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A paradigm for local conformational control of function in the ribosome: binding of ribosomal protein S19 to *Escherichia coli* 16S rRNA in the presence of S7 is required for methylation of m^2G966 and blocks methylation of m^5C967 by their respective methyltransferases

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ABSTRACT

We have partially purified two 16S rRNA-specific methyltransferases, one of which forms m^2G966 (m^2G MT), while the other one makes m^5C967 (m^5C MT). The m^2G MT uses unmethylated 30S subunits as a substrate, but not free unmethylated 16S rRNA, while the m^5C MT functions reciprocally, using free rRNA but not 30S subunits (Nègre, D., Weitzmann, C. and Ofengand, J. (1990) UCLA Symposium: Nucleic Acid Methylation (Alan Liss, New York), pp. 1–17). We have now determined the basis for this unusual inverse specificity at adjacent nucleotides. Binding of ribosomal proteins S7, S9, and S19 to unmodified 16S rRNA individually and in all possible combinations showed that S7 plus S19 were sufficient to block methylation by the m^5C MT, while simultaneously inducing methylation by the m^2G MT. A purified complex containing stoichiometric amounts of proteins S7, S9, and S19 bound to 16S rRNA was isolated and shown to possess the same methylation properties as 30S subunits, that is, the ability to be methylated by the m^2G MT but not by the m^5C MT. Since binding of S19 requires prior binding of S7, which had no effect on methylation when bound alone, we attribute the switch in methylase specificity solely to the presence of RNA-bound S19. Single-omission reconstitution of 30S subunits deficient in S19 resulted in particles that could not be efficiently methylated by either enzyme. Thus while binding of S19 is both necessary and sufficient to convert 16S rRNA into a substrate of the m^2G MT, binding of either S19 alone or some other protein or combination of proteins to the 16S rRNA can abolish activity of the m^5C MT. Binding of S19 to 16S rRNA is known to cause local conformational changes in the 960–975 stem-loop structure surrounding the two methylated nucleotides (Powers, T., Changchien, L.-M., Craven, G. and Noller, H.F. (1988) *J. Mol. Biol.* 200, 309–319). Our results show that the two ribosomal RNA MTs studied in this work are exquisitely sensitive to this small but nevertheless functionally important structural change.

INTRODUCTION

One of the major unanswered questions about the structure and function of the ribosome is the role played by the defined set of modified nucleotides which are characteristically present in all ribosomes (1,2). These include methylated nucleotides both on the base and on the 2′-OH of the ribose, pseudouridylate residues, and even more baroquely modified nucleotides (3). We have suggested that the modified nucleotides of the small ribosomal subunit may play a role in assembly (4,5), and a role in ribosome function is supported by our results as well (5), despite the fact that unmodified 30S ribosomes are not blocked in any of the partial reactions of protein biosynthesis as carried out *in vitro* (6).

In order to understand the role of modified residues in ribosomes, we have used a system which we developed previously for producing completely unmodified but biologically active 16S rRNA and 30S ribosomes of *E. coli* (7). These materials have been used as homologous substrates in order to identify and purify the enzymes involved in 16S rRNA modification. Availability of these enzymes and their genes should then allow dissection of the role of rRNA base modifications in protein synthesis and overall cellular metabolism. So far, we have identified and partially purified two 16S rRNA methyltransferases (MTs). One enzyme forms m^5C at position 967, while the other forms m^2G at the adjacent position, 966. The m^5C MT was found to use free 16S rRNA as a substrate but not the 30S subunit, while the m^2G MT had the reciprocal substrate specificity (8,9). In addition to the surprising inverse specificity observed for these two adjacent nucleotides, this site was of interest for other reasons as well. The two methylated bases, m^2G966 and m^5C967, occur

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at the same locus as the hypermodified nucleoside acp^{3}m$^1\psi$ in eukaryotes (2,3), suggesting that modification at that site is generally important. In addition, m^{2}G 966 is protected by P-site bound tRNA (10) and forms part of the P site binding pocket in the model of Stern, *et al* (11).

We have now further elucidated the substrate dependence of these two MTs and show that an entire subunit is not needed to switch MT specificity. Only S7 plus S19 is sufficient to turn off m^{5}C methylation and turn on methylation of m^{2}G. Since S7 is required for binding of S19 (12) and S7 alone has no affect on methylation, we attribute the switchover to S19 alone. Single protein-omission reconstitutions have confirmed the requirement of S19 for m^{2}G966 methylation and revealed that other proteins in addition to S19 are able to turn off m^{5}C967 methylation. These results bear not only on the question of how the MTs recognize their correct nucleotide substrate, but also on the domain character of the assembly and structure of the 30S ribosome.

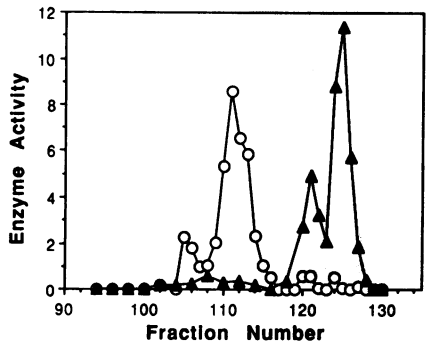

Fig. 1. Separation of m^{2}G966 and m^{5}C967 MT on a Mono S cation exchange column. A preparation containing 19,600 units of m^{2}G MT and 37,000 units of m^{5}C MT was applied to the column and eluted as described in Materials and Methods. Fractions of 0.6 ml were collected. Activity (units/μl) using either 30S subunits (○) or synthetic 16S rRNA (▲) as substrate is plotted against fraction number.

MATERIALS AND METHODS

Reagents

S-adenosyl-L-[methyl-^{3}H]methionine ([^{3}H]SAM) from Amersham was purified as described previously (8). Salts and buffer components were RNase-free or Molecular Biology grades. RNasin was from Promega. Total 30S proteins (TP30) were prepared as described previously (4). Individual proteins were prepared as described by Mandiyan *et al.* (13). A pool of all proteins except S19 were prepared by HPLC essentially as described (14, 15), using a 1×25 cm SynChroPak RP-P column (SynChrom). Some preparations of S7 and S19 were also prepared by this method. Synthetic 16S rRNA was transcribed from pWK1 and isolated as described (16).

Enzymes

Methyltransferases (MT) specific for positions 966 (m^{2}G) and 967 (m^{5}C) of 16S rRNA were purified essentially as described (8, 9) except that a cation-exchange FPLC step (Pharmacia Mono S) replaced the phosphocellulose column. FPLC on a 0.5×5 cm Mono S column used a 100 mL gradient from 50 to 1000 mM NH_4Cl in 20 mM HEPES, pH 7.5, 1mM EDTA, 2 mM DTT, 10% v/v glycerol, at a flow rate of 0.5 mL/min. A typical separation is shown in Figure 1.

Reconstitutions

Reconstitutions of 30S subunits were performed as described (4), except that the protein:rRNA ratio was varied as noted in figure and table legends. Reconstitution of complexes of 16S rRNA with S7, S9, and/or S19 was done using the annealing protocol described by Denman, et al. (4) but in a reaction mixture containing 30 mM Hepes, pH 7.5, 20 mM $Mg(OAc)_2$, 333 mM KCl/NH_4Cl, 5 mM β-mercaptoethanol, 500 units/ml RNasin, 375 nM rRNA and a 2−8 fold molar excess of the indicated proteins. Both 30S subunits and complexes were isolated by velocity centrifugation through sucrose gradients as previously described for 30S subunits (4).

Analysis of Methylation

The standard reaction mixture contained 100 nM rRNA, rRNA-protein complex, or 30S subunits, 2 μM [^{3}H]SAM (7500−10,000 dpm/pmol), 100 mM HEPES pH 7.5, 4 mM $Mg(OAc)_2$, 200 mM NH_4Cl, 5 mM β-mercaptoethanol, 300−800 units/ml RNasin, and enzyme. Incubation was at 37°C for the indicated times. Incorporated radioactivity was measured by TCA precipitation as described previously (8). One unit of activity is defined as one pmol CH_3 added per h. Where indicated, the identity of the methylated nucleotide was checked by nuclease digestion and HPLC analysis as described (8), except that the HPLC solvents were buffered with 10 mM NH_4OAc, pH 4.1, instead of sodium phosphate at pH 5.1.

Table 1. Specificity of m^{2}G966 and m^{5}C967 16S rRNA Methyltransferases

Substrate	Methyltransferase m^{2}G966	m^{5}C967
	Units per μl Enzyme	
16S rRNA	0.6 (0.03)	9.5 (0.2)
30S Ribosomes	6.8 (0.5)	0.5 (0.06)

Enzymes were prepared and assayed as described in Materials and Methods. Assays were linear with both time (10 to 40 min) and enzyme concentration. Values represent averages of 2−3 assays. Average deviation is given in parentheses.

Analysis of Protein Content

Protein content of reconstituted 30S or rRNA/protein complexes was analyzed by HPLC essentially as described previously (4), except that synthetic reconstituted subunits were used as standards in the case of the 3-protein complexes of Table 4. Mole ratios were calculated as the peak area/pmole of sample divided by the peak area/pmol of standard.

RESULTS

Substrate Specificity of the m^{2}G966 and m^{5}C967 MTs

In previous work, we described the co-purification of the m^{5}C MT and m^{2}G MT (9). Although present as a mixture, the two activities were readily distinguishable by their strikingly different substrate specificities as well as by their methylation products. We have now separated these two activities by using an alternative chromatographic procedure (Fig. 1). The smaller earlier-eluting peaks of activity are probably due to proteolysis as their size and presence varied from preparation to preparation. As shown in the figure, the activity able to methylate synthetic ribosomes eluted earlier than the activity which recognized free synthetic

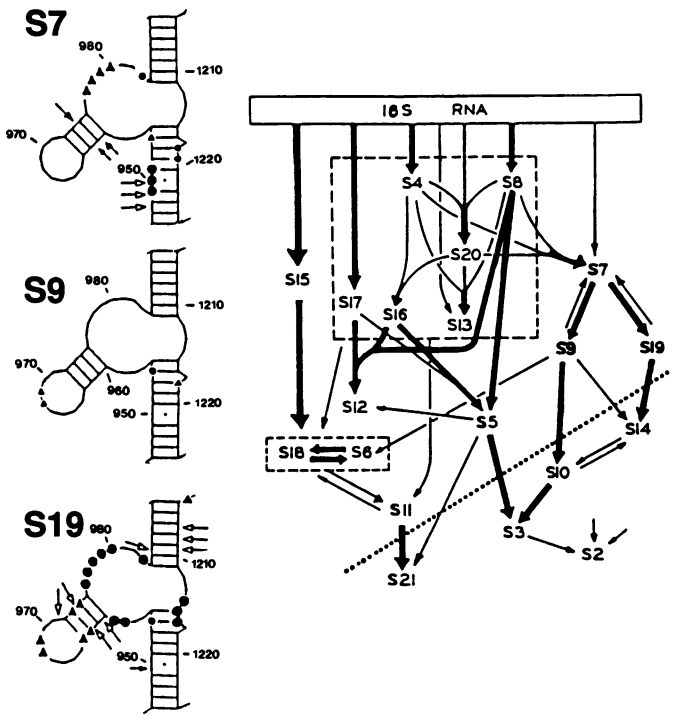

Fig. 2. Binding of ribosomal proteins to the region of 16S rRNA methylated by the m^2G966 and m^5C967 MTs. Left panel: Changes in solvent accessibility of residues upon binding of S7 alone (top) with respect to 16S rRNA, S9 plus S7 with respect to rRNA plus S7 (center), and S19 plus S7 with respect to rRNA plus S7 (bottom). Closed circles represent decreased accessibility to small molecules; closed triangles represent increased accessibility to small molecules; closed arrows represent increased susceptibility to double-stranded specific nucleases; open arrows represent decreased susceptibility to double-stranded specific nucleases. Adapted from Powers et al. (17). Right panel: assembly map of the 30S subunit. The thickness of each arrow reflects the requirement of a protein at the head of the arrow for prior binding of the component at the tail of the arrow. Thus S15, S17, S4, S20, S8, and S7 require no other proteins to bind to 16S rRNA, while S9 and S19 require prior binding of S7, S10 requires S9, and S14 requires S19 and/or S9 and/or S10. Adapted from Held et al. (12).

rRNA, and there was complete discrimination between the two substrates by both activities. As expected, the base methylated by the first activity when ribosomes were used was found to be exclusively m^2G, and that methylated by the second activity when rRNA was the substrate was only m^5C (data not shown). The specificity of the purified enzymes for substrate is shown in Table 1. The m^5C MT preferred protein-free 16S rRNA to that reconstituted into 30S subunits by 19 to 1, whereas the m^2G MT enzyme preferred 30S subunits to 16S rRNA by 14 to 1. The strong and reciprocal selectivity exhibited by these two enzymes for adjacent rRNA residues, and in particular the fact that one required free rRNA and the other an intact ribosome prompted us to examine in more detail at what level of assembly of the 30S subunit the m^5C967 MT would be turned off and the m^2G MT turned on, and whether or not the stage would be the same for both MTs.

Specific Protein Requirement for Switching of MT Recognition

Of all the 30S proteins, only S7, S9, and S19 produce unique effects in the stem-loop structure containing residues 966 and 967 (17, 18). Proteins S2 and S3 also protect residues in this

Table 2. Ribosomal Protein Requirement for Methyltransferase Specificity

Substrate	Methyltransferase m^2G966	Methyltransferase m^5C967
	moles CH_3/mole rRNA	
rRNA	0.02 (<0.01)	0.56 (0.04)
rRNA + S7	0.02 (0.01)	0.52 (0.09)
rRNA + S9	0.02 (<0.01)	0.51 (0.10)
rRNA + S19	0.02 (<0.01)	0.56 (0.04)
rRNA + S7 + S9	0.03 (<0.01)	0.58 (0.02)
rRNA + S9 + S19	0.02 (0.01)	0.51 (0.07)
rRNA +S7 +S19	0.50 (0.03)	0.17 (0.01)
rRNA + S7 + S9 + S19	0.54 (0.01)	0.12 (0.02)

rRNA–protein complexes were prepared as described in Materials and Methods using a protein:rRNA molar ratio of 2.5. The reconstitution mixtures were assayed *in situ* using the standard conditions except that 4 μM [^{3}H]SAM and 75 nM rRNA were used. Two series of methylation reactions were incubated for 30 minutes with 110 or 190 units/ml of m^2G966 MT or 180 or 400 units/ml of m^5C967 MT. Values represent the average of 2–4 determinations. The average deviation is in parentheses. The identity of the methylated nucleoside was verified by HPLC. Over 95% of the radioactivity incorporated by the m^2G MT in the (S7,9,19) sample was in m^2G, and over 95% of the radioactivity incorporated in 16S rRNA by the m^5C967 MT was in m^5C.

structure but the effects are considered to be polyspecific (19). Of these three, S19 is unique in enhancing accessibility of nucleotides in the 960–975 stem and loop to small molecules and concommittant loss of double-stranded nuclease succeptibility (Fig. 2, left). Moreover, according to the *in vitro* assembly map (Fig. 2, right), S7, S9, and S19 form a distinct assembly subdomain which is capable of direct binding to 16S rRNA. Therefore, it seemed reasonable to begin by testing the effect of these three proteins on methylation.

Table 2 shows the effects of incubating all combinations of these proteins with 16S rRNA under reconstitution conditions. Under these conditions, the combination of S7 and S19 was both necessary and sufficient to completely reproduce the change in activity of both enzymes that was observed on complete reconstitution of the 30S subunit (see Table 1). Since S7 alone had no effect, and S7 is required for binding of S19 in the absence of other proteins (12, 17), we attribute this effect to the binding of S19. The change in specificity is even more striking when the kinetics of reaction are examined (Figure 3). The loss of methylation by the m^5C MT (panel A) agrees well with the results in Table 2. Moreover, the residual activity of the m^5C MT in the S7, S19 and S7, S9, S19 incubations approaches a plateau with a rate similar to that of 16S rRNA alone, suggesting that this residual activity is due to reaction of a small population of uncomplexed rRNA rather than to slow methylation of an rRNA-protein complex. If this interpretation is correct, the complexes appear to be even more inert to the m^5C MT than the data in Table 2 would indicate. The coordinate gain of methylation by the m^2G MT is equally striking (Figure 3, panel B). The ability of the *in situ* complexes to support m^2G966 methylation was kinetically similar to 30S subunits. In preliminary experiments, the rates of methylation of the two substrates by the m^2G966 MT were within 25% of each other when compared at equal substrate concentrations.

Although these results suggested that a specific complex of 16S rRNA with S7 and S19 was responsible for the change in ability to be methylated, the possibility of direct action of the ribosomal proteins on the enzymes rather than the substrate remained to be ruled out since proteins were present in large excess. The

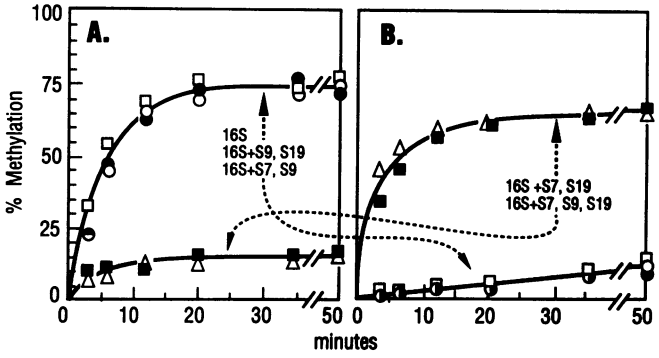

Fig. 3. Kinetics of methylation of rRNA-protein complexes. Panel A, methylation of rRNA-protein mixtures (prepared as described in the legend to Table 2) with 290 units/ml m^5C MT. (●), rRNA alone; (□), rRNA+S7 and S9; (○), rRNA+ S9 and S19; (△), rRNA+ S7 and S19; (■), rRNA+ S7, S9, and S19. Methylation was assayed under standard conditions except at 4 μM SAM, 185 mM NH_4Cl and 30 nM rRNA. Values are calculated assuming one CH_3 added per rRNA present equals 100%. Panel B, methylation of the same mixtures with 420 units/ml m^2G MT, under the same conditions as in Panel A.

Table 3. Requirement for Annealing of the rRNA-Protein Complexes

A: m^5C MT

Substrate	Annealing	m^5C Enzyme moles CH_3/mole rRNA
(1) rRNA	−	0.61
(2) rRNA	+	0.62
(3) rRNA + 8X (S7, S9, S19)	+	0.10
(4) Mixture of (3) + (1)[a]	−	0.60

B: m^2G MT

Substrate	Annealing	m^2G Enzyme moles CH_3/mole rRNA
(4) rRNA	+	0.09
(5) rRNA + 10X (S7, S19)	−	0.20
(6) rRNA + 10X (S7, S19)	+	0.52

[a]To the annealed mixture (3) was added an equal amount of rRNA (1). The mixture was assayed without further annealing, and results are expressed as mole fraction of the added rRNA only.

Annealing was performed as described in Materials and Methods. Samples (1) and (5) were incubated at 0°C for the same amount of time as the annealed samples. The annealed reaction mixtures were assayed *in situ* under standard conditions except at 75 nM rRNA, and in panel A, 185 mM NH_4Cl and 4 μM SAM. Incubation was with 220 units/ml m^5C MT enzyme for 30 min or 700 units/ml m^2G MT for 70 min.

requirement for annealing of the rRNA with the proteins under reconstitution conditions (Table 3) argues against an effect of the free proteins on the enzymes. Panel A shows that the loss of m^5C MT activity requires prior annealing of the proteins with 16S rRNA and that the excess proteins present in the annealed sample (line 3) have no effect on the ability of the methylase to act on added 16S rRNA (line 4). Panel B shows that the m^2G MT requires substrate annealing also. These results indicate that the proteins produce their effects by binding to the rRNA rather than to the enzymes.

This conclusion was more rigorously demonstrated by separating the annealed complex of S7, S9, and S19 with 16S RNA from free proteins on sucrose gradients. The complexes were then analyzed for their protein content and for their activity as substrates for each MT. The results (Table 4) show a

Table 4. Correlation of Methylation Specificity and Ribosomal Protein Content of Isolated 16S rRNA-rProtein Complexes

Protein/rRNA Ratio	0	4	8
		moles CH_3/mole rRNA	
m^5C967 MT	0.70 (0.01)	0.04[a]	0.04[a]
m^2G966 MT	<0.01(<0.01)	0.74[a]	0.72 (0.02)
		moles protein/mole rRNA	
S7	–	1.13	0.98
S9	–	0.78	0.70
S19	–	0.84	0.74
'S17'	–	0.39	0.36

[a] single determination

Complexes were formed by annealing in the presence of the indicated molar excess of proteins S7, S9, and S19 and isolated by sucrose gradient centrifugation as described in Materials and Methods. The extent of methylation was assayed under standard conditions, except with 20−30 nM rRNA or complex from the peak gradient fraction and 4 μM SAM, with 120 units/ml m^2G MT or 225 units/ml m^5C MT for 30 min. The results are averages of 2−3 determinations with average deviations in parentheses, except as noted. Protein identification by HPLC elution time and A_{278}/A_{214} ratio, and quantitation was done as described previously (4). The identity of 'S17' is tentative (see text).

convincing correlation between the gain of m^2G MT activity and loss of m^5C MT activity, assayed after removal of excess ribosomal proteins, with the measured extent of binding of S19 to 16S rRNA. As with the *in situ* assay results, the loss of m^5C MT activity and gain of m^2G MT activity were reciprocal. As expected, S7 and S9 were also found in approximately stoichiometric amounts. A small amount of material which appeared similar to S17 by HPLC analysis was also found in the complexes. Although no S17 was added, it could have been present as a minor contaminant in the protein preparations. However, the identification as S17 must be considered only tentative since the amount bound did not approach unit stoichiometry even at an 8-fold protein excess, despite it being a primary binding protein (see Fig. 2). Since all other 30S ribosomal proteins are excluded by the HPLC elution position of the material, we believe that it is a non-ribosomal protein contaminant.

Can Another Protein or Combination of Proteins Mimic the Effect of S19?

Although the complex of only S7 and S19 with rRNA mimicked the complete 30S subunit with respect to recognition and catalysis by these two methyltransferases, it is possible that another protein or combination of proteins could also do this. We tested this possibility by reconstituting 30S subunits lacking only protein S19. If the stem and loop from residues 950−975 of 16S rRNA were truly folded into a 30S-like subdomain by proteins S7 and S19 independently of the remaining 19 small subunit proteins (actually only 18 were tested: our reconstituted particles lack protein S1), then subunits containing S19 should be substrates of the m^2G MT but not the m^5C MT and those lacking S19 should be substrates of the m^5C MT but not the m^2G MT.

A pool of 30S proteins depleted in S19 (TP30-S19) was prepared, as was purified S19. 30S ribosomal subunits were then reconstituted from TP30-S19, from TP30-S19 with S19 added back , and from complete TP30 not subjected to HPLC. The ability of the three different protein preparations to reconstitute a 30S particle is shown in Figure 4. No difference in

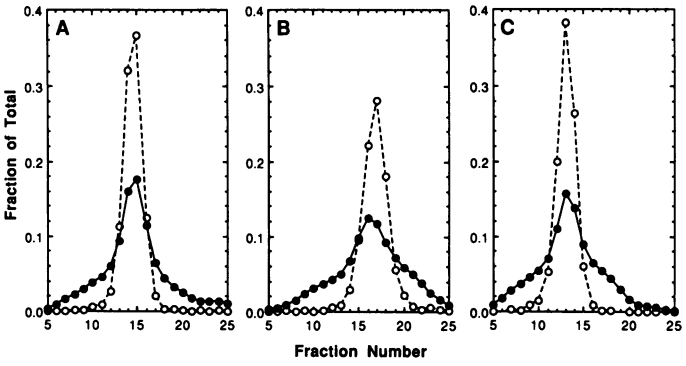

Fig. 4. Reconstitution of 30S particles with and without protein S19. Sucrose gradient centrifugation of reconstitution mixtures (preparation I of Table 5) was as described (4). Centrifugation was from right to left. (○), [^{32}P]-labelled marker 30S subunits; (●), A_{260} of reconstituted material. The vertical axis represents the fraction of the total recovered [^{32}P] or A_{260} present in each fraction. Fraction size was approximately 1.0 ml. Panel A: Reconstitution of 20 A_{260} units of synthetic rRNA with a 3.5-fold molar excess of TP30. Fractions 12–17 were pooled. Panel B: Reconstitution of 20 A_{260} units of synthetic rRNA with a 3.5-fold molar excess of TP30 minus S19. Fractions 14–19 were pooled. Panel C: Reconstitution of 20 A_{260} units of synthetic rRNA with a 3.5-fold molar excess of TP30 minus S19 and a 3.5-fold excess of protein S19. Fractions 11–15 were pooled.

Table 5. Protein content of various 30S particles

Protein	I			II		
	TP30	−S19	+S19	TP30	−S19	+S19
S2	0.41	0.16	0.24	0.65	0.50	0.53
S3	0.59	0.33	0.49	0.62	0.52	0.62
S4	1.09	1.27	1.16	0.95	0.81	0.73
S5	0.75	0.79	0.80	0.91	0.83	0.76
S6	0.75	0.85	0.73	0.97	0.91	0.32
S7	0.94	0.84	0.88	0.80	0.76	1.03
S8	1.01	0.92	0.81	0.92	0.80	0.94
S9	0.75	0.79	0.80	0.91	0.83	0.76
S10	0.97	0.63	0.96	1.22	0.54	1.10
S11	0.93	0.82	0.70	0.79	0.80	0.74
S12	0.54	0.62	0.63	0.94	0.84	0.79
S13	1.30	1.00	1.30	0.91	0.81	0.89
S14	0.66	0.41	0.65	0.62	0.58	0.65
S15	1.00	0.96	0.92	1.04	0.97	0.94
S16	0.91	0.98	0.90	0.93	0.87	0.81
S17	1.06	1.05	0.96	0.99	0.99	0.98
S18	0.83	0.94	0.99	1.06	0.99	0.97
S19	0.87	0.33	0.77	0.93	0.50	0.74
S20	1.15	1.23	1.26	1.14	1.38	1.10
S21	0.82	0.98	1.16	1.02	1.18	1.05
Average	**0.92**	**0.94**	**0.93**	**0.95**	**0.92**	**0.85**

Protein extracted from ca. 1.0 A_{260} unit (67 pmol) of the particles described in Fig. 4 (Panels A, B, and C are TP30, -S19, and +S19, respectively) was analyzed by HPLC as described in Materials and Methods. Results are expressed as moles protein/mole subunit. Only the stoichiometries of proteins whose peak areas did not vary with S19 content (shown in boldface) were averaged to obtain an average protein stoichiometry. I and II are separate preparations of particles.

sedimentation behavior due to lack of S19 (Panel B vs. C) or to HPLC treatment of the proteins (Panel A vs. C) is evident, although it was reported previously that the omission of S19 in experiments using natural 16S rRNA led to a slightly slower-sedimenting particle (20, 21). The greater dispersity in sedimentation behavior of all of the reconstituted particles in comparison to the ^{32}P-labelled isolated subunits has been noted

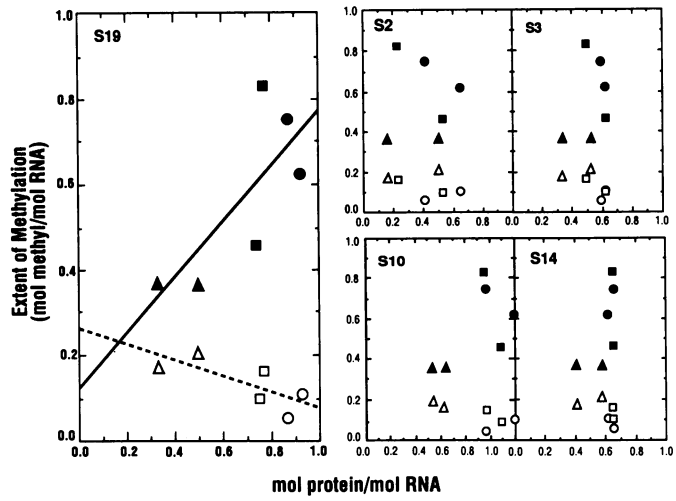

Fig. 5. Correlation of the extent of methylation with protein content. Particles isolated as described in Fig. 4 were methylated under standard conditions except at 40 nM 30S or rRNA, 500 units/ml RNasin, and 4 mM SAM with 130 units/ml m^2G MT or 115 units/ml m^5C MT by incubation at 37°C for various times until a plateau was reached. The plateau values of the extent of methylation are plotted versus the protein content of those proteins whose mole ratios vary among the reconstituted particles (Table 5). Under the same conditions, free rRNA was methylated by the m^5C and m^2G MTs to levels of 0.90 and <0.01 mol/mol, respectively. Best fit lines for the m^2G MT (solid) and m^5C MT (dashed) were obtained using a linear least-squares algorithm. Open symbols, m^5C MT; solid symbols, m^2G MT. ○,●; TP30 particles. △,▲; TP30 minus S19 particles. □,■; TP30 minus S19, plus S19 particles.

before (4) and is believed to be due to the lack of post-transcriptional modifications of the rRNA moiety (5).

The protein content of the reconstituted particles is shown in Table 5. Although it was expected that the particles reconstituted with TP30-S19 would lack S19, it is evident that a variable but considerable extent of contamination with S19 had occurred. This can in part be explained by the large molar excess of protein used, 3.5:1 in the case of preparation I and 4.5:1 in the case of preparation II. Note too that the amount of S19 in the particles increased with the excess mole ratio of proteins used. However, there is still a discrepancy between the apparent extent of removal of S19 from the TP30 (97% and 93% for preparations I and II, respectively) and the amount of S19 found in the reconstituted particles. The HPLC peak from the reconstituted particles denoted S19 was identified as S19 by sequencing of the first 10 amino acid residues of the protein.

The six reconstituted ribosomal particles, from two independent reconstitutions, were tested for their ability to be methylated by the m^2G and m^5C MTs. Assays were performed at varying times and enzyme/substrate ratios to confirm that a genuine plateau of methylation had occurred. The results are shown in Figure 5 where the plateau levels of methylation are plotted against the S19 content of the different particles. Although there is some scatter in the results, it is clear that the methylation ability of the m^2G MT is directly proportional to the S19 content of the particles. No such correlation exists with the other proteins (S2, S3, S10, S14) that also vary among the different particles. A weak correlation can be seen for S10 but it should be noted that binding of S10 depends on the presence of S19, among other proteins (Fig. 2). These results confirmed our expectation for the m^2G MT. Surprisingly, the m^5C MT could not methylate

any of the reconstituted particles, regardless of the amount of S19 present. This result implies that a protein or combination of proteins other than S19 can block methylation by the m^5C MT. Proteins S2, S3, S10, and S14 which also vary among the samples showed no correlation with the residual methylation by the m^5C MT. In other experiments (not shown), we tested a mixture of the primary binding proteins (see Fig. 2) S4, S7, S8, S15, S17, and S20, for their ability to produce a switch in the activities of the two MTs but the isolated particles contained stoichiometric amounts of only S4, S7, and S20. Preliminary results indicate that these proteins also have no effect on the activity of the m^5C MT.

DISCUSSION

Recognition of Substrate by the Methyltransferases

The most striking observation in this study is that the switch in substrate specificity from an rRNA which can be methylated at C967 by the m^5C967 MT to one able to be methylated at G966 by the m^2G966 MT can be induced by the binding of a single ribosomal protein, S19. Although S7 was also present since S7 is needed for binding of S19 to 16S rRNA (12,17), we deduce that only S19 is the critical determinant because S7 alone had no effect. We showed in two ways that formation of an rRNA-protein complex is required for the methylation switchover. First, annealing was required (Table 3) and second, isolation of the rRNA plus rRNA-protein complexes from free excess proteins (Table 4) yielded the same result. Indeed in this latter experiment, the close correspondence between moles of S19 bound and moles of m^2G966 formed is quite evident. An alternate scenario, originally proposed by Powers et al. (17) as another explanation for their protection studies, also needs to be considered. Conceivably, the binding of S19 to the S7-rRNA complex could alter the interaction of S7 with 16S rRNA in such a way as to induce the switchover in methylation specificity. In this interpretation, S7 would be responsible for the effect but only when it is complexed with S19.

If S19 is directly or indirectly responsible, what might be the mechanism of this switchover? Simple occlusion of the loop cannot be the explanation because then G966 should be hidden also. A likely explanation comes from the work of Powers et al. (17) who showed that of the 20 30S proteins examined, only S19 produced extensive increases in accessibility of nucleotides in the 960–975 stem and loop and concomitant loss of double-stranded nuclease susceptibility. These authors proposed that binding of S19 shields residues 958–959 and 976–980, but disrupts the helix formed by residues 960–963 and 972–975 accounting for the loss of double-stranded specific nuclease cuts in that region. The increase in exposure of residues 962, 963, 968, 969, 973, and 974 would then be a result of the nearly complete unfolding of this stem and loop. Such a large conformational change at the site of methylation could readily explain the switchover in methylase specificity. In fact, as mentioned above, these previously known conformational changes were the impetus for the present experiments. Although these structural changes nicely account for the switch in MT specificity, alternative explanations are also possible. For example, S19 could mask an essential part of the m^5C MT recognition site on 16S rRNA which is different from positions 966 or 967, and itself be part of the recognition site for the m^2G MT. S19 could only do this when bound to rRNA, however, since annealing was required for an effect on either MT (Table 3). Although the tertiary structure of 16S rRNA in the ribosome has long been described in terms of three or more domains (22), and the independent binding of subsets of 30S ribosomal proteins to regions of the 16S rRNA (23,24) has led to incorporation of this domain structure into current models of the 30S subunit (11,18,25), the region described here is much smaller than the 200–500 nucleotides normally ascribed to a domain. Our results suggest that some functionally important structural features of the 30S subunit may be much more local in character.

The results obtained in the single protein-omission reconstitution (SPORE) experiment indicates that the switch in the 960–975 stem-loop structure described above is not the only way that methylation by the m^5C MT can be affected. Direct occlusion of the C967 residue is unlikely unless it is postulated that subsequent addition of S19 relieves the occlusion, since G966 was methylatable in these latter particles. Protein protection studies on SPORE particles without and with S19 would be informative in this regard but have not been reported. Induction of a conformational change in the 960–975 region which is distinct from that caused by S19 such that it prevents m^5C967 formation but does not allow methylation of G966 could explain the effect. Proteins S2 and S3 are the only other proteins known to induce protection of the region in question (19), but the amounts of these proteins present in reconstituted particles (Table 5) were not sufficient to account for the decrease in m^5C MT activity observed with respect to free rRNA. Thus any postulated change in the 960–975 region induced by the binding of proteins other than S7, S9, and S19 would have to be one not detected by the chemical protection analyses. Masking by a protein or proteins other than S19 of an additional recognition site in the 16S rRNA which is required by the m^5C MT is another possibility. Thus, the recognition site for the m^5C MT may include regions distinct from the methylation site. In this regard, it should be noted that we have not yet defined the recognition requirements of the m^5C MT in terms of the size of the 16S rRNA fragment required. We may speculate on which protein(s) are responsible for the inhibition of the m^5C MT. The results described in this work eliminate all but proteins S5, S6, S8, S11, S12, S13, S15, S16, S17, S18, and S21. From considerations of proximity derived from 30S models (11,18,25,26) and an estimated size of 20–25A dia. for a spherical m^5C MT of 45 kD (P. Popieniek and J. Ofengand, unpublished results), the most likely candidates are S11, S13, S16, and S21.

Methylation and Ribosome Assembly

Is methylation required for correct assembly of the *E. coli* 30S ribosome, and reciprocally, what assembly intermediates are the correct substrates for the various methylases? With respect to the first question, it appears that methylation or other rRNA modifications such as pseudouridylation are necessary for correct assembly of the 30S subunit. Although 30S ribosomes can be constructed from unmodified rRNA, *in vitro* reconstitution requires more extreme conditions of both ionic strength and temperature and even then is not completely successful. The particles are more heterodisperse in size, and the biological activity is approximately one half that of controls (4,5). *In vivo*, although the details are murky, most base methylations are thought to occur at a relatively late stage of 30S subunit biogenesis (27–29). Formation of the ubiquitous (1,30) $m^6{}_2A$ only occurs after the 30S subunit is assembled (31).

In view of these considerations, it seemed reasonable that methylation at C967, which uses free 16S rRNA, might be a

prerequisite to more effective assembly of the unmodified rRNA. So far, however, experiments using unmodified rRNA which had been methylated at C967 by prior treatment with the m^5C MT *in vitro*, did not show any marked improvement in their ability to assemble.

When during assembly does methylation take place? Clearly m^5C967 formation is a very early event as it can occur on free rRNA but is blocked by the time S7 and S19 are added or at the point when the ill defined protein or proteins active in the SPORE experiment are bound. However, this methylation need not require free rRNA. Since S4 and S7 form two independent nucleation sites for 30S subunit assembly (24,32), it is possible that a considerable part of the S4-initiated domain can be assembled before C967 5 becomes inaccessible. On the other hand, m^5C967 formation must occur prior to that of m^2G966 which should be a relatively late event requiring as it does assembly of both S7 and S19. It is also clear from this work that neither MT requires prior methylation of the adjacent nucleotide by the other MT. Whether or not intact 30S subunits can be methylated by the m^2G966 MT is not completely clear. In preliminary experiments we have observed a) a variable degree of methylation with different 30S subunit preparations, b) an inhibition of methylation with increasing Mg^{++} concentration in the methylation reaction, and c) a decreased stability as a function of Mg^{++} concentration of synthetic 30S subunits vs. natural 30S, all of which suggest that a partial unfolding of 30S subunits may be required for G966 methylation.

The biological significance of the order of methylation, first C967 and then G966 remains as elusive as the functional role (if any) of these modifications. As noted in the Introduction, the highly modified $acp^3m^1\psi$ residue is found at the same stem-loop site in eukaryotes. This conservation of modification site, if not of modified base structure, implies some fundamental purpose, but it is not likely that an answer will be forthcoming until one can, by gene disruption experiments or other means, make a cell devoid of the ability to form m^2G966 and m^5C967. That indeed is the ultimate goal of our ongoing efforts to purify and clone these two methyltransferases.

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