An Evolutionary Study of the Methylation of Transfer and Ribosomal Ribonucleic Acid in Prokaryote and Eukaryote Organisms

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SUMMARY

The methylated bases and nucleosides of transfer RNA and ribosomal RNA isolated from a number of eukaryote and prokaryote organisms were compared. Of the many different types of methylated bases and nucleosides found, only certain ones ever appeared in each type of methylated RNA; thus, the methylation of tRNA and both types of rRNA was a highly specific process. In addition, in all cells tested the pattern of methylation for tRNA and each rRNA differed markedly from each other.

Eukaryote tRNA, whether isolated from yeast, amoeba, chicken, mouse, monkey, or human cells, always exhibited a strikingly similar pattern of methylation. In addition, a second and third pattern of methylation were found to be common to the lower and higher molecular weight species of ribosomal RNA, respectively, in the eukaryote cells tested. Each of these three eukaryote RNA species contained at least one characteristic methylated base not found (or found only in trace amounts) in any of the others. These were N2-dimethylguanine and 1-methylhypoxanthine in tRNA, N2-dimethyladenine in 18 S RNA (16 S in yeast), and 3-methyluracil in 28 S RNA (25 S in yeast).

The patterns of methylation of prokaryote tRNA and rRNA were different from those of their eukaryote RNA counterparts. Two gram-negative bacteria, Escherichia coli B and Pseudomonas aeruginosa, had identical methylation patterns for tRNA, 16 S RNA, and 23 S RNA, respectively. In addition, each of the ribosomal RNA species had a characteristic methylated base not found in the other. These were N2-dimethyladenine in 16 S RNA, and N6-methyladenine in 23 S RNA. The pattern of methylation of tRNA from gram-negative bacteria, gram-positive bacteria, and mycoplasma differed only slightly from each other.

It was concluded that great differences exist in the methylation patterns of the various RNA species in a given cell while great similarities exist in the methylation patterns of each RNA species throughout a wide range of eukaryote or prokaryote cells.

A large number of methylated bases and nucleosides have been identified as minor components of transfer RNA and ribosomal RNA (1). However, no systematic analysis of the distribution of these methylated components in the tRNA and rRNA species of different organisms is available. Thus, it is not at all clear whether the methylation of RNA is a specific process whereby different RNA species have characteristic patterns of methylation or a nonspecific process in which any of the known methylated bases and nucleosides can be found in any RNA molecule.

In this study, the methylation patterns of tRNA and rRNA isolated from prokaryote and eukaryote cells selected from different regions of the evolutionary scale were compared. Evidence is presented which suggests that the methylation of tRNA and rRNA is a highly specific process. In all cells tested, whether prokaryote or eukaryote, the patterns of methylation for tRNA and each of the rRNA species differ from each other. In addition, each of the three eukaryote RNA species contains at least one characteristic methylated base.

Furthermore, it is observed that no matter what its source is, eukaryote tRNA always exhibits a strikingly similar pattern of methylation. A second pattern of methylation is found for lower molecular weight eukaryote rRNA, and a third pattern of methylation is found for higher molecular weight eukaryote rRNA.

The patterns of methylation of tRNA and rRNA isolated from prokaryote cells are different from and less complex than those of their eukaryote tRNA and rRNA counterparts. In prokaryote cells, as is the case in eukaryote cells, a similar pattern of methylation is found for tRNA, and a second and a third pattern of methylation is found for each of the two rRNA species.

MATERIALS AND METHODS

Cell Cultures and Media—The cells used in these experiments, their growth media, and their sources are listed in Table I.

Growth Conditions—Bacillus subtilis, Escherichia coli B, Pseudomonas aeruginosa, Micrococcus lysodeikticus, and Saccharomyces cerevisiae were grown at 37° with vigorous shaking. Acanthamoeba castellanii was grown at 30° with vigorous shaking. Mycoplasma orale I and Mycoplasma laidlawii were grown aerobically in Kimler bottles at 37°. HeLa cells were grown in suspension, and all other animal cells were grown as monolayers in 32-ounce prescription bottles.

Radioactive Methylation of RNA—RNA was labeled in vivo by the addition of L-[methyl-14C]methionine (55.4 μCi per μmole)
TABLE I

<table>
<thead>
<tr>
<th>Cells, media, and their source</th>
<th>Media</th>
<th>Source of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacillus subtilis</strong> (168 Trp^*)</td>
<td>M9 (3)</td>
<td>Dr. J. M. Utting (N. I. H.)</td>
</tr>
<tr>
<td><strong>Escherichia coli B</strong></td>
<td>M9 + 0.1% leucine</td>
<td>Dr. A. V. Furano (N. I. H.)</td>
</tr>
<tr>
<td><strong>Pseudomonas aeruginosa</strong> (PA32)</td>
<td>Trypticase soy broth (B.B.L.)</td>
<td>Dr. C. F. Kulpa (N. I. H.)</td>
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<tr>
<td><strong>Micrococcus lysodeikticus</strong></td>
<td></td>
<td>Dr. C. F. Kulpa (N. I. H.)</td>
</tr>
<tr>
<td><strong>Mycoplasma orale I</strong> (CH192199)</td>
<td>Hayflick AGDV (4) + 20% horse serum</td>
<td>Dr. M. F. Barile (N. I. H.)</td>
</tr>
<tr>
<td><strong>Mycoplasma laidlawii</strong> (PG8)</td>
<td>Hayflick AGDV (4) + 20% horse serum</td>
<td>Dr. M. F. Barile (N. I. H.)</td>
</tr>
<tr>
<td><strong>Saccharomyces cerevisiae</strong> (S28S Ch mating type)</td>
<td></td>
<td>Dr. E. Calab (N. I. H.)</td>
</tr>
<tr>
<td><strong>Acanthamoeba castellanii</strong> (Neff strain)</td>
<td></td>
<td>Dr. E. D. Korn (N. I. H.)</td>
</tr>
<tr>
<td><strong>Chick embryo fibroblasts</strong></td>
<td></td>
<td>Flow Laboratories, Inc., Rockville, Md.</td>
</tr>
<tr>
<td><strong>Chick kidney fibroblasts</strong></td>
<td></td>
<td>Flow Laboratories, Inc., Rockville, Md.</td>
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<tr>
<td><strong>Mouse embryo fibroblasts</strong></td>
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<tr>
<td><strong>Mouse kidney fibroblasts</strong></td>
<td></td>
<td>Flow Laboratories, Inc., Rockville, Md.</td>
</tr>
<tr>
<td><strong>HeLa S3</strong> (human carcinoma of cervix)</td>
<td></td>
<td>Dr. B. Moss (N. I. H.)</td>
</tr>
<tr>
<td><strong>H-Ep 2</strong> (human carcinoma of larynx)</td>
<td></td>
<td>Dr. L. Bockstahler, Environmental Health Service, Rockville, Md.</td>
</tr>
<tr>
<td><strong>CV-1 clone TC7 monkey fibroblasts</strong></td>
<td></td>
<td>Dr. M. M. Rechler (N. I. H)</td>
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<tr>
<td><strong>Balb/c 3T3 clone A-31 mouse fibroblasts</strong></td>
<td></td>
<td>Dr. A. Rein, Bionetics Research Laboratory, Bethesda, Md.</td>
</tr>
</tbody>
</table>

* N. I. H., National Institutes of Health, Bethesda, Maryland.
cytoplasmic extracts of eukaryote cells by extraction with sodium dodecyl sulfate and phenol at 60°C, followed by SDS-sucrose gradient centrifugation as previously described (11).

The RNA and rRNA were isolated from bacteria and mycoplasma in a similar manner except that after harvesting cells were frozen and thawed and the SDS-phenol extraction was done at room temperature. Sucrose gradient separation of methyl-labeled RNA species from E. coli B, S. cerevisiae, and HeLa were shown in Fig. 1. The fractions of the gradient containing 4 S RNA (assumed to be all tRNA since no other cytoplasmic low molecular weight RNA species is known to be methylated) and the two rRNA species were each pooled (see legend to Fig. 1). Each RNA species was subsequently incubated with pancreatic deoxyriboonuclease (20 µg per ml) for 30 minutes at 37°C in 0.002 M MgCl₂. The tRNA samples were deacylated by incubation in 0.1 M Tris acetate, pH 8.6, for 20 minutes at 37°C.

**Chromatographic Analysis of Methylated Bases and Nucleosides**

-Samples of [³⁵S]RNA were lyophilized and resuspended in 1 ml of trifluoroacetic acid (Eastman Organic Chemicals). The samples were sealed in evacuated Carius combustion tubes and heated to 170°C for 30 minutes in a Carius bomb. After cooling, the tubes were opened and the trifluoroacetic acid removed by boiling in a steam bath for 5 minutes. The dry residues were resuspended in 10 ml of H₂O and spotted on cellulose thin layer sheets with fluorescent indicator (Eastman Organic Chemicals No. 6065). 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 RB-54 medical x-ray film. For quantitation, radioactive spots were located by autoradiography, scraped, and counted in a solution of toluene, 2,5-diphenyloxazole (POPOP, 16 g per liter), and 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPPOP, 0.8 g per liter). The trifluoroacetic acid hydrolysis method yielded free bases, except for uracil and its derivatives which were hydrolyzed to uracil nucleosides (uridine, 3-methyluridine, 2-O-methyluridine, and ribothymidine).

This technique proved to be highly sensitive in detecting trace amounts of methylated bases. A methylated base containing as little as 10 cpm over background could easily be detected in a 3-month exposure.

The following standards were used to identify the methylated compounds: N²-methylguanine, N²-dimethylguanine, 1-methylhypoxanthine, 1-methyladenine, 2-methyladenine, 3-methyladenine, 7-methyladenine, and 5-methyluridine (ribothymidine), which were purchased from the Cyclo Chemical Corporation. 1-Methylguanine, 7-methylguanine, N⁴-methyladenine, N⁴-dimethyladenine, 5-methylcytosine, 5-methylcytidine, 5-methyldeoxyuridine, 3-methyluridine, and thymine were purchased from Sigma Chemical Company; 3-methylcytosine was purchased from Mann Research Laboratories. Methylated compounds for which no standard was available were tentatively identified by extrapolation from data of Iwanami and Brown (12, 13) for HeLa tRNA and rRNA, from data of Fellner and Sanger (14) for E. coli B rRNA, and from predicted chromatographic behavior of these compounds in the solvent systems used in this report.

**RESULTS**

**Methylated Pattern of Transfer RNA Isolated from Eukaryote Cells**—Transfer RNA was isolated from mitochondria-free cytoplasmic extracts of eukaryote cells growing in L-[methyl-¹⁴C]me-

The abbreviation used is: SDS, sodium dodecyl sulfate.
Fig. 2. Autoradiography of the trifluoroacetic acid hydrolysates of six tRNA samples isolated from eukaryote cell cultures. Samples of tRNA isolated from SDS sucrose gradients like those shown in Fig. 1 were hydrolyzed in trifluoroacetic acid and chromatographed in two dimensions on cellulose (Eastman No. 6065) thin layer sheets which were autoradiographed as described under "Materials and Methods." Cold tRNA, as well as unlabeled methylated compounds used as markers, were added to [methyl-^3H]tRNA before hydrolysis. After hydrolysis, guanine, adenine, cytosine, uridine, and the markers were located on the chromatograms by ultraviolet absorption. The ^4C methylated compounds (numbered 1 to 8) were scraped off the sheets and the radioactivity measured. The identity and relative quantities of the methylated compounds in each of the six autoradiograms are given in Table II. The cells, the total counts per min recovered, and the exposure time of the autoradiograms are as follows. a, HeLa (human tRNA), 2000 cpm, 10 days; b, H-14:2 (human tRNA), 1050 cpm, 10 days; c, CV-1 (monkey tRNA), 250 cpm, 60 days; d, 3T3 (mouse tRNA), 270 cpm, 60 days; e, chick embryo tRNA, 1300 cpm, 30 days; and f, Saccharomyces cerevisiae (yeast tRNA), 2100 cpm, 10 days. The four circles in each autoradiogram indicate diagrammatically the location of G (guanine), A (adenine), C (cytosine), and U (uridine). These circles are labeled only in Panel a since they are in the same relative positions in each autoradiogram. The methylated compounds are numbered (1 to 8) only in Panel d for the same reason. O, origin; F, front.

uridine could be used as specific tests for the presence of tRNA, 18 S, or 28 S RNA, respectively, in an RNA preparation.

Methylation Pattern of Ribosomal RNA from Mouse and Yeast Cells—The methylation pattern of 18 S and 28 S RNA isolated from mouse (3T3) cells was found to be very similar, both qualitatively and quantitatively, to that of the 18 S and 28 S RNA of HeLa cells, respectively. Great similarities were also found between HeLa 18 S and 28 S RNA and their smaller ribosomal counterparts in yeast 16 S and 25 S RNA (Fig. 3, Table III). Yeast 16 S RNA had relatively large amounts of N^4-dimethyladenine (51%) and low amounts of 1-methyladenine (4%) while yeast 25 S RNA had relatively large amounts of 1-methyladenine (18%) and low amounts of N^4-dimethyladenine (4%). As in HeLa RNA, 3-methyluridine was found only in the larger ribosomal RNA species and both ribosomal RNA species contained large amounts of 2-O-methyl nucleosides (27% and 49% for 16 S and 25 S, respectively). In contrast to HeLa ribosomal RNA, yeast ribosomal RNA had no N^4-methyladenine.

From the above data it is apparent that the specificity of the enzymes used to methylate tRNA and the two ribosomal RNA species has been preserved almost intact through a wide range of evolutionary changes.

Methylation Pattern of E. coli 4.16, 16 S, and 23 S RNA—The methylation pattern of E. coli B tRNA and E. coli B 16 S and 23 S ribosomal RNA was investigated (Fig. 3, Table III) in order to compare methylation in prokaryote cells with that in the eukaryote cells described above. Only four methylated compounds were found in E. coli B tRNA. These were 1-methylguanine, 7-methylguanine, ribothymidine, and 2-methyladenine. This was in contrast to the eukaryote tRNA samples which, in addition, contained N^2-methylguanine, N^2-dimethylguanine, 1-methylhypoxanthine, and 3-methylcytosine, but which contained...
no 2-methyladenine. As in the eukaryote RNA species, each E. coli RNA species has its own distinctive fingerprint (Fig. 3). In addition, each ribosomal RNA species had at least one characteristic methylated compound not found in the other. These were (as shown in Table III): N\textsuperscript{4}-dimethyladenine which was found in large quantities in 16 S RNA (37%) and N\textsuperscript{4}-methyl adenine which was found in large quantities in 23 S RNA (29%).

The small amounts of N\textsuperscript{4}-methyladenine (1%) and N\textsuperscript{4}-dimethyl adenine (6%) (see Footnote e in Table III) found in 16 S and 23 S RNA, respectively, might be due to cross-contamination resulting from the slight degradation of 23 S RNA as well as from incomplete separation of 16 S and 23 S RNA on sucrose gradients (Fig. 1). The evidence supporting this supposition is provided by Fellner and Sanger (14) who sequenced the methylated oligonucleotides of E. coli rRNA and found no N\textsuperscript{4}-methyl adenine in 16 S RNA and no N\textsuperscript{4}-dimethyladenine in 23 S RNA. In contrast to the situation in eukaryote rRNA, very little 2-O-methylated nucleoside was found in E. coli B rRNA.

**Methylation Patterns of RNA from Other Prokaryote Cells—**The methylation patterns of E. coli B tRNA and rRNA were compared to those of another gram-negative bacteria, P. aeruginosa. Both bacteria were found to have the same tRNA methylation pattern (Fig. 4, Table IV) as well as 16 S and 23 S methylation patterns (results not shown).

The tRNA\textsuperscript{s} of two gram-positive bacteria, B. subtilis and M. lysodeikticus, were also investigated (Fig. 4, Table IV). In contrast to the two gram-negative bacteria, the two gram-positive bacteria contained 1-methylguanine in their tRNA.

The mycoplasma, M. orale (Fig. 4, Table IV) and M. luidiavisci, had a tRNA-like RNA methylation pattern, i.e. their tRNA contained a high proportion of 1-methylguanine and 7-methyl guanine and also contained 2-methyladenine which is not found in the tRNA of eukaryote cells. This observation is in accord with the idea that mycoplasma are of bacterial origin.

### DISCUSSION

The methylation of RNA is apparently a highly specific process. Thus, of the many methylated bases and nucleosides that are known, only certain ones appear in a given species of RNA. In addition, similar patterns of RNA methylation are found to be common to a wide range of organisms. This was demonstrated by analyzing methylation patterns of tRNA and rRNA isolated from a number of organisms spanning the evolutionary scale.

The technique used in the analysis of methylated RNA had several advantages over methods previously used and allowed the rapid processing and easy comparison of a large number of samples because: (a) hydrolysis of RNA was done with trifluor oacetic acid which could be completely removed in minutes by boiling; (b) chromatography of acid-hydrolyzed RNA was carried out on thin layer sheets in two dimensions which produced excellent resolution of the bases in a short period of time; and (c) the methylated compounds were located by autoradiography which not only allowed the detection of compounds containing a very small amount of radioactivity (as little as 10 epm) but also allowed rapid and unambiguous comparisons between the methylated base composition of the various RNA's.

Eukaryote tRNA, whether isolated from yeast, amoeba, chicken, mouse, monkey, or human cells, always exhibited a strikingly similar pattern of methylation. A common pattern of methylation was also observed for the smaller species as well as for the larger species of eukaryote tRNA isolated from yeast, mouse, and human cells (the only cells tested). Thus, it is apparent that the specificities of the enzymes used to methylate tRNA and each of the rRNA species have been preserved almost intact in eukaryote cells through a wide range of evolutionary changes.

Similarities were also observed in the patterns of methylation

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**TABLE II**

Relative amounts of methylated compounds in tRNA of eukaryote cells

For experimental details, see Fig. 2.

<table>
<thead>
<tr>
<th>Code No.</th>
<th>Compound</th>
<th>Percentage of total radioactivity recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HeLa (human)</td>
</tr>
<tr>
<td>1</td>
<td>1-Methylguanine</td>
<td>21.1*</td>
</tr>
<tr>
<td>2</td>
<td>7-Methylguanine</td>
<td>19.9</td>
</tr>
<tr>
<td>3</td>
<td>1-Methyladenine</td>
<td>11.9</td>
</tr>
<tr>
<td>4</td>
<td>N\textsuperscript{4}-Methylguanine</td>
<td>17.5</td>
</tr>
<tr>
<td>5</td>
<td>N\textsuperscript{4}-Dimethylguanine</td>
<td>3.1</td>
</tr>
<tr>
<td>6</td>
<td>1-Methylhypoxanthine</td>
<td>16.3</td>
</tr>
<tr>
<td>7</td>
<td>5-Methylcytosine</td>
<td>10.0</td>
</tr>
<tr>
<td>8</td>
<td>5-Methyluridine (ribotimidyl)</td>
<td>+d</td>
</tr>
<tr>
<td>14\textsuperscript{a}</td>
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<td>-</td>
</tr>
<tr>
<td>15\textsuperscript{a}</td>
<td>3-Methyluridine</td>
<td>-</td>
</tr>
<tr>
<td>16\textsuperscript{a}</td>
<td>2-O-Methyluridine</td>
<td>-</td>
</tr>
<tr>
<td>17\textsuperscript{a}</td>
<td>2-O-Methylribose\textsuperscript{b}</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3-Methylcytosine</td>
<td>-</td>
</tr>
</tbody>
</table>

* From data of Iwanami and Brown (12).

\textsuperscript{a} In another chromatogram these two compounds were resolved to yield 53% 1-methylguanine and 47% 7-methylguanine.

\textsuperscript{b} For location of these spots see Fig. 3.

\textsuperscript{d} Detectable in small amounts in fingerprints of HeLa (Fig. 3); mouse, chicken, monkey, and yeast tRNA exposed to film for a long period (90 days).

\textsuperscript{e} Tentative identification based on RF values published by Iwanami and Brown (12).
The extraction of RNA, RNA hydrolysis, chromatography with markers, identification, and quantitation of methylated compounds are as described in Fig. 2. The identity and relative quantities of the methylated compounds which are numbered 1 to 10 are given in Table III. The total counts per min recovered and the exposure time of the autoradiograms are as follows. Top left, HeLa 4 S RNA, 2000 cpm, 90 days; top center, HeLa 18 S RNA, 700 cpm, 90 days; top right, HeLa 28 S RNA, 650 cpm, 90 days; bottom left, E. coli B 4 S RNA, 500 cpm, 30 days; bottom center, E. coli B 18 S RNA, 475 cpm, 90 days; and bottom right, E. coli B 28 S RNA, 425 cpm, 90 days. The compound labeled as Z in Fig. 3 (top center) was not found in any other experiment involving 18 S RNA (or any other RNA) and was therefore disregarded.

Fig. 3. Autoradiography of the trifluoroacetic acid hydrolysates of tRNA and rRNA isolated from HeLa and Escherichia coli cells. The extraction of RNA, RNA hydrolysis, chromatography with markers, identification, and quantitation of methylated compounds are as described in Fig. 2. The identity and relative quantities of the methylated compounds which are numbered 1 to 10 are given in Table III. The total counts per min recovered and the exposure time of the autoradiograms are as follows. Top left, HeLa 4 S RNA, 2000 cpm, 90 days; top center, HeLa 18 S RNA, 700 cpm, 90 days; top right, HeLa 28 S RNA, 650 cpm, 90 days; bottom left, E. coli B 4 S RNA, 500 cpm, 30 days; bottom center, E. coli B 18 S RNA, 475 cpm, 90 days; and bottom right, E. coli B 28 S RNA, 425 cpm, 90 days. The compound labeled as Z in Fig. 3 (top center) was not found in any other experiment involving 18 S RNA (or any other RNA) and was therefore disregarded.

of various prokaryote cells which included gram-negative bacteria, gram-positive bacteria, and mycoplasma. E. coli B and P. aeruginosa, both gram-negative but with little else in common (e.g. the GC contents of these bacteria were 52% and 68%, respectively (16)), had identical methylation patterns for tRNA, 16 S RNA, and 23 S RNA, respectively. On the other hand, the two gram-positive bacteria, B. subtilis and M. lysodeikticus, differed somewhat from the gram-negative bacteria mentioned above by containing 1-methyladenine in their tRNA. The two mycoplasma tested, M. cralle and M. laidlawii, had bacteria-like methylation patterns for their tRNA. This would be in accord with the idea that mycoplasma are of bacterial origin.

The specificity of methylation was also demonstrated by comparing methylation patterns of tRNA and rRNA. Every cell line tested, whether of eukaryote or prokaryote origin, had a very different methylation pattern for tRNA and each of the two rRNA species. In addition, each of the three eukaryote RNA species contained at least one characteristic methylated base not found (or found only in trace amounts) in any of the others. These were N,N-dimethylguanine and 1-methylhypoxanthine, N,N-dimethyladenine, and 3-methyluracil, which were found in tRNA, 18 S RNA (16 S in yeast), and 28 S RNA (25 S in yeast), respectively. Furthermore, each prokaryote ribosomal RNA species had a characteristic methylated base not found in the other. These were N,N-dimethyladenine and N,N-methyladenine, which were found in 16 S and 23 S RNA, respectively.

Several other methylated bases and nucleosides were found to be restricted as to the species of RNA in which they appeared. Thus, eukaryote tRNA, but not eukaryote rRNA, contained 2-O-methyl nucleosides, 6-methyladenine, and 6-dimethyladenine. On the other hand, eukaryote tRNA contained N,N-dimethylguanine, thymine, and 5-methylcytosine. These were found...
terns of the two ribosomal RNA species; namely, N6-dimethyl- has been accompanied by vast genetic changes, the specificity
adenine occurs only in 18S RNA while I-methyladenine and of the RNA methylating enzymes has not been affected by these
might be a result of the different analytic techniques used.
lar, these authors have reported that the methylated components agreement with those of Iwanami and Brown (43% and 50%).
In particu- in 18S and 28S RNA (447, and 50%, respectively) are in good
by the other laboratories. These different quantitative values
radioactive methyl that appears as 2-O-methylated nucleosides
reported for HeLa tRNA (Table II), yeast rRNA, and
in this report are in good qualitative agreement with those
ously reported. In general, the methylation patterns described
similar to that of E. coli H.
was found in
and Sanger (14). In addition, less than 1% NG-dimethyladenine
This high value was probably due to contamination by 16S
and may contain a modification (possibly a methyl) on the ribose.
It is probably identical to "mu" described by Fellner and Sanger
This compound displays properties very similar to thymidine
This spot might contain some Compound 9.
This compound might be N4-methyl (2-O.methyl)cytidine
Thymine is derived from DNA and not RNA since tri-
fluoroacetic acid hydrolysis of deoxythymidine phosphate yields
the free base while ribothymidine is not hydrolyzed under these
Quantitative data are for autoradiogram of Fig. 2a.
A + indicates base was present in small amounts and was not
measured quantitatively.
This compound displays properties very similar to thymidine
and contain a modification (possibly a methyl) on the ribose.
It is probably identical to "mu" described by Fellner and Sanger
For experimental details, see Fig. 3.
only in trace amounts in eukaryote RNA. Prokaryote RNA,
but not prokaryote tRNA, contained N4-methylguanine, N6-di-
methyladenine, and 2-O-methyl nucleosides.
Methylation patterns for HeLa tRNA (12), HeLa RNA (13),
yeast RNA (17), and E. coli K-12 RNA (14) have been previ-
ously reported. In general, the methylation patterns described
in this report are in good qualitative agreement with those
reported for HeLa tRNA (Table II), yeast RNA, and E. coli
K-12 RNA, although the quantitative values that we find for a
few of the methylated compounds are different than those found
by the other laboratories. These different quantitative values
might be a result of the different analytic techniques used.
On the other hand, there are some significant discrepancies in
the methylation patterns we have found for 18S and 28S RNA
and those found by Iwanami and Brown (13). In particu-
lar, these authors have reported that the methylated components
of 18S and 28S RNA are qualitatively identical. However, we
have found some qualitative differences in the methylation pat-
terns of the two ribosomal RNA species; namely, N4-dimethyl-
adenine occurs only in 18S RNA while 1-methyladenine and
3-methyluracil occur only in 28S RNA. It is possible that the
qualitative differences that we find in the methylation of 18S
and 28S RNA are due to better resolution of these two RNA
species on sucrose gradients which results in less overlap. An-
other discrepancy is our failure to find any N4-methylcytosine
in HeLa ribosomal RNA while Iwanami and Brown (13) have
found large amounts of this compound in both 18S and 28S
RNA. However, these authors mention that N4-methylcytosine
is labile in 85% formic acid heated at 175° for 30 min. There-
fore, it is possible that this compound was destroyed during our
hydrolysis procedure which used trifluoroacetic acid heated to
170° for 30 min. It should be pointed out that our results for the percentage
of radioactive methyl that appears as 2-O-methylated nucleosides
in 18S and 28S RNA (44% and 50%, respectively) are in good
agreement with those of Iwanami and Brown (43% and 50%).
It is apparent from the results presented above that although
the evolution of eukaryotes from single cell to multicell organisms
has been accompanied by vast genetic changes, the specificity
of the RNA methylating enzymes has not been affected by these

<table>
<thead>
<tr>
<th>Code No.</th>
<th>Compound</th>
<th>HeLa</th>
<th>Yeast</th>
<th>E. coli</th>
</tr>
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<td>1-Methylguanine</td>
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<td>2.4</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
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<td>8.2</td>
<td>2.4</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
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<td>2.4</td>
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<td>13</td>
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<td>16</td>
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<tr>
<td>17</td>
<td>2-O-Methyl ribose</td>
<td>8.2</td>
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<td>19</td>
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<td>8.2</td>
<td>2.4</td>
<td>+</td>
</tr>
</tbody>
</table>

* A + indicates base was present in small amounts and was not
measured quantitatively.
* Tentative identification based on RF values published by
Iwanami and Brown (12) and on predicted chromatographic be-
behavior of 2-O-methyl nucleosides in this solvent system. 2-O-
Methyl ribose is presumably the hydrolysis product of 2-O-methyl-
urine and 2 O methylcytidine.
* This spot might contain some Compound 0.
* This compound might be N4-methyl(2-O-methyl)cytidine
which has been reported to be in E. coli 16S RNA (14).
TABLE IV

Relative amounts of methylated compounds in tRNA of prokaryote cells

For experimental details, see Fig. 4.

<table>
<thead>
<tr>
<th>Code No.</th>
<th>Compound</th>
<th>Percentage of total radioactivity recovered</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td>1</td>
<td>1-Methylguanine</td>
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<td>2</td>
<td>7-Methylguanine</td>
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<td>3</td>
<td>1-Methyladenine</td>
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<td>8a</td>
<td>5-Methyluridine (ribothymidine)</td>
<td>27.0</td>
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<tr>
<td>9</td>
<td>2-Methyladenine</td>
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</table>

* A significant amount of labeling of adenine and guanine was observed probably due to the lack of uptake of adenosine (Fig. 4). The radioactivity associated with adenine and guanine was not included in calculating the percentage of total radioactivity recovered.

* In another experiment with better resolution these two compounds were resolved to yield 42% 1-methylguanine and 58% 7-methylguanine.

Changes in the enzymes and their functions have been preserved relatively intact as a group. Thus, the preservation of the specificity of RNA methylation through the evolutionary scale implies that methylation might have an important biological role. The nature of this role is as yet unclear. It is known, however, that (a) unmethylated RNA species like messenger RNA and heterogeneous nuclear RNA have shorter half-lives than do tRNA or rRNA (18); (b) methylation of DNA is involved in the restriction by bacteria of phage infection (19); and (c) in the processing of 45S ribosomal precursor to mature 18S and 28S RNA, it is the unmethylated regions that are preferentially lost (20). Therefore, methylation may play a role in RNA degradation by protecting certain RNA sequences from cell nucleases. Furthermore, the characteristic methylation patterns of the 18S and 28S regions of the 45S precursor RNA might play a role in producing the mature ribosomal RNA product.

Whatever the function of RNA methylation may be, it is suggested here that this function is expressed in a similar manner throughout the evolutionary scale.

REFERENCES

An Evolutionary Study of the Methylation of Transfer and Ribosomal Ribonucleic Acid in Prokaryote and Eukaryote Organisms
Michael Klagsbrun


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