Methylation Sites in *Escherichia coli* Ribosomal RNA: Localization and Identification of Four New Sites of Methylation in 23S rRNA†

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ABSTRACT: Four previously undetermined sites of methylation are mapped in *Escherichia coli* 23S rRNA employing a novel combination of methods. First, using a double-isotope approach, the total number of methyl groups in 23S rRNA was determined to be 14.9 ± 1.6. Second, hybridization of methyl-labeled rRNA to complementary DNA restriction fragments and PAGE analysis were used to purify RNA–DNA heteroduplexes and to quantify methyl groups within specific 23S rRNA fragments. Third, the methylated nucleosides in these fragments were identified and quantified using HPLC, confirming the presence of 14 methylation sites in 23S rRNA, four more than had been previously identified. In contrast, a similar set of analyses conducted on 16S rRNA gave evidence for 10 sites of methylation, all at approximate locations consistent with published 16S methylated nucleoside identities and locations. Selected regions of the 23S rRNA molecule containing previously unidentified methylated nucleosides were released by site-directed cleavage with ribonuclease H and isolated by PAGE. Sites of methylation within the RNA fragments were determined by classical oligonucleotide analyses. The four newly identified methylation sites in 23S rRNA are m^2G-1835, m^5C-1962, m^6A-2503, and m^2G at one of positions 2445–2447. Together with previously described sites of modification, these new sites form a group that is clustered in a current model for the three-dimensional organization of the 23S rRNA in the 50S ribosomal subunit, at a locus congruent with nucleotides previously implicated in ribosomal function.

Mature 16S and 23R ribosomal RNAs from *Escherichia coli* contain small numbers of methylated nucleotides. Methylated rRNA sequences were first characterized by oligonucleotide fingerprinting and related sequencing techniques (Fellner & Sanger, 1968; Fellner, 1969). The locations of methyl groups within 16S rRNA have since been well documented (Carbon et al., 1979; Van Charldorp et al., 1981) and are reaffirmed by our current results. In contrast, the assignment of methyl groups observed in the 23S rRNA (Hsuchen & Dubin, 1980; Gehrke & Kuo, 1989) to the primary sequence (Brosius et al., 1980; Branlant et al., 1981) has remained incomplete. The distributions of hitherto localized methyl groups in 16S rRNA and 23S rRNA secondary structures (Carbon et al., 1979; Van Charldorp et al., 1981; Branlant et al., 1981; Noller, 1984; Gutell & Fox, 1988; Raué et al., 1988; Brimacombe et al., 1990a) correlate well with those of universally conserved nucleotides (Gutell & Fox, 1988) and are clustered within the proposed three-dimensional structures for these molecules (Brimacombe et al., 1988a; Stern et al., 1988; Mitchell et al., 1990) at sites implicated in ribosomal function. In order to gain further insight into the processes and functional significance of rRNA methylation in *E. coli* it is clearly necessary to map the remaining methyl groups in the 23S rRNA sequence. Here we first determine the number of methyl groups within *E. coli* 23S rRNA to be 14 and then localize four previously unidentified methylated nucleosides within the primary sequence.

EXPERIMENTAL PROCEDURES

Materials

Plasmid pSK42, a derivative of plasmid pKK3535 carrying the *rrnB* 23S and 5S coding regions (Doutwaite et al., 1985) was obtained from Dr. Steve Douthwaite (University of California, Santa Cruz). Plasmid pBRS-A9, carrying the *rrnB* 16S coding region, was obtained from Professor David Draper (Johns Hopkins). Nucleoside standards were purchased from Sigma (m^5C and UpA) or obtained from Dr. J. McCloskey (University of Utah; m^4Cm and m^5Cm) and Dr. Phoebe Leboy, University of Pennsylvania (all others). The following materials were purchased from the commercial sources indicated: [2-^{14}C]uracil (50 Ci/mol), [*methyl*-^{3}H]-methionine (85 Ci/mmol), and [*methyl*-^{14}C]methionine (56 Ci/mol), Amersham. Restriction enzymes were from Boehringer Mannheim (BMB) and Bethesda Research Laboratories. Nuclease P1 (specific activity 200 units/mg) was purchased from BMB. Bovine alkaline phosphatase (BAP;[1] 30–45 units/mg) and RNases A and T_2 were from Sigma and RNase T_1 was from Sankyo.

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[1] Abbreviations: ACN, acetonitrile; BAP, bovine alkaline phosphatase; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PEI, poly(ethylenimine); TLC, thin-layer chromatography.

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Methods

Radioactively Labeled rRNAs. Methyl-labeled rRNA was prepared by phenol extraction (Amils et al., 1979; Hall et al., 1985) of crude ribosomes (S-100 pellet) isolated from *E. coli* strain Q13 grown for two doublings in M10 medium (Schleif & Wensink, 1981) in the presence of either [*methyl*-^{3}H]-methionine (4.5 μCi/mL, 0.3 mg/mL, final specific activity 0.36 $\mu Ci/A_{260}$) or [*methyl*-^{14}C]methionine (1 μCi/mL, 0.3 mg/mL, final specific activity 0.05 $\mu Ci/A_{260}$). ([^{14}C]Uracil + [^{14}C]cytosine)-labeled rRNA (0.09 $\mu Ci/A_{260}$) was prepared by the same extraction procedure from *E. coli* grown to saturation in the presence of [2-^{14}C]uracil (Moore & Boylen, 1955).

[*methyl*-^{14}C]-labeled large subunit rRNA for fingerprinting experiments was extracted from 50S ribosomal subunits prepared by sucrose density centrifugation and digestion with proteinase K (Merck), followed by phenol extraction and ethanol precipitation, as previously described (Brimacombe et al., 1990b). The specific radioactivity of the rRNA was then adjusted to ca. 4×10^4 dpm/A_{260} by the addition of unlabeled 23S RNA.

Uniformly [^{32}P]-labeled large subunit rRNA was isolated from cultures of *E. coli* strain MRE 600 grown in a medium containing [^{32}P]orthophosphate (obtained carrier-free from Amersham), as previously described (Brimacombe et al., 1990b).

RNA–DNA Hybridization. Hybridization of [*methyl*-^{3}H]-labeled rRNA to restriction-digested pSK42 or pBRS-A9 was performed as described previously (Hall et al., 1985; Smith, 1990) with the addition of ([^{14}C]uracil + [^{14}C]cytosine)-labeled rRNA, in a molar ratio of 1–2 with respect to [*methyl*-^{3}H]-labeled rRNA. Following hybridization, any remaining single-stranded DNA or rRNA was digested by both S1 nuclease (16 units/mg of total nucleic acid) and T1 nuclease (250 units/mg of total nucleic acid).

PAGE Analysis. The specific activity of full-length rRNAs was determined on samples prepared by PAGE in the presence of 8 M urea (Hall et al., 1985). Determination of ^{3}H and ^{14}C in RNA–DNA hybrids was performed on samples prepared by nondenaturing PAGE (Hall et al., 1985). In both cases, following electrophoresis, the gels were cut into 1-mm slices, solubilized using NCS solubilizer (Amersham), and counted as described previously (Hall et al., 1988).

Methylation Stoichiometry in Hybrids. The number of methyls in a given hybrid is calculated from the observed ^{3}H and ^{14}C activities

$$Me_x = R_x/R_{std} \quad (1)$$

where Me_x is the total number of methyls in hybrid x, R_{std} is the $^3H/^{14}C$ ratio for one cytidine plus one uridine with one methyl group, and R_x is the observed $^3H/^{14}C$ ratio for hybrid x multiplied by the sum of the numbers of cytidines and uridines in hybrid x inferred from the *rrnB* DNA sequence. The standard ratio (R_{std}) was generally determined by setting the total number of methyls for all hybrids derived from 23S rRNA to be 14.9. In some cases, a particular hybrid was chosen as the standard, assuming a methyl stoichiometry for that hybrid.

Nucleoside Analysis by HPLC. Identification and quantification of methylated nucleosides in rRNA were performed using HPLC on a Zorbax C-18 column (DuPont) essentially as described (Buck et al., 1983; Gehrke et al., 1983) for modified nucleosides in tRNA. Full-length rRNAs or RNA–DNA hybrids, purified by electrophoresis and electroeluted from excised gel slices, were ethanol-precipitated and dissolved in 25 μL of water. After the samples were heated to 100 °C for 15–30 s and quickly cooled on ice, 2 μL of 20 mM $ZnSO_4$ and 10 μL of P1 nuclease (200 units/mL in 30 mM sodium acetate, pH 5.3) and 10 μL of BAP (3.8 units/mL in water, specific activity 30–45 units/mg) were added. Digestion was carried out for (1–2 h at 37 °C, at which time 15 μL of 0.5 M Tris-HCl (pH 7.9) was added, followed by continued digestion for another hour. The digestion mixture was then subjected to HPLC, without further purification. In some cases, nucleoside mixtures from digested rRNA were separated by boronate affinity chromatography (Bio-Rad Affi-Gel 601, 0.3-mL bed volume) following the manufacturer's recommended protocol. Nucleosides applied to the HPLC column were eluted at 0.7 mL/min with a gradient of acetonitrile in buffer A (0.25 M ammonium acetate, pH 6.0) in three linear segments: initial conditions 0:100:0 (ACN/buffer A/water), 3 min; 2:95:3, 7 min; 10:75:15, 30 min; 40:0:60, 15 min. For analysis of labeled samples, 0.5-min fractions were collected and counted with 4 mL of Triton–toluene scintillation cocktail (Jaynes et al., 1978). The radioactive nucleoside peaks were identified by comparison with the UV absorbance profile of cochromatographed nucleoside standards. The yields of modified nucleoside standards (Ψ, m^5C, m^7G, m^3U, m^2G, and m^6_2A) in this system were 77% ± 4%. The identifications were further checked by subjecting lyophilized material from HPLC peak fractions to TLC in solvent system A, as described (Wei & Moss, 1975).

For double-labeled RNA–DNA hybrids analyzed by HPLC, the observed dpm in the collected fractions was converted to methyl stoichiometry using eq 1, except Me_x represents the total number of methyls in peak x, and the ^{14}C component is determined by the sum of the cytidine and uridine peaks.

Site-Directed Cleavage of 23S RNA. Decadeoxynucleotides were synthesized on an Applied Biosystems synthesizer (381A) employing phosphoramidite chemistry and used without further purification. RNase H was isolated from *E. coli* strain MRE 600 as described (Brimacombe et al., 1990b). For double-label analyses, approximately 8×10^4 cpm (Cerenkov radiation) of [^{32}P]-labeled rRNA (specific activity ca. 10^8 cpm/A_{260}) were added to 1 A_{260} unit of [*methyl*-^{14}C]-labeled rRNA. Partial digestions with RNase H of rRNA–oligodeoxynucleotide hybrids, prepared as described (Brimacombe et al., 1990b; Mitchell et al., 1990), were performed on 1-A_{260} aliquots of either the double-labeled or [^{32}P]-labeled rRNA. In the latter case, ca. 10^7 cpm of Cerenkov radiation was diluted to give a total of 1 A_{260} by addition of unlabeled rRNA.

Isolation of RNA Fragments. The RNase H-digested rRNA was either treated with proteinase K and extracted with phenol as described previously (Mitchell et al., 1990) or loaded directly onto a 5% polyacrylamide gel containing 7 M urea and 0.1% (w/v) SDS (Brimacombe et al., 1990b; Mitchell et al., 1990). Two-dimensional PAGE was performed as described (Brimacombe et al., 1988b, 1990b; Mitchell et al., 1990). After electrophoresis in the second dimension, the selectively excised RNA fragments were visualized by autoradiography. The appropriate gel sections were cut out and phenol-extracted. RNA was recovered by precipitation with ethanol.

Oligonucleotide Analysis of rRNA Fragments. Uniformly [^{32}P]-labeled and [^{32}P + *methyl*-^{14}C]-double-labeled 23S rRNA fragments were totally digested with either RNase T_1 or RNase A and subjected to fingerprint analysis on poly(ethylenimine) (PEI)–cellulose thin-layer plates (Volkaert & Fiers, 1977b). Fingerprints of [^{32}P]-labeled material were

Table I: Distribution of Radioactivity in PAGE-Purified 16S and 23S rRNA

isotopic label	23S/16S ratio expected	observed[a]	corrected
^{14}C (U + C) (U*-RNA)	1.88	1.72 ± 0.04 (2)	
$^{3}H-CH_3$ (M*-RNA)		1.10 ± 0.04 (4)	1.20 ± 0.06

[a] Number of determinations is shown in parentheses.

visualized by autoradiography (Brimacombe et al., 1990b). Autoradiography of [^{32}P + ^{14}C]-double-labeled fingerprints was conducted according to the method of Maden and Salim (1974). Chromatograms were exposed to two sheets of film (Kodak XAR 500, exposure time ca. 3 weeks), layered one on top of the other. A ^{32}P-only fingerprint was obtained upon development of the distal film, whereas the proximal film provided a ^{32}P + ^{14}C double-label fingerprint. After an additional period of approximately 10 weeks, during which the ^{32}P radiation had significantly decayed, the chromatograms were reexposed for a further 3 weeks to provide essentially ^{14}C-only fingerprints.

Secondary analyses of [^{32}P]-labeled and [*methyl*-^{14}C]-labeled oligonucleotides resulting from RNase T_1 and RNase A digestion were performed using the complementary enzyme (i.e., RNase T_1 products were digested with RNase A, and vice versa) and the double-digestion products were resolved by thin-layer chromatography on PEI–cellulose plates (Volkaert & Fiers, 1977b; Brimacombe et al., 1990b). In one case, double-digestion analyses revealed a methylated dinucleotide product. The [^{32}P]-labeled material was further analyzed by digestion with RNase T_2 (Mitchell et al., 1990). Nucleotides released were identified by chromatography on PEI–cellulose plates (Volkaert & Fiers, 1977a). Oligonucleotide data were fitted to the known sequence of 23S rRNA (Brosius et al., 1980; Branlant et al., 1981).

RESULTS

Methylated Nucleosides in 23S and 16S RNA. The ratio of radioactivities found in 23S and 16S rRNA, resolved by urea–PAGE from preparations of either total [*methyl*-^{3}H]-labeled (M*-rRNA) or total ([^{14}C]uracil + [^{14}C]cytosine)-labeled tRNA (U*-rRNA) are presented in Table I. From the base composition of 16S and 23S rRNA (666 pyrimidines and 1230 pyrimidines, respectively), the expected value of the ratio U*-23S/U*-16S is 1.88. This is higher than the observed value of 1.72 ± 0.04 because of the contamination of the 16S rRNA band with partially degraded 23S rRNA (contamination of 23S rRNA with degraded 16S rRNA is obviously not a problem). The observed ratio M*-23S/M*-16S, 1.10 ± 0.04, is a lower limit value, again because of the contamination of the 16S rRNA band. Taking this contamination into account gives a corrected value for the M*-23S/M*-16S ratio of 1.20 ± 0.06.

23S rRNA, purified by PAGE, was subjected to digestion by nuclease P1 and BAP. One portion of the resulting mixture of nucleosides was fractionated on a boronate column and both the retained fraction (containing unmodified riboses) and the nonretained fraction (containing 2′-OMe riboses) were separately analyzed by HPLC. Control experiments with standard nucleosides showed that the recovery of both retained and nonretained nucleosides from the boronate column was the same. A second portion of the original digestion mixture was analyzed directly by HPLC. The results are presented in Figure 1 and Table II. There is good evidence that each of the well-resolved nonretained 2′-OMe nucleosides, Cm, Um, and Gm, are present in one copy per 23S rRNA (Nichols & Lane, 1967; Hsuchen & Dubin, 1980; Branlant et al., 1981). Accordingly, we used these nucleosides as standards in converting percentage of radioactivity into stoichiometry. Setting the percentage of radioactivity in these three peaks (6.9 + 5.7 + 7.6 = 20.2) as corresponding to 3.0 methyl groups gives a value of 6.7% ± 0.7% per methyl group, or a total of 14.9 ± 1.6 Me groups/23S rRNA, and allows calculation of the apparent stoichiometries of each of the other methylated nucleosides present in 23S rRNA. Compared to the methylated nucleosides previously identified within 23S rRNA, the results in Table II present evidence for one additional m^5C, one additional m^6A, and 2–3 additional m^2Gs.

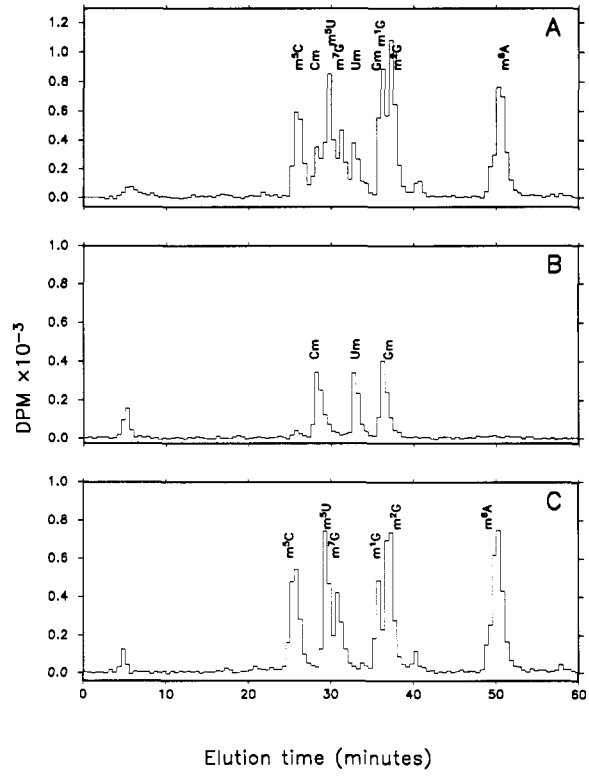

FIGURE 1: Methylated nucleoside composition of 23S rRNA. PAGE-purified M*-23S rRNA was digested to nucleosides and analyzed by HPLC. Panels B and C show nucleoside analyses of the nonretained (B) and retained (C) boronate column fractions of the material analyzed in panel A.

Table II: Stoichiometries of Methyl Groups in Methylated Nucleosides of 23S rRNA and 16S rRNA

nucleoside	23S rRNA[a] −[c]	23S rRNA[a] +[d]	16S rRNA[b] −[c]	16S rRNA[b] −[e]	16S rRNA[b] +[d]
m^5C	2.0 ± 0.1	2.0	2.1 ± 0.3	2.2	1.9
Cm	0.8 ± 0.1	1.1			
m^5U	2.0 ± 0.2	1.8			
m^7G	1.1 ± 0.1	1.3	1.2 ± 0.2	1.3	1.2
m^3U			1.3 ± 0.3[f]	1.1	1.0
m^4Cm					0.6
Um	1.0 ± 0.1	0.9			
Gm	2.3 ± 0.2[g]	1.2			
m^1G		1.1			
m^2G	2.7 ± 0.1	2.4	4.3 ± 0.1	4.3	3.8
m^6A	3.1 ± 0.1	3.1			
m^6_2A			3.7 ± 0.2	3.6	4.0

[a] Normalized to 14.9 methyls per 23S rRNA. [b] Normalized to 12.4 methyls per 16S rRNA. [c] Without boronate. Average of three determinations. PAGE-purified from total rRNA. [d] With boronate. One determination. PAGE-purified from total rRNA. [e] Without boronate. One determination. PAGE-purified from 16S rRNA–DNA hybrid. [f] m^3U and m^4Cm are not resolved. [g] Gm and m^1G are not resolved.

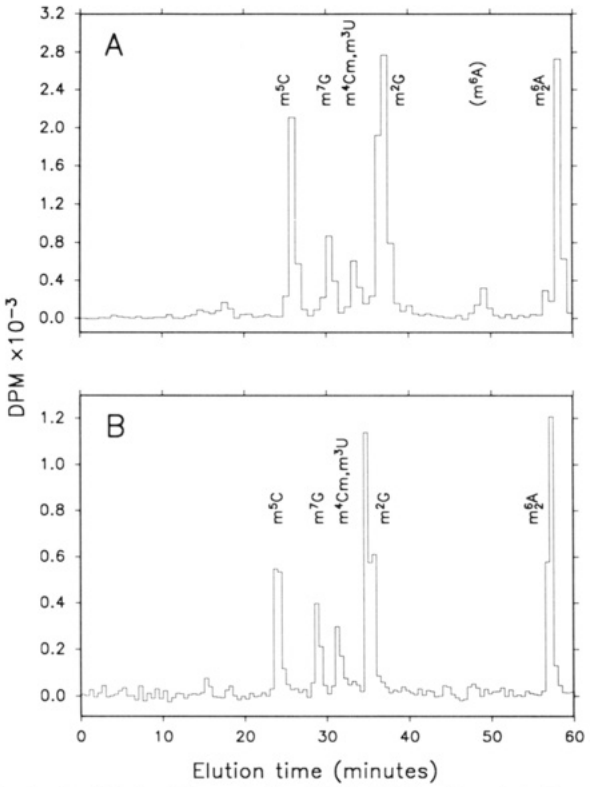

FIGURE 2: Nucleoside analysis of 16S rRNA. Panel A shows a nucleoside analysis of M* plus U*-16S rRNA purified directly from total rRNA. Panel B shows the same analysis of M* plus U*-total rRNA hybridized to *Bam*HI-digested pBRS-A9. The heteroduplex band was purified by PAGE, digested to its constituent nucleosides, and analyzed by HPLC. The ^{14}C in cytidine and uridine are not shown in this figure.

The value of 14.9 ± 1.6 Me groups/23S rRNA and the M*-23S/M*-16S ratio of 1.20 ± 0.05 results in a calculated level of 12.4 ± 1.3 methyls/M*-16S rRNA. This value is in good agreement with the expected number of 13, based on the 10 methylated nucleosides reported within 16S rRNA, of which three are dimethylated [two m^6_2A (positions 1518 and 1519) and one m^4Cm (position 1402)].

Nucleoside analyses, similar to those performed on 23S rRNA, were also performed on 16S rRNA, purified by PAGE either from total rRNA or from a full-length hybrid of 16S rRNA with its complementary DNA. Typical analyses are shown in Figure 2. All of the results obtained are collected in Table II. The major obvious difference between the two chromatograms in Figure 2 is the presence of the m^6A peak for the 16S RNA prepared directly from total rRNA, reflecting the contamination of such a sample with degraded 23S rRNA, amounting to 10–15% of the total. This agrees with the estimate from the PAGE analysis presented above. The size of the m^6A peak and the known methylated nucleoside composition of 23S rRNA (Table II) permitted estimation of the contribution of 23S rRNA nucleosides to the observed peaks in Figure 2A, assuming that degradation of the 23S rRNA is essentially random. The nucleoside stoichiometries collected in Table II include these corrections. Clearly there is very good agreement between the values obtained for 16S M*-rRNA prepared in the two different manners.

Despite the agreement of our results with prior studies on the overall stoichiometry of methyl groups within 16S rRNA, there is an apparent discrepancy with respect to distribution of methyl groups within 16S rRNA. For analyses conducted in the absence of a boronate column separation, only 1.1–1.3 methyls instead of the expected three show up in the unresolved peak corresponding to m^3U and m^4Cm, and 4.3 methyl groups instead of the expected three show up in m^2G. Similarly, when a boronate column was employed, one methyl group is found in m^3U, as expected, but only 0.6 methyl instead of the expected two coelute with m^4Cm, and about 4 methyl groups show up in m^2G. This apparent discrepancy is an artifact resulting from two circumstances: first, P1 nuclease digestion of 16S rRNA results in the formation of the dinucleotide pm^4CmpC, corresponding to nucleotides 1402 and 1403, which is unusually resistant to P1 nuclease digestion (Negre et al., 1989; Edmonds et al., 1991); second, the elution volume and mobility of the phosphatase-treated dinucleoside phosphate, m^4CmpC, are very similar to those of m^2G under the HPLC (Takeda et al., 1991; C. Weitzmann, personal communication) and TLC conditions we employ. When it is considered that methyl groups eluting with m^2G may correspond to m^4CmpC, the apparent discrepancy between our results and those in the literature disappears.

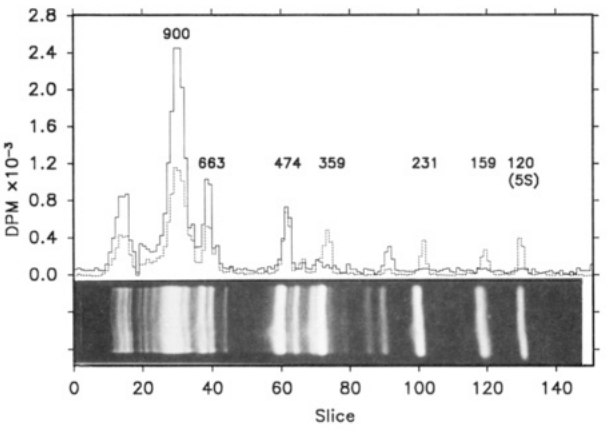

FIGURE 3: PAGE analysis of M*-plus U* rRNA hybridized to a *Hin*fI digest of pSK42. A photo of the ethidium bromide-stained gel track is shown at the bottom. ^3H dpm are indicated by solid lines, ^{14}C dpm by broken lines. Number of base pairs is indicated at the top.

Approximate Locations of Methylated Nucleosides in 23S rRNA. Our results with 23S rRNA indicated the presence of methylated nucleosides not previously localized in the literature [Branlant et al., 1981; see also Fellner and Sanger (1968), Fellner (1969), Hsuchen and Dubin (1980) and Gehrke and Kuo (1989)]. We used the restriction fragment hybridization strategy (Hall et al., 1985, 1988), coupled with HPLC analysis, to obtain approximate locations for these bases within rRNA. In this approach, heteroduplexes are formed between a combination of M*-rRNA and U*-rRNA and restriction fragments of the corresponding DNA, the heteroduplexes are resolved according to size by PAGE, and the radioactivity and nucleoside composition of each heteroduplex is determined.

For the heteroduplexes, the ^3H content provides a measure of the methylation level, while the ^{14}C content provides a measure of the yield. Thus, the $^3H/^{14}C$ ratio provides a measure of the stoichiometry of methylation of the heteroduplex. Sample data showing the results of PAGE analysis of heteroduplexes formed on hybridizing the *Hin*fI digestion of pSK42 with labeled rRNA are shown in Figure 3. Here three of the heteroduplexes, corresponding to 23SrRNA regions 2005–2904, 1342–2004, and 637–1110, are seen to be methylated.

Digestion of the extracted heteroduplexes with nuclease P1 and BAP, followed by HPLC analysis, allows identification of the labeled nucleosides within each heteroduplex, as well as a refinement in the value of the stoichiometry, since minor

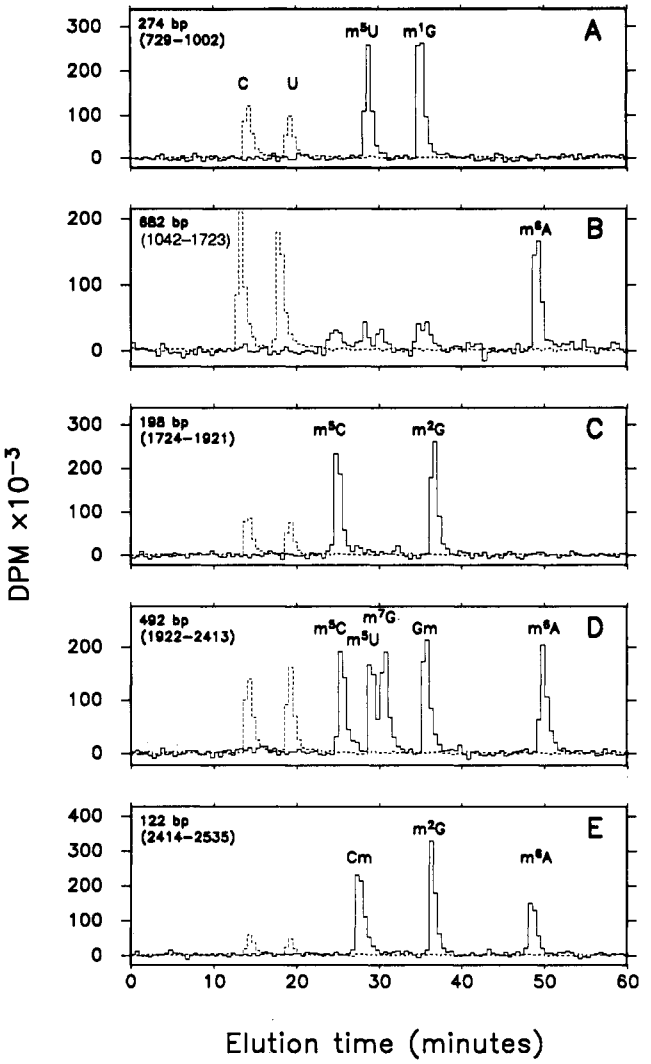

FIGURE 4: Nucleoside analysis of pSK42/*Sau*96I hybrids. M* plus U*-rRNA hybridized to *Sau*96I-digested pSK42 was electrophoresed, the resulting heteroduplex bands (named in the upper left of each panel) were excised and digested to nucleosides, and the nucleosides were analyzed by HPLC. ^3H dpm are indicated by solid lines, ^{14}C dpm by broken lines. ^3H dpm in each fraction was normalized using the ^{14}C dpm recovered in cytidine and uridine to calculate the number of methyls per mole of hybrid.

contaminating nucleosides are readily apparent. The results of such an analysis of the *Sau*96I-derived heteroduplexes (which show methylation of fragments 2414–2535, 1922–2413, 1724–1921, 1042–1723, and 729–1002) are displayed in Figure 4; they allow approximate placement and identification of 13 methylated nucleosides within 23S rRNA. Of these, only m^6A within fragment 2414–2535 is present at a stoichiometry < 1.0 (we estimate 0.6–0.7).

The levels of methylation obtained by hybridization with *Hin*fI and *Sau*96I restriction fragments are summarized in Figure 5. The *Hin*fI results are more consistent with a total of 14 nucleosides. Furthermore, there is good evidence for a Um residue at position 2552 (Branlant et al., 1981; vide infra). This would have been present in a 72-nt heteroduplex in the *Sau*96I experiment, and we have found heteroduplexes of that size difficult to recover in reasonable yield. Our conclusion is that there are 14 methylated nucleosides within 23S rRNA, which is in excellent accord with the value of 14.9 ± 1.6 estimated from the PAGE-purified 23S rRNA.

As discussed further below, the presence of what appear to be m^5C and m^2G nucleosides in 23S rRNA fragment 1724–1921 and m^2G and m^6A nucleotides in 23S rRNA fragment 2414–2535 (Figure 4) is new information, which prompted fingerprinting studies to identify the precise nucleotides methylated.

Approximate Locations of Methylated Nucleosides in 16S rRNA. Hybridization and HPLC studies essentially identical to those described above for 23S rRNA (Figures 3 and 4) were also carried out on 16S rRNA. The partial localization results obtained were fully consistent with the published identities and locations of methylated nucleosides (Carbon et al., 1979; Van Chaldorp et al., 1981; Noller, 1984). However, evidence was obtained for somewhat less than complete stoichiometries of $m^6{}_2A$ and of m^4Cm. This may account for the corrected M*-23S/M*-16S ratio of 1.20 ± 0.06, which is slightly higher than the expected ratio of 1.08 for 14 methyls in 23S rRNA and 13 methyls in 16S rRNA.

Identification of Methylated Nucleosides in 23S rRNA. Site-directed cleavage of 23S rRNA with complementary oligo DNAs and RNAse H (Donis-Keller, 1979; Mitchell et al. 1990) and PAGE purification was used to prepare several [^{32}P]- or [^{32}P/*methyl*-^{14}C]-labeled 23S rRNA fragments containing the sequences of interest. Further analysis of these fragments on a second dimension 12% polyacrylamide gel (Brimacombe et al., 1988b, 1990b) showed them to be pure.

23S rRNA fragment 1703/1712–1962/1971 was subjected to RNase T_1 fingerprint analysis (Figure 6). Four spots in the $^{32}P/^{14}C$ double-label fingerprints (panel B) are observed at a higher relative intensity in comparison with the ^{32}P-only fingerprints (panel A) due to the presence of ^{14}C label. These are denoted by asterisks and are clearly identifiable as comprising methylated oligonucleotides in the ^{14}C-only fingerprints (panel C), where they are numbered 1–4. Spot 3 corresponds to Ψ-A-A-C-mU-A-Ψ-A-A-C-Gp (positions 1911–1921). An mU at position 1915 has been previously reported (Branlant et al., 1981). Our RNase A fingerprinting results, yielding a ^{14}C-only spot corresponding to mU-A-Ψp, support some type of methylated nucleoside at this position. When this spot was eluted from the gel, subjected to nuclease P1 and BAP digestion, and analyzed by HPLC, the ^{14}C radioactivity eluted precisely with m^5C (data not shown). However, it is unlikely that the ^{14}C-labeled nucleotide corresponds to m^5C, since RNase A treatment of m^5C-A-Ψ_p should have released m^5C directly, without requiring further digestion by nuclease P1. Thus, the identification of an m^5C residue within the 1724–1921 sequence on the basis of the results presented in Figure 4 is likely to be in error, due to coelution of the labeled nucleoside at position 1915 with m^5C. We return to this point under Discussion.

Spot 4 contains the oligonucleotide A-A-A-m^5U-U-C-C-U-U-Gp (positions 1936–1945). The m^5U residue was also observed in RNase A analysis in the end product G-A-A-A-m^5Up. An m^5U at position 1939 has been previously reported (Fellner, 1969; Branlant et al., 1981).

Spots 1 and 2, on the other hand, correspond to newly located methylated oligonucleotides. The chromatographic mobility of spot 1 (Figure 6C) is consistent with that of a tetranucleotide containing a single uridine residue [cf. Figure 3 in Brimacombe et al. (1990b)]. Secondary analysis yielded a methylated product which exhibited a characteristic mobility slightly faster than Gp in both dimensions of the chromatographic system. An identical product was observed upon secondary analysis of a spot adjacent to the G-Cp spot in the corresponding RNase A fingerprint. From the 23S RNA sequence between positions 1703 and 1971, three or four oligonucleotides (due to an observed sequence heterogeneity at position U/C-1865; P. Mitchell, unpublished results) would be expected to coelute

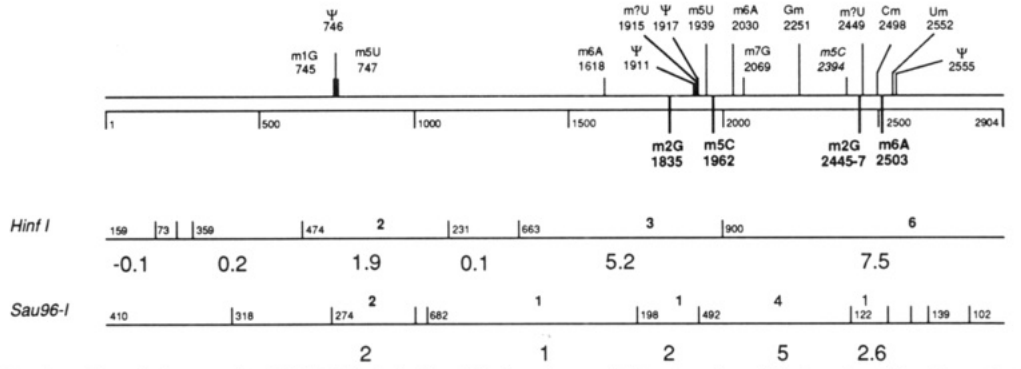

FIGURE 5: Distribution of methyl groups in 23S rRNA hybrids. The location and identity of modified nucleosides above the top line are based on prior results (Brosius et al., 1980; Hsuchen & Dubin, 1980; Branlant et al., 1981; Noller, 1984; Gehrke & Kuo, 1989). For both *Hin*fI and *Sau*96I, line show predicted sites of cleavage in 23S rRNA. The size in base pairs for each of the major restriction fragments is indicated. The number of methyls based on previously reported results is given in boldface type above the line. The number of methyls we observed in various regions of 23S rRNA is given below the line. For *Hin*fI the observed values are based on PAGE analyses, performed in triplicate. For *Sau*96I, the observed values are based on an HPLC analysis. The hybrid containing Um-2552 was not recovered in the *Sau*96I hybridization.

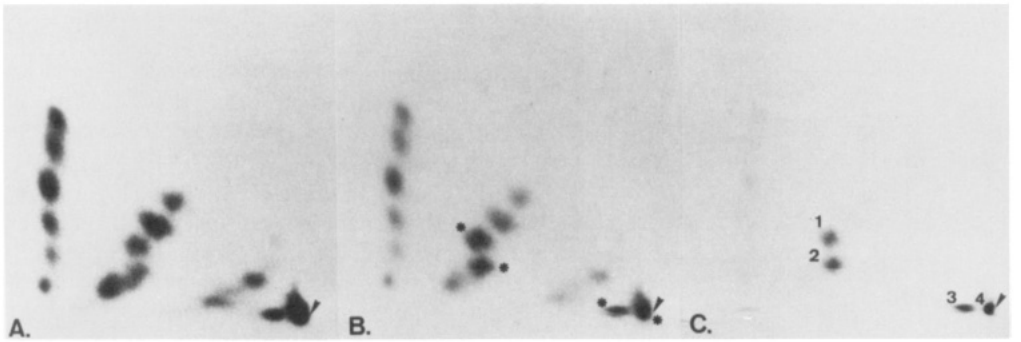

FIGURE 6: $^{32}P/^{14}C$ double-label RNase T_1 fingerprint analysis of fragment 1703/1712–1962/1971 in 23S RNA. ^{32}P-only (panel A), $^{32}P/^{14}C$ double-label (panel B), and ^{14}C-only (panel C) fingerprints of the chromatogram are shown. The direction of chromatography was from right to left in the first dimension and from bottom to top in the second dimension. Arrowheads indicate the points of sample application. Spots containing ^{14}C label are denoted by asterisks in the double-label fingerprint (panel B) and are numbered 1–4 in the ^{14}C-only fingerprint (panel C).

with spot 1. However, the presence of mG-Cp in the RNase A analysis is compatible only with methylation at G-1835.

This interpretation was confirmed by analysis of a shorter RNA fragment, from positions 1767/1776 to 1841/1850, using [^{32}P]-labeled and [$^{32}P/^{14}C$]-double-labeled material. In this fragment, the spot corresponding to spot 1 in Figure 6C comprises only the sequence C-C-U-G (positions 1832–1835). [$^{32}P/^{14}C$]-Double-label fingerprint analysis demonstrated that this oligonucleotide contains ^{14}C label. Secondary analysis of the [^{32}P]-labeled oligonucleotide released 2 Cps, Up, and the same characteristic modified guanosine residue. These findings are in complete agreement with the earlier report of the methylated (but unlocalized) 23S RNA oligonucleotide G-C(C,U)-m^2G (Fellner & Sanger, 1968; Fellner, 1969), as well as with the placement of an m^2G within 1724–1921 (Figure 4).

Spot 2 (Figure 6C) exhibits a chromatographic mobility consistent with a pentanucleotide containing a single uridine residue. Secondary analysis released a methylated product indistinguishable from Cp. Methyl label was also observed in the Cp spot in the RNase A fingerprint. Two RNase T_1 oligonucleotides would be expected to coelute with spot 2: C-A-C-U-Gp (positions 1793–1797) and A-C-C-U-Gp (positions 1960–1964). However, the presence of mCp in the RNase A fingerprint is consistent only with methylation at position 1962. The remaining cytosine residues in these oligonucleotides are released in end products other than Cp upon digestion with RNase A.

Confirmatory evidence was obtained by separate fingerprint analyses of double-labeled fragments where the spot equivalent to spot 2 comprised only one of the two putatively methylated RNase T_1 oligonucleotides. ^{14}C label was observed in the oligonucleotide A-C-C-U-Gp upon analysis of an RNA fragment comprising positions 1841/1850–2084/2093, whereas no ^{14}C label could be detected in the corresponding spot (comprising C-A-C-U-Gp) upon analysis of a fragment from positions 1767/1776 to 1841/1850. These results and those reported in Figure 4 establish the identity of position 1962 as m^5C.

Analysis of the 2354/2363–2568/2577 fragment gave a total of three ^{14}C-only spots (Figure 7C). Spot 6, C-Um-Gp (positions 2551–2553), and spot 7, C-A-Cm-U-U-Gp (positions 2496–2502), contain the previously known methylation sites Um-2552 and Cm-2498. However, secondary analysis of spot 6 revealed the presence of an additional methylated product with a characteristic mobility similar to that of A-Up. The same methylated A-Up spot was observed upon secondary analysis of the RNase A oligonucleotide G-A-Up. As the position of spot 6 in the fingerprint (Figure 7C) shows it to comprise trinucleotides containing a single uridine residue, the "new" methylated oligonucleotide is inferred to have the sequence A-U-G. This oligonucleotide is expected form only one site in the fragment analyzed, namely, at positions 2503–2505.

The methyl group was further localized to position A-2503 in two ways. Tertiary analysis of the methylated AUp spot from [^{32}P]-labeled material by digestion with RNase T_2 released Up and a methylated product with a chromatographic mobility differing from that of Up or Ap. In addition, digestion of the A-Up spot with nuclease P1 and BAP followed by HPLC

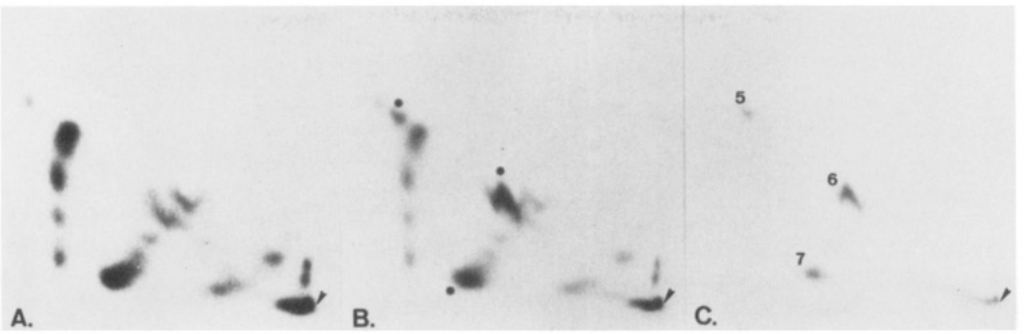

FIGURE 7: $^{32}P/^{14}C$ double-label RNase T_1 fingerprint analysis of fragment 2354/2363–2568/2577 of the 23S RNA sequence (cf. Figure 6). Panel A ^{32}P-only (panel A), $^{32}P/^{14}C$ double label (panel B), and ^{14}C-only (panel C) fingerprints of the chromatogram are shown. Oligonucleotides containing ^{14}C label are denoted by asterisks in the double-label fingerprint (panel B) and are numbered 5–7 in the ^{14}C-only fingerprint (panel C).

Table III: Analysis of Newly Identified Methylated Nucleosides in 23S rRNA

		double-digestion products[a]				
spot	label	(T_1/A)	(A/T_1)	fragments analyzed[b]	derived sequence	methyl site
1	^{14}C	mGp	mGp	1703–1971 1767–1850	G-(C,C,U)-mG-C	mG-1835
	^{32}P	Cp, Up, mGp	Cp, mGp	1703–1971 1767–1850		
2	^{14}C	mCp	mCp	1703–1971 1841–2093	G-(mC,U,A-C)-G	mC-1962
	^{32}P	Cp, Up, Gp, ACp	Cp	1841–2059 1904–2059		
5	^{14}C	mGp	mGp	2354–2577	G-(G,G,mG)-A-U	mG-(2445–7)
	^{32}P	mGp	Gp, mGp, AUp	2438–2577 2438–2528		
6	^{14}C	mAUp	mAUp	2354–2577	G-A-U-G; G-mA-U-G	mA-2503
	^{32}P	Gp, AUp, mAUp	Gp, AUp, mAUp	2438–2577 2438–2528		

[a] T_1/A: RNase T_1 primary digestion, RNase A secondary digestion. A/T_1: RNase A primary digestion, RNase T_1 secondary digestion. The relative intensities of ^{32}P-labeled double-digestion products were assessed by visual inspection of the autoradiograms and are indicated by the underlining of spots observed in higher yield, in the manner of Brownlee and Sanger (1967). [b] As defined by the positions complementary to the deoxyoligoribonucleotides used to direct the RNase H digestions in each case.

analysis showed coelution of the [*methyl*-^{14}C]label with authentic m^6A. These results, taken together with those presented in Figure 4, identify position 2503 as m^6A. This identification corrects earlier work on 23S rRNA, which properly identified the sequence G-X-U-G-U-C-G, corresponding uniquely to positions 2502–2508 in 23S rRNA (Brosius et al., 1980; Branlant et al., 1981) but erroneously identified nucleotide X as a modified cytosine (Herr & Noller, 1978).

The final newly located site comes from analysis of spot 5 (Figure 7C). This spot runs adjacent to the Gp spot. Secondary analysis revealed a methylated product with the same mobility as that released from spot 1: mGp. The same methyl product was also observed on secondary analysis of a pair of [*methyl*-^{14}C]-labeled oligonucleotides detected upon RNase A fingerprint analysis (due to incomplete modification of U-2449; see also Discussion). Comparison with the RNase A oligonucleotide data from parallel analyses with [^{32}P]-labeled RNA mapped the mG residue to the sequence G-G-G-G-A-U at positions 2444–2449. Methylation at position G-2444 can be discounted, since this would not result in the release of mGp upon digestion with RNase T_1. The presence of three consecutive guanosine residues at positions 2445–2447 prevented further localization of this methyl group. These results, taken together with those presented in Figure 4, identify one of positions 2445–2447 as m^2G.

A summary of the analyses used to locate the four new methylated nucleoside positions is presented in Table III. Of the four, only methylation of A-2503 was found to be nonquantitative, in agreement with HPLC analysis of the 2414–2535 fragment (Figure 4).

Table IV: Location of Modified Nucleosides in 23S rRNA

position	nucleoside	previously identified	position	nucleoside	previously identified
745	m^1G	yes	2030	m^6A	yes
746	Ψ	yes	2069	m^7G	yes
747	m^5U	yes	2251	Gm	yes
1618	m^6A	yes	2445–7	m^2G	no
1835	m^2G	no	2449	U*[a]	yes
1911	Ψ	yes	2498	Cm	yes
1915	mU[a]	yes	2503	m^6A	no
1917	Ψ	yes	2552	Um	yes
1939	m^5U	yes	2555	Ψ	yes
1962	m^5C	no			

[a] Structure unknown.

DISCUSSION

The results presented in this work demonstrate the power of coupling newer methods of partial localization with traditional fingerprinting methods to identify and place modified nucleosides within long RNA chains. Our results, taken together with the previously known identify and locations of 10 other methylated nucleosides within 23S rRNA, essentially completes the identification of the 14 methylated nucleosides in 23S rRNA, as summarized in Table IV. The one remaining ambiguity concerns the nature of the methylated nucleoside at position 1915. The evidence that it is a modified

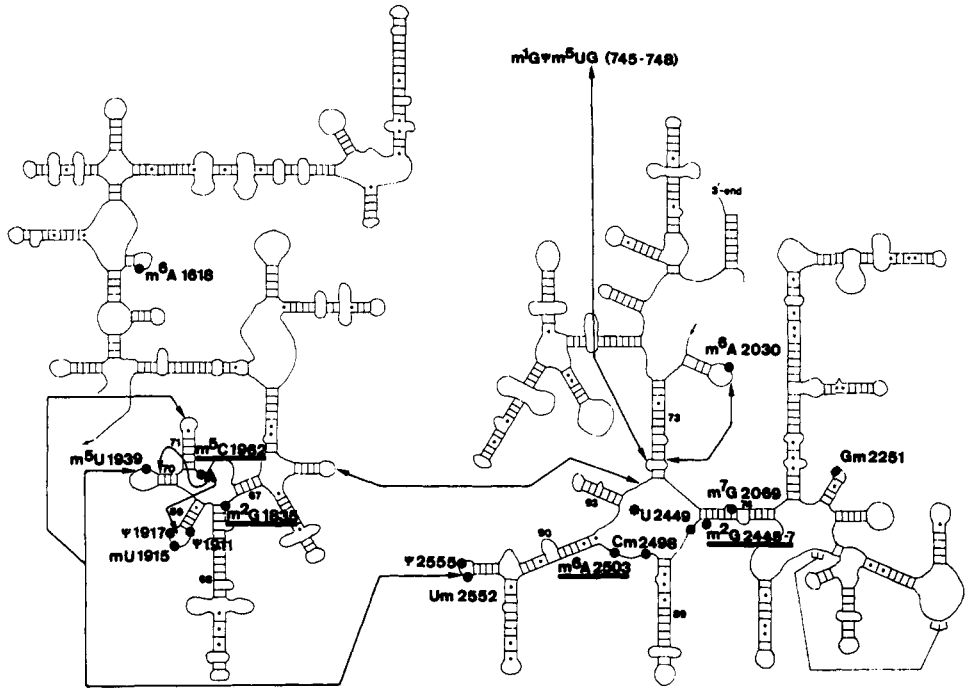

FIGURE 8: Secondary structure of positions 1271–2904 in 23S RNA (Brimacombe et al., 1990a) showing the locations of known methylated nucleotides (Branlant et al., 1981; this study) and pseudouridine residues (Noller, 1984). This portion of the secondary structure is depicted schematically in two sections, with small arrows indicating points of interconnection. The methyl groups localized in these studies are denoted by underlining. Intramolecular cross-linking data (Stiege et al., 1983; Mitchell et al., 1990; Döring et al., 1991) demonstrating the topographical proximity of the modified nucleotides in situ in the 50S ribosomal subunit are indicated by double-headed arrows. The sequence (745–748) including all three modified nucleotides lying outside this region of the secondary structure is indicated, along with the 748–2614 cross-link that demonstrates the proximity of nucleosides 745–747 to the other modified nucleotides in 23S rRNA. Helices are numbered according to Brimacombe et al. (1990a). The unidentified uracil modification at position 2449 is indicated by an asterisk.

uridine comes from the DNA sequence for 23S rRNA (Brosius et al., 1980), direct RNA sequence analysis (Branlant et al., 1981), and the allocation of methylated T1 oligonucleotide sequences to the rDNA sequence (Brosius et al., 1980). As U-1915 falls within an evolutionarily highly conserved sequence (Gutell & Fox, 1988) it is unlikely that there is sequence heterogeneity at this position. Our HPLC results would appear to rule out m^3U, m^5U, and Um as possibilities. Moreover, incomplete digestion with nuclease P1 to give the type of artifact already noted above in the case of 1402-m^4Cm in 16S rRNA is unlikely, since no methylated ribose is involved. We believe it most likely that mU corresponds to a uridine derivative, as yet unidentified, that contains a substituted methyl group (or groups) derived from methionine, is a poor substrate for RNase A, and coelutes with m^5C on HPLC analysis. Clearly, further work will be required to resolve this question.

Our results do not support the placement by Noller (1984), presumably based on early work of Fellner (1969), of an m^5C at position 2394. We find no ^{14}C-only spot corresponding to an oligonucleotide including this position. In addition, it is clear from the results presented in Figure 4 that the 1922–2413 fragment contains a single m^5C that we have placed definitively at position 1962. Fellner (1969) reported the sequence G-A-U-m^5C-C-G, which corresponds to positions 2391–2396 in 23S rRNA and is an isomer of the sequence G-A-m^5C-C-U-G that includes m^5C-1962. We believe Fellner's sequence to be in error, possibly due to contamination of his T_1 oligonucleotides with products released from other regions of the RNA molecule. The same explanation may also account for the related G-C-A-m^5C-(C,U)-G sequence reported by Sri Wadada et al. (1979).

The positions of all of the modified nucleosides within the sequence of 23S rRNA, including the four methylated nucleosides newly identified in this work, are listed in Table IV. The locations of these 19 modified nucleosides within the secondary structure of 23S rRNA (Branlant et al., 1981; Noller, 1984; Gutell & Fox, 1988; Raué et al., 1988; Brimacombe et al., 1990a) are indicated in Figure 8. What is remarkable about their placement is that 17 of the 19 fall within one of two specific regions of 23S rRNA. The first region, the central loop of domain V (which draws together helices 73, 74, 89, 90, and 93), is clearly identified with the peptidyltransferase center [a recent review of the pertinent evidence is presented in Cooperman et al. (1990)]. Nine modified nucleosides are directly associated with this region: three (U*-2449, Cm-2498, and m^6A-2503) fall within the loop, two (m^7G-2069 and m^2G-2445-7) are in helix 74, and four others (m^1G-745, ψ-746, m^5U-747, and m^6A-2030) may be placed close to helix 73 on the basis of intra-RNA cross-links. The second region draws together helices 67–71 and has eight associated modified nucleosides, six in helices 68–71 or their associated loops (m^2G-1835, ψ-1911, mU-1915, ψ-1917, m^5U-1939, and m^5C-1962) and two (Um-2552 and ψ-2555) placed close to helix 70 via an intra-RNA cross-link.

These two regions are themselves clearly linked both functionally and structurally. Thus, both are important for tRNA binding [(Wower et al., 1989; Cooperman et al., 1990; Mitchell et al., 1990, 1992) and may be juxtaposed, at least approximately, on the basis of three cross-links: 1777/1792 to 2607/2609, 1936/1945 to 2552/2554, and 1952/1955 to 2552/2554 (Figure 8) [it should also be noted that an 18th modified nucleoside, Gm-2251, has been linked to tRNA binding by chemical footprinting experiments (Moazed & Noller, 1989)]. In fact, our recent model (Mitchell et al., 1990) for the tertiary structure of part of the 23S RNA suggests that these two regions are clustered, at the interface of the 50S subunit with the 30S subunit. The RNA within these

regions is highly conserved (Gutell & Fox, 1988), so that there is a striking correlation between the distribution of modified nucleotides, phylogenetically conserved structural elements, and sites implicated in 50S–tRNA interactions. An analogous correlation has been noted (Brimacombe et al., 1988a) between the spatial distribution of modified nucleotides and conserved elements in the tertiary structure of 16S RNA (Brimacombe et al., 1988a; Stern et al., 1988), where they were postulated to line the "neck" of the 30S subunit. Furthermore, recent cross-linking results [16S to 23S RNA (Mitchell et al., 1990) and mRNA to ribosomes (Dontsova et al., 1991; Rinke-Appel et al., 1991)] provide evidence that the clusters of methylated nucleosides in the 30S and 50S subunits are proximal to one another.

The functional significance of nucleoside methylation remains largely speculative, however. Ribosomes reconstituted from unmodified RNA transcripts synthesized in vitro and isolated ribosomal proteins (Krzyzosiak et al., 1987; Cunningham et al., 1990) have been shown to exhibit protein synthesis activity in translational assay systems, albeit at a somewhat reduced rate, but little is known about the effect of methylation on such processes as RNA maturation and the control of translation rates. Methylation is clearly important for the interaction of some antibiotics with ribosomes (Cundliffe, 1990) and it is not unreasonable to expect that the binding of other ligands will be affected as well. It has been suggested that rRNA methylation may serve to optimize ribosomal assembly or function, rather than being indispensable for these processes (Krzyzosiak et al., 1987; Cunningham et al., 1990). The location of a number of modified nucleotides at the loop ends of helices, or in single-stranded regions presumably arranged in a highly ordered manner in situ (Figure 8), favors a suggestion that such an optimization may be afforded, at least in part, by modulation of tertiary interactions within rRNA, or of contacts between rRNA and ribosomal protein, tRNA, mRNA, or protein translation factors.

However, it is important to emphasize that sites of modification do not appear to be conserved evolutionarily. A phylogenetic comparison (Raué et al., 1988) of the limited sequence data available on posttranscriptional modifications in large rRNAs (Veldman et al., 1981; Maden, 1988) has shown that, with the exception of Um-2552, sites of modification in *E. coli* are not conserved in yeast or higher eukaryotes. The methylation sites newly identified in the present study are also not conserved in the known eukaryotic methylation patterns. Thus, were the above suggestion correct, the modulating effects of rRNA modifications would have to be different between prokaryotic and eukaryotic organisms. Clearly, experiments to determine the temporal pattern of rRNA modification in *E. coli* during subunit assembly and the functional significance of these processes are important objectives for future research.

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