The Methylation of Transfer and Ribosomal Ribonucleic Acid in Human Fibroblasts: Normal Methylation of Ribonucleic Acid in Cystic Fibrosis

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Extract

A comprehensive study of tRNA and 18 S and 28 S rRNA methylation in human fibroblasts is presented. Cells from normal volunteers and patients with cystic fibrosis (CF) were examined during exponential growth by incubation with L-(methyl-¹⁴C)methionine and (³H)uridine. The ratio of ¹⁴C to ³H counts per minute in isolated RNA serves as a measure of the extent of methylation and was found to be similar in normal and CF fibroblasts for tRNA, 18 S rRNA, and 28 S rRNA. Hydrolyzed samples of (¹⁴C)-methyl-labeled RNA species were analyzed by two dimensional chromatography and autoradiography. The methylated base and nucleoside compositions of tRNA and rRNA agree well with data for HeLa cells and other eukaryote cells such as those from yeast, mouse, and chicken. RNA methylation patterns in CF fibroblasts were qualitatively and quantitatively similar to those observed in normal cells for 4 S, 18 S, and 28 S RNA. There is no evidence that RNA species from patients with cystic fibrosis are deficient in any methylated components.

Speculation

Methylation of RNA by tissue-cultured cells from cystic fibrosis patients is qualitatively and quantitatively normal and will not prove to be useful as a marker for identification of carriers of cystic fibrosis.

Introduction

Rennert *et al.* [13, 14] have reported that tissue-cultured fibroblasts and lymphocytes from patients with CF as well as obligatory heterozygotes for CF show a decreased methylation of RNA. If undermethylation of CF cells could be consistently demonstrated, a valuable and easily measurable marker for the detection of CF carriers would be available.

Therefore, a comprehensive study was undertaken of tRNA and 18 S and 28 S rRNA methylation in human fibroblasts by methods established previously

in normal cells from various nonhuman organisms [6-8]. Accordingly, fibroblasts were isolated from normal volunteers and patients with CF, and methylation of RNA was examined both *in vivo*, *i.e.*, in tissue culture, and *in vitro* with fibroblast extracts. The results on methylation of tRNA *in vitro* and preliminary data on the methylation of tRNA *in vivo* are summarized briefly elsewhere [9]. Those findings indicated that all tRNA methylating enzymes that are present in normal human cells are also active in CF fibroblasts. In the present report, comprehensive data are

presented on the *in vivo* methylation of tRNA and rRNA for fibroblasts from normal volunteers and CF patients.

Materials and Methods

Cell Cultures and Media

Skin biopsies were obtained from normal volunteers and from several patients with cystic fibrosis. The normal group (age range 21–23 years) included one pure blooded Chinese subject (*PJ*) who was almost certainly not a carrier for cystic fibrosis [16]. A broad spectrum with respect to age (10–24 years) and severity of disease was represented by the cystic fibrosis patients. Derived fibroblasts were cultured in 75 cm² flasks containing Eagle's minimal essential medium plus a mixture of nonessential amino acids, 10% fetal calf serum, and neomycin sulfate (50 µg/ml). Both normal and CF cell lines had been serially subcultured seven to nine times before determination of RNA methylation. Cultures remained mycoplasma free as demonstrated by routine screening of the cell sheets and the media [17]. The mean generation time of fibroblasts in this laboratory based on cell counts is essentially the same for cystic fibrosis (26 ± 6 hr) and control (27 ± 9 hr) cells [11].

Radioactive Methylation of RNA

When the monolayers reached 25% confluence (*i.e.*, during the exponential growth phase), RNA was labeled with L-(methyl-¹⁴C)methionine and (³H)uridine. To accomplish this, the above growth medium was replaced by 12.5 ml labeling medium for approximately 24 hr. The latter was identical with growth medium except for the lack of unlabeled methionine and the presence of (1) L-(methyl-¹⁴C)methionine (0.25 µCi/ml, 50 mCi/mmol [18]); (2) (³H)uridine (0.5 µCi/ml, 27.7 Ci/mmol [19]); (3) uridine (5×10^{-6} M), and (4) adenosine (10^{-4} M). Uptake of isotopic precursors was found to be similar in a limited number of control and cystic fibrosis lines examined.

Isolation of RNA

Fibroblasts were removed from tissue culture flasks by trypsinization. The cells were harvested by centrifugation at $4,000 \times g$ for 5 min, washed twice with Earle's basic salts, resuspended in 3 ml RSB medium (0.01 M Tris, pH 7.5, 0.01 M NaCl, and 0.0015 M MgCl₂) for 15 min to allow swelling, and then broken by 10 strokes in a tight-fitting Dounce homogenizer. Nuclei

and large particulate matter were removed by two successive centrifugations of $1,000 \times g$ for 5 min and $10,000 \times g$ for 15 min. The supernatant fraction was extracted twice with an equal volume of 88% phenol at 60° for 3 min, after addition of sodium dodecyl sulfate (SDS), to a final concentration of 0.25%. The RNA was then precipitated from the aqueous phase by adding 2.5 volumes ethanol which contained 0.1 M sodium acetate buffer, pH 5.1, and allowing to stand at –20° overnight. The precipitate was washed twice with 70% ethanol, resuspended in H₂O, and kept frozen at –20°.

Samples of tRNA and rRNA were prepared by centrifugation of the total cellular RNA on a 15% to 30% linear sucrose gradient made up in SDS buffer (0.1 M NaCl, 0.01 M Tris, pH 7.5, 0.001 M ethylenediaminetetraacetate, and 0.5% SDS) at 20° for 17 hr at 27,000 rpm in the SW 41 rotor of the Spinco ultracentrifuge [1]. The distribution of RNA species was determined by continuous monitoring at 260 nm of the optical density of the sucrose gradient during collection. Tubes containing 4 S, 18 S, and 28 S RNA were pooled, and the RNA was precipitated with 2.5 volumes ethanol. The pooled RNA was redissolved in water, and the radioactivity in each sample was determined by the method of Furano [3]. The samples were counted in a scintillation counter [20] with approximate efficiencies of 17% for ³H and 40% for ¹⁴C; the crossover of ¹⁴C into the low energy channel was about 10%.

All RNA fractions isolated from the sucrose gradient were incubated with pancreatic deoxyribonuclease (20 µg/ml) in 0.002 M MgCl₂ for 30 min at 37°. These fractions were then incubated in 0.1 M Tris acetate, pH 8.6, for 30 min at 37°. Because only 28 S, 18 S, and 4 S RNA are methylated, all the methyl radioactivity in the 4 S region was considered to be tRNA [2].

As presented elsewhere [9], examination of the sucrose density gradients reveals similar profiles for ultraviolet absorbance and labeling with (³H)uridine. Specific activities of RNA preparations were as reported previously [9]. (Approximately 80% of the total RNA is rRNA and 20% is tRNA.) Thus, it is evident in these experiments that (³H)uridine provides a uniform label for RNA. On the other hand, about 40% of the L-(methyl-¹⁴C)methionine incorporation is found in rRNA and 60% is found in tRNA.

Two Dimensional Chromatography of Methylated Bases

Samples of (¹⁴C)tRNA were lyophilized and resuspended in 1 ml trifluoroacetic acid [21]. The samples

were sealed in evacuated Carius combustion tubes and heated to 170° for 30 min in a Carius bomb [12]. After cooling, the tubes were opened and the trifluoroacetic acid removed by evaporation with a current of air. The dry residues were resuspended in 10 μ l H₂O and spotted on cellulose thin layer sheets with fluorescent indicator [22]. The sheets were chromatographed in the first direction in methanol-hydrochloric acid-water (70/20/10) and in the second direction in 1-butanol-acetic acid-water (4/1/1). Thin layer sheets were autoradiographed with Kodak RB-54 medical x-ray film [23]. For quantitation, radioactive spots were located by autoradiography, scraped, and counted in toluene which contained 2,5-diphenyloxazole and 1,4-bis[2-(5-phenyloxazolyl)]benzene. The compounds were identified by cochromatography with methylated compounds purchased from Cyclo Chemical [24].

Results

Extent of Methylation of tRNA and rRNA

Exponentially growing human fibroblasts isolated from four normal volunteers and six patients with cystic fibrosis were labeled for 24 hr with L-(methyl-¹⁴C)-methionine and (³H)uridine. Analysis of the incorporation of L-(methyl-¹⁴C)-methionine and (³H)uridine into RNA indicates that both cystic fibrosis and normal human fibroblasts incorporate either isotope into RNA equally well. Thus, approximately the same number of ³H or ¹⁴C counts per minute are found in each sample of total RNA.

The ratio of ¹⁴C to ³H counts per minute in tRNA, 18 S ribosomal RNA, and 28 S ribosomal RNA is a measure of the extent of methylation of each of these RNA species. As is shown in Table I, the mean values

Table I. The extent of methylation of 4 S, 18 S, and 28 S RNA in human fibroblasts

	Ratio of ¹⁴ C to ³ H cpm		
	4 S RNA	18 S RNA	28 S RNA
Normal			
BH	0.322	0.107	0.075
DL	0.526	0.217	0.072
HS	0.400	0.116	0.070
HS (experiment 2)	0.485	0.113	0.078
Mean	0.433	0.138	0.073
Cystic fibrosis			
PG	0.526	0.111	0.066
AO	0.434	0.117	0.069
RM	0.416	0.125	0.068
RM (experiment 2)	0.540	0.145	0.092
Mean	0.479	0.124	0.073

for ¹⁴C to ³H ratio of each of the RNA species in four samples of normal human cells are similar within experimental error to the ratios of each corresponding RNA species in four samples of cystic fibrosis cells. Thus, the extent of methylation of RNA *in vivo* is similar for cystic fibrosis and normal human fibroblasts.

Base Composition of tRNA Methylated *in Vivo*

Despite the normal extent of methylation observed in the RNA of cystic fibrosis fibroblasts, it is possible that an altered distribution of methylated bases exists in the RNA of these cells. Samples of (¹⁴C)-methyl-labeled tRNA were hydrolyzed in trifluoroacetic acid and analyzed by two dimensional chromatography on thin layer cellulose sheets, which were then subsequently autoradiographed. Autoradiograms of the methylated bases and nucleosides of tRNA isolated from one normal human line (DL) and from one cystic fibrosis line (AO) are shown in Figure 1. The identities and the relative amounts of the methylated compounds found in the tRNA of four normal human lines and six cystic fibrosis lines are shown in Table II. In addition, repeat data for one normal (HS) and one cystic fibrosis (RM) sample, are given as evidence of reproducibility. It is apparent from Figure 1 and Table II that the patterns for the methylation of tRNA *in vivo* are qualitatively and quantitatively similar in normal human lines and cystic fibrosis lines. There are no extra or missing bases in either cell type. Thus, there is no evidence that tRNA samples from patients with cystic fibrosis are deficient in any of the methylated bases or nucleosides.

Base Composition of rRNA Methylated *in Vivo*

The 18 S and 28 S RNA methylation patterns were examined in two normal and two cystic fibrosis lines. (The relatively small amount of methylation that occurs in rRNA compared with tRNA [8] makes the processing of large numbers of samples difficult.) Autoradiograms of the methylated bases and nucleosides of 18 S and 28 S rRNA isolated from one normal human line (BH) and one cystic fibrosis line (AO) are shown in Figure 2. The identities and the relative amounts of the methylated compounds found in the 18 S and 28 S rRNA of two normal lines and two cystic fibrosis lines are presented in Table III. As is the case with the methylation of tRNA *in vivo*, the patterns for the methylation of each of the ribosomal RNA species *in vivo* are qualitatively and quantitatively similar in normal human lines and cystic fibrosis lines. In addition, the data shown in Tables II and III for the methylation of tRNA and rRNA *in vivo* in human fibro-

Fig. 1. Autoradiography of the methylated bases and nucleosides of tRNA. Samples of tRNA isolated from normal (DL) and cystic fibrosis (AO) fibroblasts as described in *Materials and Methods* were hydrolyzed in trifluoroacetic acid and chromatographed in two dimensions on cellulose thin layer sheets which were then autoradiographed. Guanine (G), adenine (A), cytosine (C), and uridine (U) markers were added to tRNA samples before hydrolysis and located on the chromatogram by ultraviolet absorption. The methylated compounds are identified and quantitated in Table II with use of the same identification numbers published previously [8]. The total ^{14}C activities recovered by scraping the thin layer sheets were 566 cpm in the normal sample (DL) and 699 cpm in the cystic fibrosis sample (AO).

Table II. Relative amounts of methylated compounds recovered from 4 S RNA

Identification No. ¹	Compound	Total radioactivity recovered, %													
		Normal						Cystic fibrosis							
		PJ	BH	DL	HS	HS ²	Mean $\pm$ SD	GF	JS	HO	PG	AO	RM	RM ²	Mean $\pm$ SD
1	1-Methylguanine														
2	7-Methylguanine														
3	1-Methyladenine	19.7	20.8	21.5	20.8	20.5	20.6 $\pm$ 0.65	19.9	19.7	19.5	23.3	23.7	20.2	21.0	21.0 $\pm$ 1.7
4	N ² -Methylguanine	17.2	18.0	17.9	16.0	18.6	17.5 $\pm$ 0.99	16.8	19.4	19.1	17.9	17.0	17.3	17.6	17.9 $\pm$ 1.0
5	N ² -Dimethylguanine	16.7	17.3	17.0	15.6	17.0	16.7 $\pm$ 0.66	16.2	19.4	17.8	17.4	16.8	15.3	15.9	16.9 $\pm$ 1.5
6	1-Methylhypoxanthine	15.1	14.8	13.9	14.3	14.4	14.5 $\pm$ 0.46	16.0	15.7	14.4	13.1	13.5	15.1	15.0	14.6 $\pm$ 1.1
7	5-Methylcytosine	3.0	3.7	3.2	3.4	2.7	3.2 $\pm$ 0.38	3.1	2.3	2.5	2.5	2.5	2.7	3.6	2.7 $\pm$ 0.46
8	5-Methyluridine	6.3	5.9	7.9	6.8	6.4	6.7 $\pm$ 0.77	6.3	6.0	5.1	6.4	8.4	7.2	8.7	6.9 $\pm$ 1.2
		21.9	19.0	18.4	22.7	20.5	20.5 $\pm$ 1.8	22.0	17.4	21.2	19.1	17.7	22.0	17.8	19.6 $\pm$ 2.1

¹ Corresponds to numbers in Figure 1 as well as to numbers used previously [8].

² Second experiment.

blasts agree well with data reported previously for HeLa cells and other eukaryote cells [8].

Discussion

Various nucleic acids such as DNA, tRNA, and rRNA are found to be methylated in animal cells [2]. Methyla-

tion is accomplished at the polynucleotide level with S-adenosylmethionine serving as the methyl donor. This process is catalyzed by a group of enzymes known as methylases which are highly specific with respect to individual bases and type of nucleic acid. Although the biologic role of nucleic acid methylation is as yet un-

Fig. 2. Autoradiography of the methylated bases and nucleosides of 18 S and 28 S ribosomal RNA. Samples of 18 S and 28 S RNA isolated from normal (BH) and cystic fibrosis (AO) fibroblasts as described in *Materials and Methods* were hydrolyzed in trifluoroacetic acid and chromatographed in two dimensions on cellulose thin layer sheets which were then autoradiographed. Guanine (G), adenine (A), cytosine (C), and uridine (U) markers were added to 18 S and 28 S samples before hydrolysis and located on the chromatogram by ultraviolet absorption. The methylated compounds are identified and quantitated in Table III with the use of the same identification numbers previously published [8]. The total ^{14}C activities recovered by scraping the thin layer sheets were 272 cpm for 18 S RNA and 94 cpm for 28 S RNA in the normal sample (BH), and 322 cpm for 18 S RNA and 219 cpm for 28 S RNA in the cystic fibrosis sample (AO). The small amount of radioactivity recovered in the case of BH 28 S RNA (D) makes the spots in the print of the autoradiogram difficult to discern; the original x-ray film, however, is quite clear. A: cystic fibrosis 18 S RNA (AO); C: cystic fibrosis 28 S RNA (AO); B: normal 18 S RNA (BH); D: normal 28 S RNA (BH).

Table III. Relative amounts of methylated compounds recovered from 18 S and 28 S RNA

Identification No. ¹	Compound ²	Total radioactivity recovered, %									
		18 S RNA					28 S RNA				
		Cystic (AO)	Cystic (RM)	Normal (BH)	Normal (DL)	HeLa ³	Cystic (AO)	Cystic (RD)	Normal (BH)	Normal (DL)	HeLa ³
2	7-Methylguanine	6.0	9.0	8.5	5.7	7.2					2.3
3	1-Methyladenine					1.4	9.5	13.2	9.2	11.0	11.1
7	5-Methylcytosine	2.2	3.5	4.5	3.3	2.8	5.3	4.7	5.2	6.9	3.4
10	N ⁶ -Methyladenine	8.5	7.7	9.2	6.0	8.2	7.6	7.3	8.0	8.2	10.5
11	N ⁶ -Dimethyladenine	22.7	21.5	18.0	19.8	23.9					2.4
15	3-Methyluridine						11.6	8.5	8.6	12.0	6.8
16	2-O-Methyluridine	9.2	10.1	7.9	9.1	5.6	22.1	23.3	29.2	21.0	19.4
17	2-O-Methylribose	35.8	35.9	34.9	42.1	38.6	29.1	27.0	28.4	30.6	30.6
18	Unidentified	15.6	11.9	14.2	13.7	10.7	14.7	16.4	10.9	10.8	11.1

¹ Corresponds to numbers in Figure 2 as well as to numbers used previously [8].

² Tentative identification based on chromatographic data for HeLa, mouse, and yeast ribosomal RNA previously published [8].

³ From data of Klagsbrun [8].

clear, it is possible that methylation of nucleic acids confers some protection against degradation by deoxyribonucleases and ribonucleases [4, 15].

It has been reported by Rennert *et al.* [13, 14] that a defect in the methylation of RNA is a characteristic of cells isolated from cystic fibrosis patients as well as obligatory heterozygotes. Thus, measurement of RNA methylation might be used as a test for cystic fibrosis carriers. In light of the importance that such a test could have in identification of cystic fibrosis heterozygotes, a comprehensive study of the tRNA and rRNA methylation has been performed with cultured fibroblasts isolated from six CF patients.

The results presented herein fail to demonstrate any significant differences in the methylation of RNA in CF fibroblast lines as compared with human fibroblasts isolated in an identical manner from four normal volunteers. Specifically, it was found that fibroblasts from CF patients were normal with respect to: (1) the extent of methylation *in vivo* of tRNA, 18 S RNA, and 28 S RNA, and (2) the methylated base composition of tRNA and rRNA methylated *in vivo*.

Additionally, it has previously been observed by Klagsbrun and Farrell [9] that cytoplasmic extracts from cystic fibrosis fibroblasts have the same capacity for methylation of *E. coli* tRNA *in vitro* as do extracts from normal human fibroblasts. Furthermore, the distribution of tRNA methylating enzymes observed in normal fibroblasts was the same in CF extracts [9], and thus an absent methylase could not be detected in the latter cells. Therefore, the *in vivo* methylation data of this report combined with the *in vitro* findings from fibroblast extracts [9] strongly suggest that RNA methylation is unaltered in cystic fibrosis.

A possible explanation for the discrepancy between these results and those of Rennert *et al.* [13, 14] involves differences in the methods used for measuring the extent of RNA methylation. We have measured the extent of RNA methylation by determining the number of methyl groups per RNA molecule, whereas Rennert and associates have reported measurements on the number of methyl groups per cell without measuring RNA synthesis in those cells. Thus, the observation of the latter group of undermethylated RNA in cystic fibrosis fibroblasts might actually reflect a decrease in total RNA synthesis in these cells. This could be the case if the contact-inhibited, confluent fibroblasts from CF patients, as used by Rennert *et al.* [14], were growing more slowly than confluent normal fibroblasts. In order to eliminate the variable of differential growth rates, we have utilized exponentially dividing fibroblasts; such cells from CF patients show normal generation times in this laboratory. Another possible explanation for the differences between these findings and those of Rennert and associates relates to transport of (¹⁴C)-methionine. If confluent CF fibroblasts had a reduced uptake (or activation) of methionine, RNA might then appear undermethylated in these cells. However, Hodes and Merritt [5] have found in a recent study of confluent fibroblasts that RNA methylation was unaltered in cells from CF patients.

Although there are minor differences in Rennert's tissue culture medium as compared with ours, it is unlikely that such variables could account for the discrepant rates of RNA methylation. Rather, based on results obtained with cultured cells in other inborn errors of metabolism [10], it is likely that a fundamental chemical defect (as claimed for RNA methylation in

CF) would be readily and reproducibly identified, despite minor differences in tissue culture techniques.

The analytical methods employed in this investigation were well characterized and established in previous studies of RNA methylation in tissue-cultured cells from various prokaryote and eukaryote organisms [6-8]. These techniques would readily identify an aberrant RNA methylation pattern. In fact, a strikingly similar pattern of methylation has been observed [8] for eukaryote tRNA, whether isolated from: (1) yeast cells, (2) chick kidney fibroblasts, (3) mouse kidney fibroblasts, (4) CV-1 monkey fibroblasts, or (5) HeLa cells. The results of the present study indicate that tRNA from human skin fibroblasts exhibits the same distribution of these enzymatically controlled, methylated components. Because patterns of RNA methylation are highly specific and preserved throughout a wide variety of disparate organisms, it is improbable that cells from cystic fibrosis patients would show a basic alteration in such an apparently important process. Our comprehensive investigation supports the proposal that methylation of transfer and ribosomal nucleic acid is normal in cystic fibrosis.

Summary

Methylation of tRNA and rRNA was evaluated in normal human fibroblasts and in fibroblasts from patients with cystic fibrosis. The methylated base and nucleoside composition of 4 S, 18 S, and 28 S RNA species from these cells was found to closely resemble the patterns noted previously for other eukaryote cells. The RNA methylation patterns in the CF fibroblasts were qualitatively and quantitatively similar to those observed in normal fibroblasts. It is concluded that: (1) CF cells are not deficient in RNA methylation, and (2) the latter cannot be employed as a marker for identifying carriers of cystic fibrosis.

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