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In vitro methylation of *Escherichia coli* 16S ribosomal RNA and 30S ribosomes

(5-methylcytidine/2-methylguanosine/3-methyluridine/HPLC of nucleosides)

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ABSTRACT **Treatment of synthetic 30S particles lacking all of the normally methylated nucleotides with *S*-adenosyl-[^{3}H]methionine and either an S100 or ribosomal high salt wash extract resulted in ribosome-dependent incorporation of [^{3}H]methyl groups into trichloroacetic acid-insoluble material. No incorporation was observed when naturally methylated isolated 30S particles were used, showing that methylation at unnatural sites did not occur. Enzymatic hydrolysis of the labeled RNA to nucleosides followed by HPLC analysis identified the [^{3}H]methylated residues. Activities for the formation of N^6-methyladenosine, N^6-dimethyladenosine, 5-methylcytidine (m^{5}C), 3-methyluridine, and N^2-methylguanosine were found. Fractionation by ammonium sulfate partially resolved the different activities. All of the fractions with m^{5}C activity were 6–8 times more active on synthetic unmethylated 16S RNA than on synthetic 30S ribosomes, whereas the N^2-methylguanosine activity preferred 30S ribosomes to 16S RNA by a factor of more than 10. The N^6-methyladenosine and N^6-dimethyladenosine activities were 30S ribosome-specific. The m^{5}C activity present in the 55–85% ammonium sulfate fraction of the high salt wash yielded a maximum of 1.0 mol of m^{5}C per mol of 16S RNA, although two m^{5}C residues, positions 967 and 1407, are found *in vivo*. RNase protection by hybridization with the appropriate oligodeoxynucleotide identified the methylated residue as C-967. Methylation of m^{5}C-967 did not require prior methylation of G-966, and methylation of A-1518 and A-1519 was not dependent on prior methylation of G-1516.**

There are 10 known methylated nucleotides in *Escherichia coli* 16S rRNA which together contain a total of 13 methyl groups (1, 2). These modified residues are clustered in the 3′ third of the RNA with three (m^{4}Cm-1402, m^{5}C-1407, and m^{3}U-1498) in the two highly conserved sequences, positions 1393–1408 and 1492–1505, three (m^{2}G-1516, m$^6_2$A-1518, and m$^6_2$A-1519) in the 3′-terminal stem, and three (m^{2}G-966, m^{5}C-967, and m^{2}G-1207) positioned elsewhere in the 3′ third of the molecule. m^{7}G-527 is the only residue found in a different region.

The function of these modified bases is largely unknown. The m$^6_2$A residues are not essential for normal ribosomal function (3, 4), although their absence does affect translational fidelity (5), modifies the stability of the adjacent stem structure (6), and renders the ribosome kasugamycin resistant (7). None of the other methylated bases appear essential for protein synthesis, since ribosomes constructed from unmethylated 16S RNA are able to carry out all of the partial reactions of *in vitro* protein synthesis, albeit at a somewhat lower efficiency (8–13).

With regard to a role in assembly of the ribosome, it is believed that methylation occurs late in this process (14, 15), but little or nothing is known about the detailed temporal or sequential nature of the methylation reactions. Nothing is known about the substrate specificity either, except that m$^6_2$A formation requires a 30S particle as substrate (16).

The availability of 16S RNA and 30S ribosomes that are completely unmethylated by virtue of being made *in vitro* (8) has now made it possible to explore these and other questions relating to the methylation of ribosomes. In this work, we have identified four discrete activities that are specific for the methylation of *E. coli* 16S RNA. We show that one methylation occurs specifically on 16S RNA and that others specifically require the 30S ribosome. We also identify the specific site of methylation for one of these reactions.

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MATERIALS AND METHODS

Chemicals. Methylated nucleotides were obtained from the following sources. m^{4}C was from Jack Fox (Sloan–Kettering Institute, Rye, NY); Cm was from ICN; m^{4}CmpCp was from Byron Lane (University of Toronto); m^{7}G, m^{5}C, m^{3}U, m^{2}G, m^{6}A, m$^6_2$A, and CmpC were from Sigma. m^{4}CmpC and m^{4}Cm were prepared from m^{4}CmpCp by enzymatic digestion. m^{4}CmpCp was eluted from the column described in Fig. 1 at 3.5% (vol/vol) acetonitrile as two closely spaced peaks, probably the 2′ and 3′ isomers. Treatment with alkaline phosphatase converted both peaks to a single peak eluting at the position labeled as m^{4}CmpC in Fig. 1. Digestion of m^{4}CmpCp with venom phosphodiesterase plus alkaline phosphatase yielded the m^{4}CmpC peak plus approximately equal amounts of cytidine and a second peak. This second peak must be m^{4}Cm and is so labeled in Fig. 1. *S*-Adenosyl[^{3}H]-methionine ([^{3}H]SAM; 60 Ci/mmol; 1 Ci = 37 GBq) was obtained from Amersham and unlabeled SAM was from Sigma. The [^{3}H]SAM was diluted to a specific activity of ≈2000 dpm/pmol and purified from *S*-adenosylhomocysteine by the method of Shapiro and Ehninger (17). The specific activity was determined at pH 2 by using an ε_{256} (6 M HCl) of 14,700 (17). DNase (RNase-free) and RNase A, RNase T1, RNase P1, and bacterial alkaline phosphatase were obtained from Worthington, Sankyo, Boehringer Mannheim, and Sigma, respectively. RNasin was from Promega Biotec. Synthetic and natural 16S RNA and 30S ribosomes were obtained as described (8, 18). Deoxyoligomers complementary to 16S RNA residues 958–977, 1197–1216, 1398–1417, and 1506–1525 were prepared on an Applied Biosystems model 381A synthesizer, manually deprotected, and purified by Sephadex G-25 gel filtration. RD buffer is 20 mM Hepes, pH 7.5/100 mM NH_4Cl/20 mM $Mg(OAc)_2$/5 mM 2-

Abbreviations: m^{6}A, N^6-methyladenosine; m$^6_2$A, N^6-dimethyladenosine; m^{7}G, 7-methylguanosine; m^{2}G, N^2-methylguanosine; m^{1}G, 1-methylguanosine; m^{3}U, 3-methyluridine; m^{5}C, 5-methylcytidine; m^{4}C, N^4-methylcytidine; Cm, 2′-*O*-methylcytidine; m^{4}Cm, N^4,$O^{2'}$-dimethylcytidine; m^{4}CpC, N^4-methylcytidylylcytidine; m^{4}CmpC, N^4,$O^{2'}$-dimethylcytidylylcytidine; CmpC, 2′-*O*-methylcytidylylcytidine; m^{4}CmpCp, N^4, $O^{2'}$-dimethylcytidylylcytidine 3′-phosphate; SAM, *S*-adenosylmethionine; HSW, high-salt-wash fraction from ribosomes.

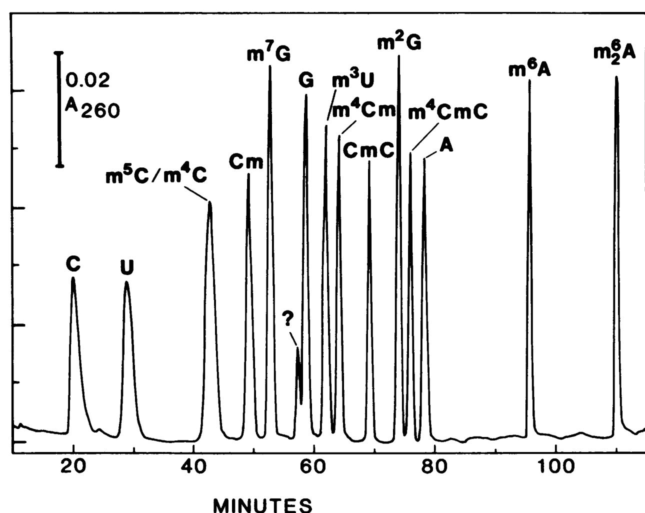

FIG. 1. HPLC analysis of methylated and nonmethylated nucleosides. Standards were obtained as described in the text. ?, Unidentified contaminant.

mercaptoethanol. A(x) buffer is 20 mM Tris·HCl, pH 7.5/x mM NH_4Cl/1 mM EDTA/6 mM 2-mercaptoethanol/10% (vol/vol) glycerol. Formamide (Fluka, puriss.) was freshly deionized (19) before use.

Enzymes. S100 cell-free extract and high salt wash (HSW) were prepared from 120 g of *E. coli* MRE600 as described by Poldermans *et al.* (16) for strain Q13 except that 4560 units of RNase-free DNase was added to the initial extract and the mixture was incubated at 0°C for 15 min before centrifugation at 30,000 × *g*. To either the S100 (217 ml) or HSW (55 ml), $(NH_4)_2SO_4$ was added to a final concentration of 35% saturation at 0°C over a 2- to 3-hr period. After stirring overnight, the precipitate was removed by centrifugation (15 min, 10,000 × *g*), dissolved in the original volume of buffer A(50), and dialyzed for 18 hr versus buffer A(50) with three changes. An equal volume of glycerol was added for storage at −20°C. The supernatant was brought to 55% saturated $(NH_4)_2SO_4$ and the precipitate was collected, dissolved, and dialyzed in a similar fashion. This supernatant was then adjusted to 85% saturated $(NH_4)_2SO_4$ and the resulting precipitate was recovered. After glycerol addition, the final volume of the S100 and HSW fractions was 40 ml and 20 ml, respectively. Thus, 1 vol of the HSW fractions came from twice as many cells as 1 vol of the S100 fractions. Further separation of one-half of the 35–55% saturated $(NH_4)_2SO_4$ HSW [HSW(35–55)] fraction (before glycerol addition) was done on DEAE-Sepharose CL-6B. The column (2.5 × 30.0 cm) was pretreated separately with 3 vol each of buffer A(50), buffer A(50) + 0.1% bovine serum albumin, buffer A(1000), and buffer A(50). After loading the sample and washing with 300 ml of buffer A(50), elution was done with a 50–1000 mM NH_4Cl gradient. Fraction 14, which had only m^6_2A methylase activity, was eluted at the beginning of the gradient. A more dilute S100 preparation made from 12.1 g of cells was concentrated by 85% $(NH_4)_2SO_4$ precipitation, dissolved in 4 ml of buffer A(50) plus 50% glycerol, and stored at −20°C. One milliliter of this extract (S100C) was equivalent to 1 ml of the $(NH_4)_2SO_4$ fractions of S100.

Methylation. Reaction mixtures contained 100 mM Tris·HCl or Hepes, pH 7.5, 40 mM NH_4Cl, 2 mM $Mg(OAc)_2$, 6 mM 2-mercaptoethanol, RNasin (800 units/ml), 4.3 μM [^{3}H]SAM, 20 nM 16S RNA or 30S ribosome, and enzyme as indicated. Incubation was at 37°C for the times indicated. The reaction was stopped by addition of trichloroacetic acid at 0°C to 5% (wt/vol). After 10 min, the precipitate was collected on BA85 cellulose nitrate filters (Schleicher & Schuell) and dissolved in Bray's solution, and the radioactivity was measured. For nucleoside analysis, the methylation of 1–10 pmol of RNA or ribosomes was terminated by chilling to 0°C and adding $NaDodSO_4$ to 1%. Natural 30S (1A_{260} unit) was added as carrier, and the mixture was extracted with phenol. After re-extraction of the phenol phase, the combined aqueous phases were extracted four times with ether. The RNA was precipitated with ethanol three times and dissolved in water. For the nuclease protection analysis, the methylation reaction contained 40 nM RNA (0.6 A_{260} unit), 2.4 A_{260} units/ml of carrier yeast $tRNA^{Phe}$ was added at the end of the reaction, and phenol/$NaDodSO_4$ extraction was carried out as above.

Nucleoside Analysis. The methylated 16S RNA plus carrier (1.0–1.15 A_{260} units in 250 μl of water) was digested with 10 μg of RNase P1 in 1 ml of 20 mM NaOAc, pH 5.3/0.5 mM $ZnSO_4$ at 37°C for 2 hr. Incubation was continued for 2 hr longer after addition of Tris (pH 8.0) to 56 mM and 1.7 units of bacterial alkaline phosphatase. The reaction was terminated by the addition of HOAc to 28 mM. For HPLC analysis, the digest was supplemented with 0.2 A_{260} unit of a mixture containing equimolar amounts of the methylated nucleosides as internal standards. HPLC was on a Waters μBondapak C_{18} column (4 × 300 mm) run at 23°C in 10 mM sodium phosphate adjusted to pH 5.1 with NH_4OH (buffer A) changing to a 1:1 (vol/vol) mixture of buffer A plus acetonitrile (buffer B). The gradient was 0% buffer B for 5 min, 0–9% buffer B from 5 min to 7 min, 9–50% buffer B from 70 min to 90 min, and 50–100% buffer B from 90 min to 100 min at a flow rate of 0.5 ml/min. The absorbance was monitored with a Waters 990A diode array detector. Fractions (0.5 ml) were collected for radioactivity analysis.

Nuclease Protection. [^{3}H]Methyl-labeled 16S RNA (0.25–0.53 pmol) was hybridized with a 50-fold excess of deoxyoligonucleotide in 45 μl of a mixture containing 40 mM Mes (pH 6.4), 400 mM NaCl, 1 mM EDTA, and freshly deionized 80% (vol/vol) formamide. After heat denaturation for 10 min at 90°C, the sample was removed to room temperature. After 15 min, the mixture was diluted with 9 vol of ice-cold 10 mM Tris·HCl, pH 7.5/300 mM NaCl/5 mM EDTA. RNase T1 (0.4–3.6 Sankyo units/pmol of 16S RNA) was then added. Digestion was carried out for 30 min at 30°C and stopped with 9 vol of ice-cold 10% trichloroacetic acid. After 10 min at 0°C, the mixture was filtered on a BA85 nitrocellulose filter and dissolved in Bray's solution, and the radioactivity was measured.

RESULTS

Nucleoside Analysis. The HPLC system used for nucleoside analysis was adapted from that of Gehrke *et al.* (20). All of the expected methylated bases were separated from each other as well as from unmethylated bases, except for m^5C and m^4C, which coeluted (Fig. 1). The dinucleotide m^4CmpC could not be further digested by the standard conditions that employ nuclease P1 plus alkaline phosphatase. This is presumably because of the m^4C moiety since CmpC is readily split into cytidine and Cm by the same digestion conditions (data not shown).

Methylation by Crude Extracts. To determine if *in vitro* methylation of synthetic RNA or ribosomes would occur at all, incubation with [^{3}H]SAM and an S100 extract from *E. coli* was performed. As shown in Table 1, activity was readily detected for synthesis of m^5C, m^3U, m^2G, and m^6_2A. The m^6A is most likely produced as an intermediate in m^6_2A synthesis as no m^6A is known to occur in *E. coli* 16S RNA. The synthesis of m^3U appeared highly dependent on the Mg^{2+} concentration, being stimulated 3-fold when the Mg^{2+} was raised from 2 to 9 mM. The use of naturally methylated 30S ribosomes as a control showed that no methylation occurred

Table 1. Methylation activities in a crude *E. coli* extract

Ribosome	Mg^{2+}, mM	Methylated bases, mol/mol				
		m^5C	m^3U	m^2G	m^6A	m^6_2A
Syn	2	0.04	0.07	0.86	0.14	1.02
Nat	2	<0.02	<0.02	<0.02	<0.02	<0.02
Syn	9	0.09	0.21	0.51	0.21	0.40
Nat	9	<0.02	<0.02	<0.02	<0.02	<0.02

Values are expressed as mol of nucleoside per mol of substrate added to the reaction mixture and thus assume 100% recovery at all steps. Analysis was by HPLC. Syn, 30S ribosomes containing synthetic 16S RNA; Nat, isolated 30S ribosomes. The standard methylation conditions were modified to contain 8.3 μM SAM, 42 nM ribosomes, and S100C (80 μl/ml). Reaction was at 37°C for 1 hr.

at incorrect sites, since such sites should also have been available in the control ribosomes.

Methylation by Partially Fractionated Extracts. On the basis of these results, the crude extract was fractionated by $(NH_4)_2SO_4$ precipitation. The HSW extract was similarly fractionated. Assays were done on free unmethylated RNA in addition to the 30S ribosomes to explore the substrate specificity. A typical chromatogram is shown in Fig. 2, and the results are summarized in Table 2. Only ribosomes were used in the assay of the 0–35% enzyme fraction because trichloroacetic acid precipitation assays showed that incorporation of $[^3H]CH_3$ with RNA as the substrate was barely above the enzyme blank value. Since the purpose of this analysis was to survey the distribution of methylase activities, only a single enzyme concentration and a fixed reaction time were used in these experiments. This explains the nonstoichiometric amount of methylated products. The assumption of 100% recovery in the extraction and precipitation steps may also act to reduce the mole ratio. However, the effect cannot be large since 90% of the expected amount was obtained for m^6_2A synthesis with S100(35–55). Enzyme blanks were not determined in this series because each of the substrates served as a control for the other. From a comparison of the results, it is clear that most or all of the reported methylations were substrate-dependent. The data in Table 2 were obtained at 2 mM Mg^{2+}, but 10 mM Mg^{2+} was also tested with the HSW(55–85) fraction and both RNA and 30S substrates with no major differences being found (data not shown). Note also that m^5C and m^4C cannot be distinguished by this column procedure. Although in the following discussion the base will be referred to as m^5C for convenience, we have not actually identified it as such except for HSW(55–85) acting on free RNA (see Fig. 5 below).

Activities specific for the methylation of the various bases were clearly separable from each other. Similar distributions

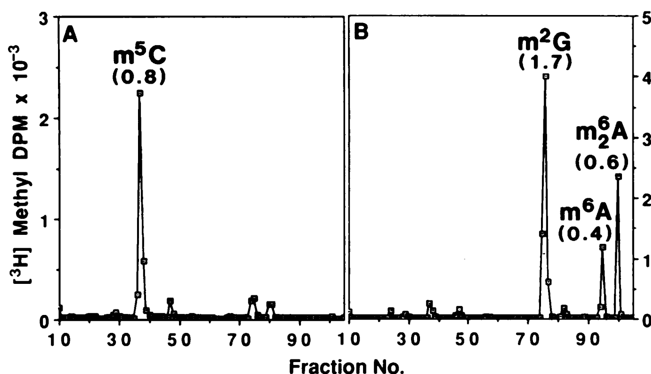

FIG. 2. HPLC analysis of nucleotides methylated by cell-free extracts of *E. coli*. (*A*) Synthetic 16S RNA. (*B*) Synthetic 30S subunits. Methylation using the HSW(55–85) fraction and nucleoside analysis were as described in Table 2. The identity of each peak is indicated. Values in parentheses are mole ratios from Table 2.

Table 2. Distribution of methylation activities in fractionated *E. coli* extracts

Enzyme	Substrate	Methylated bases, mol/mol			
		m^5C/m^4C	m^2G	m^6A	m^6_2A
HSW(0–35)	Rib	<0.1	**1.0**	**0.2**	**1.3**
HSW(35–55)	Rib	<0.1	**0.8**	0.1	**0.9**
HSW(35–55)	RNA	**0.2**	**0.2**	<0.1	<0.1
HSW(55–85)	Rib	0.1	**1.7**	**0.4**	**0.3**
HSW(55–85)	RNA	**0.8**	0.1	0.1	<0.1
S100(0–35)	Rib	<0.1	**0.6**	**0.3**	**0.3**
S100(35–55)	Rib	0.1	**1.1**	0.1	**1.8**
S100(35–55)	RNA	**0.6**	0.1	<0.1	<0.1
S100(55–85)	Rib	0.1	**1.4**	<0.1	<0.1
S100(55–85)	RNA	**0.7**	<0.1	<0.1	<0.1

Values are expressed as mol of nucleoside per mol of 30S ribosome or 16S RNA added to the reaction mixture and thus assume 100% recovery at all steps. Analysis was by HPLC. The $(NH_4)_2SO_4$ fractions are indicated by the numbers in parentheses. They were used at 80 μl/ml of reaction mixture in a 1-hr incubation. RNA, synthetic 16S RNA lacking all methylated bases; Rib, 30S ribosomes reconstituted from synthetic 16S RNA. Values greater than 0.1 are shown in boldface type.

were found in both crude extracts although there appeared to be more activity in the ribosome-free S100 than in the HSW considering that 1 ml of HSW comes from twice as many cells as does 1 ml of S100. However, the main feature of interest was the substrate specificity. The m^5C activity preferred RNA to 30S by up to 8-fold whereas the m^2G activity was the reverse, preferring ribosomes to RNA by as much as 17-fold. This striking effect is illustrated in Fig. 2 where the same enzyme fraction and reaction conditions produced completely different products depending on the nature of the substrate.

Stoichiometry of Methylation. As mentioned above, only m^6_2A formation approached the expected level. To determine if any of the other enzyme fractions could also produce stoichiometric quantities of methylated product, HSW(55–85) was chosen for further study. This fraction made less than half the expected amount of m^5C with RNA as the substrate and did not make any other methylated base. The kinetics of this reaction is shown in Fig. 3. The dependence on added RNA was high, and there was a clear plateau of reaction, which reached a value of 0.96 mol of methyl per mol of RNA. Note that twice as much enzyme was needed in this exper-

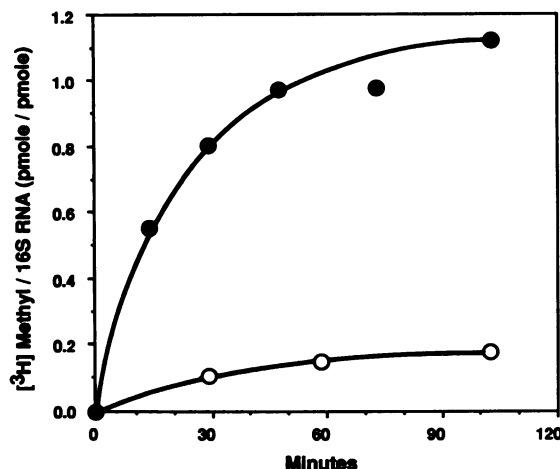

FIG. 3. Kinetics of m^5C formation with synthetic 16S RNA as substrate. Methylation was performed with the HSW(55–85) enzyme fraction at 160 μl/ml for the indicated times. Analysis was by trichloroacetic acid precipitation. Solid circles, 16S RNA added; open circles, no RNA added.

iment to reach the same level of reaction as was obtained in Table 2. This is likely due to the 2 months that elapsed between the two experiments, as methylases are known to be unstable in the absence of substrate.

Identification of the Site of m^{5}C Formation. HSW(55–85) was only capable of forming m^{5}C on free 16S RNA and only to the extent of 1 mol/mol. On the reasonable assumption that all of the methylation was, therefore, occurring at one m^{5}C or the other, rather than being somehow distributed between the two sites, the location of the methylated base was investigated. Since the two m^{5}C sites are well-separated from each other, at positions 967 and 1407 (Fig. 4), it was possible to design a hybridization-protection assay to determine which m^{5}C was being made. Deoxyoligomers complementary to sequences of 16S RNA spanning each of the m^{5}C sites were made, as indicated in Fig. 4. After hybridization to the [^{3}H]CH_3-containing 16S RNA, the complex was digested with RNase T1, and the amount of protected [^{3}H]CH_3 was measured by trichloroacetic acid precipitation of the undigested fragments. As shown in Fig. 5*A*, deoxyoligomer 958–977 strongly protected the [^{3}H]CH_3 from RNase digestion, whereas deoxyoligomer 1398–1417 was totally ineffective. Identical results were obtained when RNase A was used for the digestion (data not shown). To verify the specificity of deoxyoligomer 958–977 protection, 16S RNA methylated exclusively at A-1518 and A-1519 was used in a parallel experiment (Fig. 5*B*). Deoxyoligomer 958–977 was completely ineffective in protecting this site of methylation, as was another deoxyoligomer 1197–1216, although very high protection was obtained by deoxyoligomer 1506–1525. These results show that HSW(55–85) contains an m^{5}C methylase that is specific for position 967, but only when that site is present in free RNA.

DISCUSSION

Previous work on ribosome methylation has been hampered by the general lack of a defined phenotype for the isolation of methylase-deficient mutants. In only one case, that of the m$^6_2$A methylase, has the enzyme been characterized (16), and the gene been cloned and sequenced (22). This was possible because mutants lacking this enzyme are kasugamycin-resistant, thus providing not only a defined phenotype but a selectable marker. Such a situation is not known to exist for any of the other methylases that use 16S RNA as a substrate, and no other methylases specific for 16S RNA have been described. No phenotype or selectable markers are known for methyl-deficient 23S RNA either, although a mutant

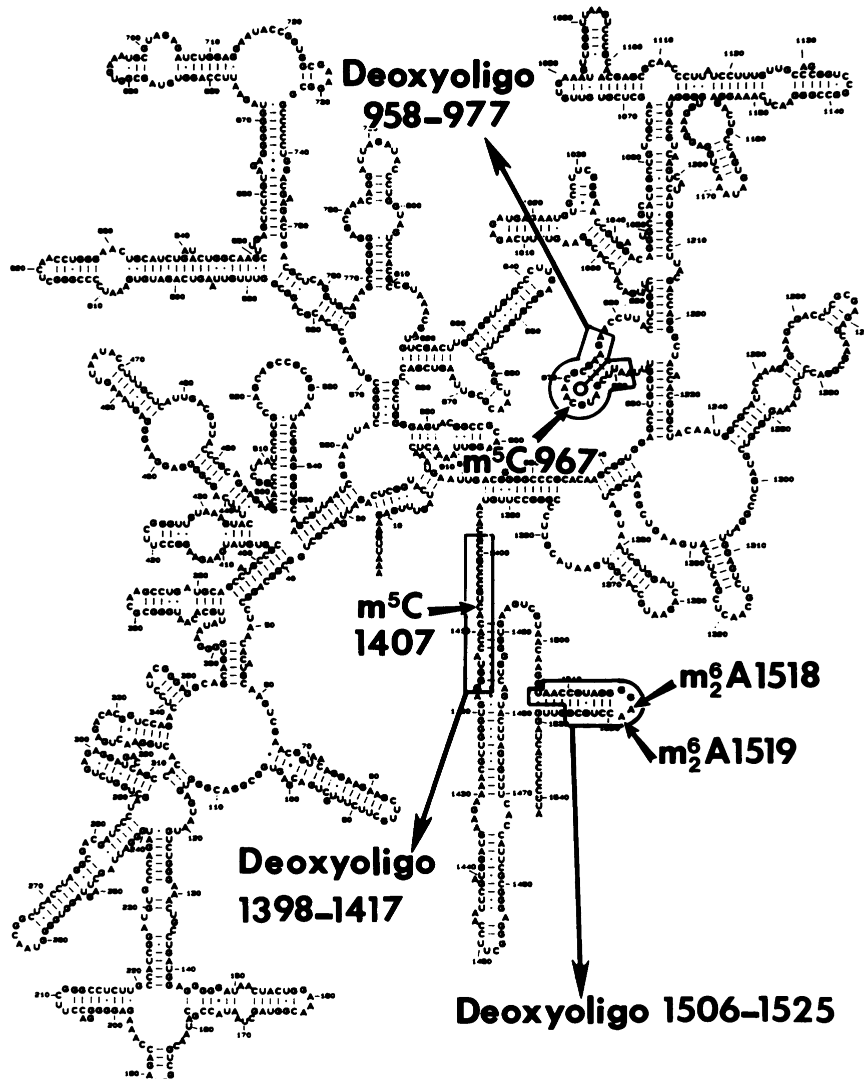

FIG. 4. Location of m^{5}C, m$^6_2$A, and the protecting deoxyoligomers in the secondary structure of 16S RNA. The methylated residues are indicated by arrows and the regions expected to base pair with the deoxyoligomers are boxed. The secondary structure and numbering of residues are according to Moazed *et al.* (21).

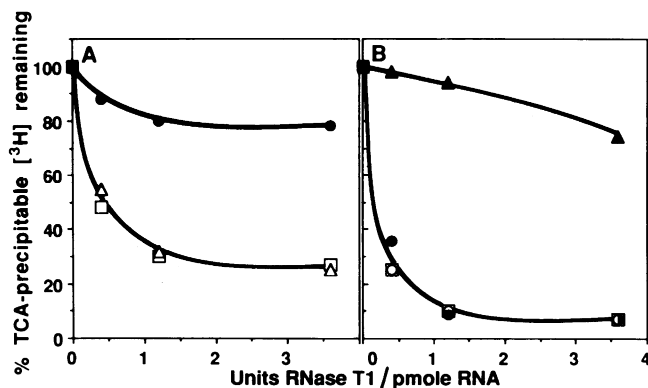

FIG. 5. Determination of the sites of formation of m^5C and m^6_2A by RNase protection. (A) m^5C. Synthetic 16S RNA (40 nM) was methylated with enzyme fraction HSW(55–85) at 320 μl/ml for 1 hr at 37°C and the RNA was isolated. We obtained 1.1 methyl groups per RNA of which 74% was m^5C. RNase protection was done with deoxyoligomer 958–977 (●), deoxyoligomer 1398–1417 (△), or without oligomer (□). (B) m^6_2A. Synthetic 30S ribosomes (40 nM) were methylated with 0.5 ml of fraction 14 from the DEAE-Sepharose column per ml of reaction mixture for 1 hr at 37°C and the RNA was isolated. We obtained 2.2 methyl groups per ribosome of which 86% was m^6_2A and 14% was m^6A. Deoxyoligomers 958–977 (●), 1197–1216 (○), or 1506–1525 (▲) or no oligomer (□) were used for protection.

lacking m^1G-synthesizing activity and potential mutants defective in m^2G and m^5C synthesis were identified by the use of a labor-intensive non-selective screening method (23–25).

Except for m^6_2A formation, the stage of ribosome assembly at which methylation occurs and the possible need for precursor forms of rRNA is not well understood (14, 15, 26). Irrespective of the natural course of events, our results show clearly that unmethylated but mature length forms of free RNA and of 30S ribosomes can be methylated *in vitro* in reactions that show specificity, both for the substrate and for the residues methylated.

Activities for m^5C, m^3U, m^2G, and m^6_2A were detected, but not those for m^7G or Cm. As the m^4C activity was only distinguished from m^5C formation in one case (Fig. 5), we do not know whether some of the other fractions that made m^5C/m^4C might actually possess m^4C activity instead of, or in addition to, the m^5C activity. Secondary analysis to separate m^5C from m^4C will be required. Nevertheless, the following indirect evidence suggests that m^4C activity may be absent from all of the extracts in Table 2. The expected product after m^4C methylation and nuclease digestion is m^4CpC since this dinucleotide should be resistant to P1 nuclease cleavage, like m^4CmpC but unlike CmpC. m^4CpC should be eluted slightly before CmpC, if the elution behavior of m^4C versus Cm is any criterion, yet no $[^3H]CH_3$ was detected in this region of the chromatogram. The enzymatic activities that were not detected may not have survived the fractionation procedure, may require different partially assembled forms of 30S ribosomes, or may need different reaction conditions.

The specificity of the m^5C and m^2G activities for RNA and ribosome, respectively, may simply reflect the relative exposure of that segment of rRNA to solvent as a result of alternative folding due to the presence of ribosomal proteins. Alternatively, for m^5C simple steric hindrance by the proteins in the ribosome may occur, while for m^2G the enzyme may specifically require the presence of nearby ribosomal proteins. In any event, the methylases appear to be not only site-specific but also substrate-specific.

The fact that m^5C-967 can be made in the absence of any other methylation means that m^2G-966 formation is not a prerequisite for this reaction, although the converse could be true. Similarly, since the enzyme fraction used to make m^6_2A had no m^2G activity, m^6_2A-1518, -1519 formation does not require the prior synthesis of m^2G-1516. Why m^5C-967 is made in free RNA but not at the ribosome level is not known. It may reflect only steric effects, as noted above, but it could also reflect a specific need for this methylation in the assembly process.

The system described in this paper should now make it possible to isolate and characterize all of the methylases involved in 30S formation. The ability to make mutations in and around the sites of methylation should prove invaluable in this effort. The system also has the ability to generate subparticles and RNA fragments to delineate the substrate requirements precisely. With purified enzymes in hand, the role of methylation in assembly and function of the ribosome can be examined to a degree not possible earlier.

An approach similar to that taken in this work has been described for the specific *in vitro* methylation of mRNA (27).

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