The methyltransferase that forms m^2G1207 in *Escherichia coli* small subunit rRNA has been purified, cloned, and characterized. The gene was identified from the N-terminal sequence of the purified enzyme as the open reading frame (SWISS-PROT accession number P39406). The gene, here renamed *rsmC* in view of its newly established function, codes for a 343-amino acid protein that has homologues in prokaryotes, Archaea, and possibly also in lower eukaryotes. The enzyme reacted well with 30 S subunits reconstituted from 16 S RNA transcripts and 30 S proteins but was almost inactive with the corresponding free RNA. By hybridization and protection of appropriate segments of 16 S RNA that had been extracted from 30 S subunits methylated by the enzyme, it was shown that of the three naturally occurring m^2G residues, only m^2G1207 was formed. Whereas close to unit stoichiometry of methylation could be achieved at 0.9 mM Mg^{2+}, both 2 mM EDTA and 6 mM Mg^{2+} markedly reduced the level of methylation, suggesting that the optimal substrate may be a ribonucleoprotein particle less structured than a 30 S ribosome but more so than free RNA.

The methyltransferase that specifically forms m^5G residues has recently been described. The first, *rsmA* (more commonly known as *ksgA*), makes the two m^5G residues at positions 1518 and 1519 (8). Loss of these four methyl groups has little effect on ribosome function (9). The synthase for the single pseudouridine at position 516 (6) has been deleted, and the cell still grows well, some function (9). The synthase for the single pseudouridine at position 516 (6) has been deleted, and the cell still grows well, suggesting that the optimal substrate may be a ribonucleoprotein particle less structured than a 30 S ribosome but more so than free RNA.

The modified nucleosides of RNA remain one of the enigmas of RNA biology. Despite being widely distributed among all classes of RNA in a bewildering plethora of shapes and forms (1), little or no functional role, and certainly no unifying concept, has yet to emerge. Although certain modulating effects on cellular processes have been described, usually under special circumstances and so far only in tRNA (2), no modified nucleoside has yet been shown to be of major importance for cell growth and/or survival. In rRNA, which next to tRNA contains the widest known assortment of modified nucleosides, no function has so far been ascribed to them, although a potential role in peptide bond formation for a subset of the modified nucleosides of *Escherichia coli* 23 S RNA has been postulated (3). In *E. coli* 16 S RNA, in which all of the modified nucleosides are known and their locations precisely determined, none was essential for any of the known ribosomal functions, although ribosomal efficiency was reduced to approximately half when all were absent (4, 5). Evidence has been presented that this lowered efficiency of the 30 S subunits may be attributable to the lack of one or more of the 10 methylated residues and/or the single pseudouridine (6) in the RNA (7). To explore the role of individual modified nucleosides of 16 S RNA in 30 S subunit structure and function, a way is needed to specifically block their formation, one at a time. The most straightforward way to do this is by inactivating the enzymes responsible for their synthesis by gene deletion or disruption, because mutation of the parent nucleoside in the RNA could have unforeseen consequences. So far, three genes and enzymes for the modified nucleosides of *E. coli* 16 S RNA have been described. The first, *rsmA* (more commonly known as *ksgA*), makes the two m^5G residues at positions 1518 and 1519 (8). Loss of these four methyl groups has little effect on ribosome function (9). The synthase for the single pseudouridine at position 516 (6) has been deleted, and the cell still grows well, although precise growth rates have not yet been determined. A methyltransferase that specifically forms m^5C967 has recently been described, but results of deletion experiments are not yet available. In this work, we describe the characterization of another methyltransferase, that for m^2G1207. The gene was identified by N-terminal amino acid sequence analysis of the isolated enzyme and confirmed by overexpression with His tag, affinity purification, and *in vitro* biochemical characterization.

**EXPERIMENTAL PROCEDURES**

*Materials—[3H]-Adenosyl-methionine ([3H]-SAM)* was from Amer sham Pharmacia Biotech. Plasmid pET-15b, the BL21/DE3 strain of *E. coli*, and His-Bind resin were from Novagen, Inc. The XLI-Blu Epic rian strain of *E. coli* was from Stratagene. RNase T1 was from Calbio chem, and RNase P1 was obtained from Life Technologies, Inc. DNase I was from Worthington. T4 DNA ligase and restriction enzymes were from New England Biolabs. Wizard DNA purification kits and RNA sin were from Promega. BA85 cellulose nitrate filters were obtained from Schleicher & Schuell, polyvinylidene difluoride membranes were from Millipore, and omega cells were from Filtron. DEAE-Sepharose CL-6B and MonoS fast protein liquid chromatography columns were obtained from Amersham Pharmacia Biotech. Alumina type A-5 was from Sigma. Deoxyoligonucleotides for protection studies were those available.

*Buffers—Buffer A* is 20 mM Hepes, pH 7.5, 10 mM Mg(OAc)_2, x mM NH_4Cl, and 2 mM dithiothreitol. Buffer B is 20 mM Hepes, pH 7.5, 1 mM EDTA, x mM NH_4Cl, and 2 mM dithiothreitol. Buffer C is 20 mM Hepes, pH 7.5, 10 mM Mg(OAc)_2, x mM NH_4Cl, and 2 mM dithiothreitol.

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1. Niu and J. Ofengand, unpublished results.
2. J. S. Tischerne, K. Nurse, P. Popienick, H. Michel, M. Sochacki, and J. Ofengand, submitted for publication.
3. The abbreviations used are: SAM, S-adenosyl-methionine; HPLC, high performance liquid chromatography; ORF, open reading frame; Mes, 4-morpholinoneethanesulfonic acid.
purification of the nG methyltransferase—The purification procedure was similar to that previously described (13, 14). One hundred grams of *E. coli* MR600 cells harvested in mid-log phase were ground with 200 g of aluminia. Then 3000 units of DNase I and 100 ml of Buffer A containing 0.2 mM phenylmethylsulfonfyl fluoride were added, the volume was adjusted to 275 ml with water, and the mixture was sonicated in 6 ml of either 20 mM Hepes, pH 8.0, or 0.1 M NaCl, and 5 mM phenylmethylsulfonfyl fluoride. The sonicated mixture was centrifuged at 15,000 × g at room temperature for 15 min. They were then diluted with 9 volumes of ice-cold RNase Buffer (10 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 5 mM EDTA), and freshly denatured 80% (v/v) formaldehyde. The samples were heat-denatured at 90 °C for 10 min and then placed at 20 °C.

Cloning and Overexpression of the nG Methyltransferase Gene—The putative gene was amplified by polymerase chain reaction. The N-terminal primer extended from −13 to +14, where A of the initiating ATG is +1, with changes at −1, −2, and −3 to create an Ndel site adjacent to the initiating ATG. The C-terminal primer, in the reverse orientation, extended from +1061 to +1067, where the last sense nucleotide is 1029, and contained mismatches at 1073 and 1074 to create a BamHI site. After polymerase chain reaction, the amplified product was digested with BamHI and Ndel and ligated into a 0.2-kb phenylmethylsulfonyl fluoride to create an BamHI/Ndel site. After polymerase chain reaction, the amplified product was digested with BamHI and Ndel and ligated into a plasmid DNA purification kit, concentrated by membrane filtration (Amicon Microcon 100), and digested with Ndel and BamHI. The pET-15b vector, also digested with Ndel and BamHI, and the gene insert were ligated by standard methods using T4 DNA ligase. The resulting plasmid was transformed into XL-Blue E. coli cells and the transformants were selected on LB containing 0.2 mM phenylmethylsulfonfyl fluoride and Xgal/IPTG/1 mM inducer. The transformant was confirmed by restriction analysis.

Affinity Purification—The S15 supernatant containing fractions were dialyzed against Buffer D with decreasing concentrations of urea from 6 to 3 M at 1.0 M intervals and then from 3 to 0 M at 0.5 M intervals for 1 h each. Both protein solutions were adjusted to contain 50% glycerol and stored at −20 °C.

Nucleoside Assays—Reaction mixtures contained 100 mM Heps, pH 7.5, and 2 mM Mg(OAc)2 except where otherwise indicated, 200 mM NH4Cl, 5 mM dithiothreitol or BME, 2 μM [3H]SAM, 500 units/ml RNase, and 100 mM 16 S RNA transcript (prepared as described in Ref. 15) or 80 mM 30 S ribosomal subunits reconstituted from 16 S RNA transcripts and ribosomal proteins (4, 16), and enzyme as indicated. Incubation was at 37 °C. The reaction was monitored by the addition of ice-cold 5% A280-containing fractions were pooled and used directly, or dialyzed 4 M urea, and then chilled. For preparation of recombinant protein, cells from 100 ml of culture were washed in 10 mM Tris, pH 8, plus 6 M urea. The pooled A280-containing fractions were dialyzed against Buffer D and dialyzed sequentially against Buffer D or in Buffer containing 6 M urea.

RESULTS Purification of the Methyltransferase and Identification of the Gene—Previous work from this laboratory had identified two *E. coli* 16 S RNA methyltransferases, one specific for mG926 and another specific for mG927 (11, 13, 14). The mG926 enzyme reacted with unmodified 30 S subunits (11, 13) or unmodified 16 S RNA complexed with 30 S proteins S7 and S19 (14) but not with free 16 S RNA, whereas the mG927 enzyme had the reciprocal specificity. The purpose of the present work was to identify and clone the gene for the mG methyltransferase. Following a purification scheme similar, but not identical, to that previously used, a preparation that reacted with 30 S but not 16 S RNA was obtained. Gel electrophoresis showed a single strong band at ~37 kDa. N-terminal sequencing of this band yielded the following sequence: ASPA/S1EY/S1R/HSDDQQ. A search of the GenBank data base showed an exact match to ORF yqT (SWISS-PROT accession number P39406). The calculated molecular mass was 37.6 kDa, in good agreement with the experimental value. The presence of a strong 7-nucleotide Shine-Dalgarno sequence nucleotides upstream from the initiating ATG was observed.

Cloning of the Gene, Overexpression, and Affinity Purifica-
tion—The putative gene was cloned into pET-15b by standard methods. Induced cells containing clones with this insert produced large amounts of protein ~37 kDa in size, whereas no such band was visible in the pET control (data not shown). The overexpressed protein was distributed between the S15 supernatant fraction and S15 pellet. Distribution between these two fractions varied somewhat from preparation to preparation, and renaturation of the enzyme in the pellet produced variable results. Therefore, all experiments were conducted using the soluble supernatant fraction of the enzyme. This fraction was purified by affinity chromatography on a Ni<sup>2+</sup>-containing resin, because the overexpressed protein contained an N-terminal His tag. The eluted protein product gave a single band on gel electrophoresis at the expected size (data not shown). The finding of methyltransferase activity in the affinity-purified protein (see below) is definitive proof that the gene cloned is a methyltransferase gene.

Substrate and Product Specificity of the m<sup>2</sup>G Methyltransferase—The overexpressed enzyme possessed the same preference for 30 S particles over free RNA as described previously (14) for the partially purified m<sup>2</sup>G<sub>966</sub> methyltransferase (Fig. 1). The m<sup>2</sup>G enzyme was able to incorporate ~0.5 pmol of methyl groups/pmol of particles in 20 min, whereas 0.02 pmol of methyl groups was incorporated/pmol of free 16 S RNA in the same time interval. The nature of the methylated nucleoside was examined by HPLC. [<sup>3</sup>H]methyl 30 S ribosomes were prepared using the recombinant m<sup>2</sup>G methyltransferase. 1.05 pmol of CH<sub>3</sub>/pmol of 30 S were obtained. The enzyme was extracted with phenol, isolated, digested with RNase P1 and alkaline phosphatase, and analyzed by HPLC. The position of the m<sup>2</sup>G added as an internal standard was determined by its UV absorption and is marked by the arrow.

Localization of the Site of Methylation—E. coli 16 S RNA contains three m<sup>2</sup>G residues, at positions 966, 1207, and 1516 (Ref. 18 and Fig. 3). To determine which G was methylated by the recombinant enzyme, hybridization-protection studies were conducted using deoxyoligonucleotides that were complementary to the RNA sequence spanning each of the m<sup>2</sup>G sites (13). Oligomer 958 spanned the region from residue 958 to residue 977, which included m<sup>2</sup>G<sub>966</sub>; oligomer 1197 spanned the region from residue 1197 to residue 1216, including m<sup>2</sup>G<sub>1207</sub>; and oligomer 1506 spanned the region from residue 1506 to residue 1525, which included m<sup>2</sup>G<sub>1516</sub> (Fig. 3). The m<sup>2</sup>G methyltransferase previously characterized was specific for G<sub>966</sub> (13). Because the purification scheme for the native enzyme used in this work was similar, and the recombinant enzyme had the same substrate specificity, we supposed that the same methyltransferase had been cloned. Unexpectedly, the hybridization-protection experiment showed clearly that this was not the case (Fig. 4). Oligomer 958 was as ineffective in protecting the label in the RNA from RNase digestion as was oligomer 1506 or no oligomer. On the other hand, oligomer 1197 efficiently protected the RNA from degradation. To control for artifacts, the deoxyoligonucleotides used in this experiment were the identical preparations used previously (13). In that work, oligomer 1197 did not protect either the m<sup>2</sup>G- or m<sup>6</sup>A-containing RNAs. Therefore, its protection here cannot be attributable to some generalized inhibitory effect on RNase T1. In that work, oligomer 1506 did protect both the m<sup>2</sup>G- or m<sup>6</sup>A-containing RNAs. Thus its failure to protect here cannot be attributable to some failure of hybridization. Likewise, because oligomer 1506 did protect the m<sup>6</sup>A-containing RNA, its inactivity here cannot be attributable to a hybridization failure. We conclude from these results that the recombinant m<sup>2</sup>G methyltransferase described here specifically modifies G<sub>1207</sub> rather than G<sub>966</sub> or G<sub>1516</sub>.

Mg<sup>2+</sup> Dependence of the m<sup>2</sup>G Methyltransferase—Because of the fact that this methyltransferase recognized 30 S ribosomes but not free RNA, we expected that Mg<sup>2+</sup> would be required for this reaction, even though a similar methyltransferase specific for m<sup>2</sup>C967 and for 16 S RNA functioned equally well in 1 mM EDTA as in 10 mM Mg<sup>2+</sup>. Moreover, because the m<sup>2</sup>G<sub>966</sub> methyltransferase studied previously was inhibited in its
methyltransferase of 30 S subunits by increasing concentrations of Mg$^{2+}$ (14), we tested two concentrations, 0.9 mM, which should cause partial unfolding of the particle, and 6 mM, which is known to be sufficient to stabilize natural 30 S particles in this buffer. Fig. 5 shows that at the lower Mg$^{2+}$ concentration, methylation approaches unit stoichiometry (0.93 mol/mol), whereas at 6 mM Mg$^{2+}$, the reaction levels off at 0.3 mol of methyl/mol of ribosomes. Some Mg$^{2+}$ is required, however, because in other experiments, 2 mM EDTA decreased the plateau level from 0.74 mol of methyl incorporated/mol of 30 S in 0.9 mM Mg$^{2+}$ to 0.14 mol/mol in EDTA. In these experiments, the substrate was not preincubated under the specified Mg$^{2+}$ conditions before the methylation reaction was initiated. In a single preincubation experiment in which the ribosomes were incubated in reaction buffer without SAM for 10 min at 37 °C before initiation of the reaction with SAM, the presence of 2 or 10 mM EDTA reduced incorporation levels further to 0.1 or <0.1 mol of methyl/mol of ribosomes, respectively, whereas the control in 0.9 mM Mg$^{2+}$ still yielded 0.8 mol/mol.

**DISCUSSION**

**Substrate Specificity and Mg$^{2+}$ Requirements of the Enzyme**—Because the m$^2$G1207 methyltransferase reacts with 30 S particles but barely at all with 16 S RNA, it seems likely that methylation of the G residue occurs after the 16 S RNA has associated with some ribosomal proteins. The m$^2$G966 methyltransferase, which has similar specificity, has been shown to only require the presence of proteins S7 and S19 with the 16 S RNA to be recognized by the enzyme (14). Whether the m$^2$G1207 methyltransferase can also efficiently recognize 16 S RNA complexed with only a few ribosomal proteins, and what these proteins are, must await further study.

The Mg$^{2+}$ requirements for methylation are also consistent with the hypothesis that G1207 is methylated after the association of an unspecified number of ribosomal proteins but before assembly of the 30 S subunit is complete. At 0.9 mM Mg$^{2+}$, virtually all of the G1207 residues in the 30 S subunit were available for reaction, whereas when the Mg$^{2+}$ concentration was raised to 6 mM, only ⅓ were still available. On the other hand, in EDTA, only Ⅵ were reactive, and preincubation reduced that amount to less than Ⅵ. The structure of the 30 S particle at 0.9 mM Mg$^{2+}$ is probably partially disordered and thus may mimic the structure of the ribonucleoprotein intermediate, which may be the true substrate. In EDTA, this structure may be lost, and at 6 mM Mg$^{2+}$, the 30 S particle may be too compact for reactivity. The observed partial reaction at 6 mM Mg$^{2+}$ may be attributable to the fact that 30 S ribosomes assembled from an RNA transcript are less stable than ones reconstituted from natural RNA (4, 16). We emphasize a role for Mg$^{2+}$ in maintaining the proper substrate structure rather than a role at the catalytic center of the enzyme solely by analogy with the m$^3$C967 methyltransferase, which had no Mg$^{2+}$ requirement. Clearly, the current experiments do not...
allow us to distinguish between the two possibilities.

The substrate preference for 30 S ribosomes versus free RNA shown here for the m²G1207 enzyme and previously for the m²G966 methyltransferase (13, 14) is shared by the KsgA enzyme, which also requires a 30 S subunit for reaction (20). Perhaps the ability of the m⁵C967 methyltransferase to react with free RNA, but not with 30 S subunits, is the exception to the rule, and most or all of the other 16 S RNA-modifying enzymes require either a ribonucleoprotein or a complete 30 S subunit. RsuA, which makes the single pseudouridine in 16 S RNA, also has a specific requirement for a particular RNP particle (15).

How did two similar purification procedures yield an m²G966 enzyme previously and an m²G1207 enzyme in the current work? We suspect this occurred during the DEAE column purification step. Previously the eluate was only assayed with free RNA in the belief that the m²G activity, measured free RNA, but not with 30 S subunits, is the exception to the rule, and most or all of the other 16 S RNA-modifying enzymes require either a ribonucleoprotein or a complete 30 S subunit. RsuA, which makes the single pseudouridine in 16 S RNA, also has a specific requirement for a particular RNP particle (15).

The gene coding for the m²G1207 methyltransferase is yjjT. This ORF codes for a 343-amino acid protein with a calculated molecular mass, 37.6 kDa, that agrees well with the value of 37 kDa for the native enzyme. We propose that the ORF be renamed rsmC for (r)ibosomal (s)mall subunit (m)ethyltransferase, because the function of the gene product has been identified as the enzyme for formation of m³G1207. C denotes that this is the third gene sequence for an rRNA methyltransferase to be described, the first one being ksgA, the gene for the m²G methyltransferase (8), and the second one being rsmB, the gene for the m³C methyltransferase.² In keeping with this nomenclature, we suggest that rsmA would be a suitable alternate name for ksgA.

The RsmC sequence contains the SAM-binding motif DXGXXGXXXL (21) at residues 201–209 as well as the somewhat longer motif in which this is embedded, which has been described by Koonin et al. (22) at residues 199–215. A search of the GenBank data base using BLAST 2.0.4 (23) identified proteins with highly significant similarity to the m²G1207 methyltransferase in seven organisms. These included the prokaryotes E. coli, Hemophilus influenzae, Bacillus subtilis, Staphylococcus aureus, and even the extremely divergent Thermotoga neapolitana as well as the archaeabacteria Pyrococcus horikoshii and Methanococcus jannaschii. Somewhat less related were proteins from Streptomyces anulatus, Streptomyces pristinaeaepiralis, and Chlamydia trachomatis. No strong similarities were found with any higher eukaryotic ORFs.

The protein most similar in sequence to RsmC according to this analysis is from H. influenzae (P44453, HI0012). This protein is 330 amino acids long compared with 342 for RsmC. They are 46% identical and 65% similar along 328 residues. Given the high level of similarity between the two sequences, it is reasonable to suppose that this gene codes for an equivalent methyltransferase in H. influenzae and, therefore, that this organism also has an equivalently located m³G in its 16 S RNA. No information is available as to the presence of such an m³G in H. influenzae or for that matter in any of the other prokaryotes or archaeabacteria. The next most similar amino acid sequence to RsmC is the hypothetical protein product of the E. coli ygiO gene. This 43.4-kDa protein is 33% identical and 50% similar over 173 residues to RsmC. We suspect that this protein is the m³G966 methyltransferase that we described previously (13, 14).

Role of Methylation of G1207 in the Ribosome—m³G1207 is
located in the helix 34 stem (see Fig. 3) in a region believed to be involved in recognition of peptide chain termination codons (24, 25). This region has been directly linked to the decoding site on the ribosome by cross-links from U1052, which is adjacent to m²G1207 on the opposite strand to the A site codon of mRNA (26) and from A1196, 11 nucleotides away on the same strand, to the next downstream mRNA codon (10). However, no specific role for m²G1207 is known in chain termination or codon recognition or, for that matter, in any other function of the ribosome. Moreover, it cannot be essential for ribosome function because, as noted in the introduction, functional 30 S ribosomes have been prepared lacking all modified nucleosides. However, as also noted there, the assembly ability as well as function relative to modified controls was reduced.

To study the reasons for this reduction in assembly and function, we have embarked on a program to identify all of the genes responsible for the modifying enzymes that act on E. coli 16 S RNA. So far, we have identified three such genes, rsuA (15), rsmB, and in this work, rsmC. rsmA (ksgA) was already known (8). There should be at most seven more genes to identify. Two putative genes have already been identified by their strong sequence similarity to RsmB and RsmC, respectively. Gene inactivation, one or more at a time, to block selected function relative to modified controls was reduced.

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