine fragments are shown in Fig. 3 and polypurine fragments are shown in Fig. 4. Polypyrimidine fragment g, C_6U_4G, of hamster has a sequence of CUUCCUUCCCG and polypyrimidine fragment j, C_7U_2G, of man has a sequence of CCCCUCUCCG.

	Nucleotide Sequence	Nucleotide Composition	Source	Spot	Possible Mutation
Polypyrimidine	CCCCCCG	C_6G	mouse,hamster,man	f,i,l	C ↕ U
	$(C_5U)G$	C_5UG	rat	c	
	C CCCCCUUCCCG ↕ ↕ PuCCCCCUUCG	C_9U_2G	rat,mouse	a,d	C ↕ Pu C ↕ G
		C_6U_2G	hamster,man	h,k	
	CCCCCUCCCG ↕ CUCCCUCCCG	C_8UG	rat	b	C ↕ U
		C_7U_2G	mouse	e	
Polypurine	GGGGGGGAC ↕ PyGGGGGGGAU ↕ G GGGGGGGAU	AG_7C	rat	a	C ↕ U
		AG_7U	hamster,man	c,e	Py ↕ G
		AG_8U	mouse	b	
	GG GGGGGGC ↕ PyGGGGGGC	G_8C	hamster	d	G ↕ Py
		G_6C	man	f	

ment c or e and their nucleotide sequence is GGGGGGGAU. Fragment GGGGGGGAC of rat 18S rRNA represents a single mutational difference, C ⟷ U, from fragment GGGGGGGAU of Chinese hamster or man. Fragment GGGGGGGGAU of mouse 18S rRNA could also represent a single mutational change from the fragment GGGGGGGAU of Chinese hamster or man,

PyPyGGGGGGGAU
↕
PyG GGGGGGGAU.

DISCUSSION

The size of the high molecular weight ribosomal RNA of a wide range of species were compared by several workers (26,27). It was found that the molecular weight of the smaller rRNA, 18S rRNA, is well kept constant among animals but the larger rRNA, 28S rRNA, has increased in size in more evolved species. This suggests 18S rRNA structure is more highly conserved than 28S rRNA structure. Bendich and

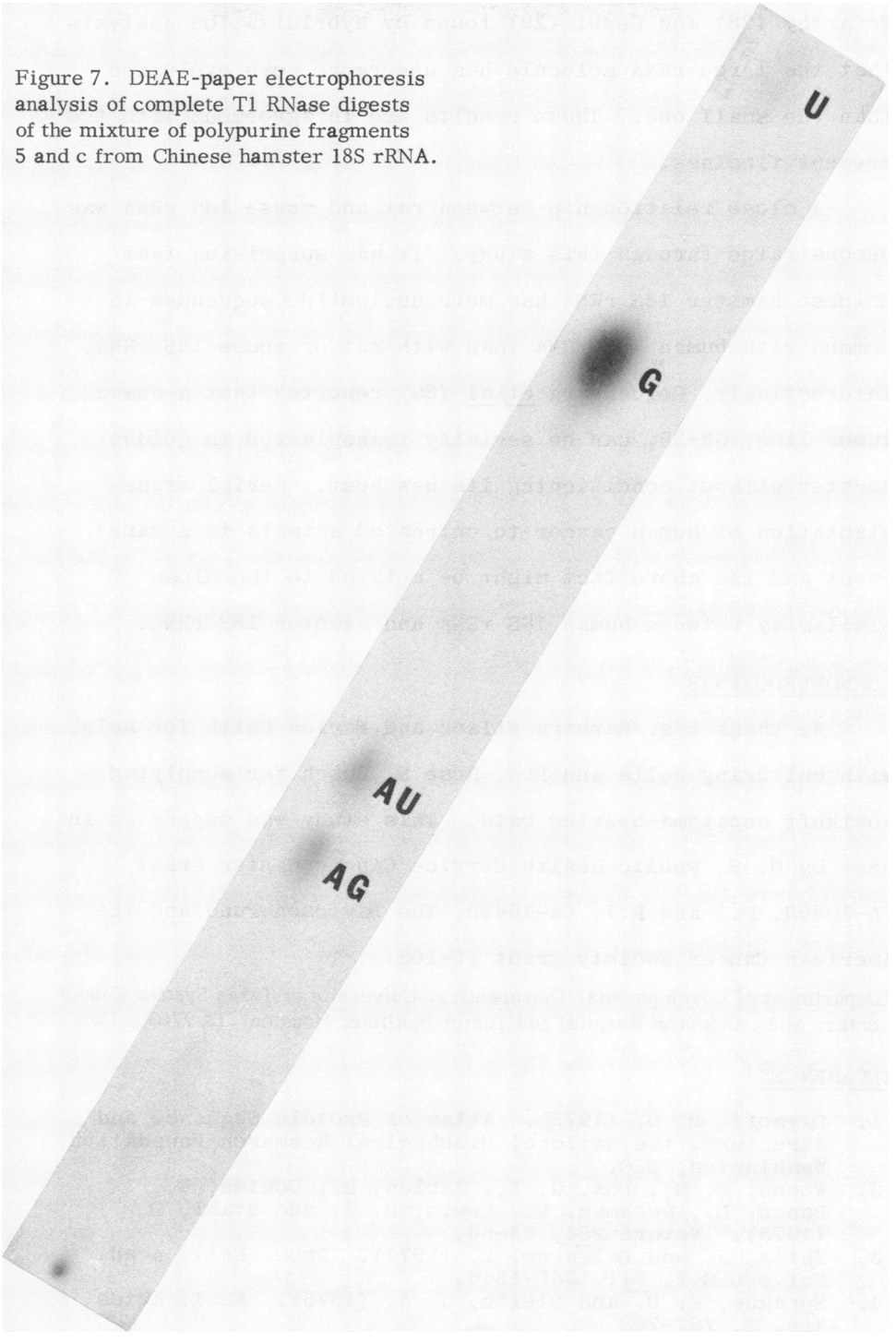

Figure 7. DEAE-paper electrophoresis analysis of complete T1 RNase digests of the mixture of polypurine fragments 5 and c from Chinese hamster 18S rRNA.

McCarthy (28) and Gerbi (29) found by hybridization analysis that the large rRNA molecule has undergone more evolution than the small one. These results are in agreement with the present findings.

A close relationship between rat and mouse 18S rRNA was demonstrated through this study. It was surprising that Chinese hamster 18S rRNA has more nucleotide sequences in common with human 18S rRNA than with rat or mouse 18S rRNA. Interestingly, Goldenberg et al (30) reported that a human tumor line, GW-39, can be serially transplanted in golden hamster without conditioning its new host. Serial transplantation of human cancer to untreated animals is a rare event and the above fact might be related to the close similarity between human 18S rRNA and hamster 18S rRNA.

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