688–693 Nucleic Acids Research, 1996, Vol. 24, No. 4

Structural characterization of U*-1915 in domain IV from *Escherichia coli* 23S ribosomal RNA as 3-methylpseudouridine

Jeffrey A. Kowalak[1], Eveline Bruenger[2], Takeshi Hashizume[2], John M. Peltier[2], James Ofengand[3] and James A. McCloskey[1,2,*]

[1]Department of Biochemistry and [2]Department of Medicinal Chemistry, University of Utah, Salt Lake City, UT 84132, USA and [3]Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110, USA

Received October 12, 1995; Revised and Accepted December 8, 1995

ABSTRACT

Mass spectrometry-based methods have been used to study post-transcriptional modification in the 1900–1974 nt segment of domain IV in 23S rRNA of *Escherichia coli*, a region which interacts with domain V in forming the three-dimensional structure of the peptidyl transferase center within the ribosome. A nucleoside constituent of M_r 258 (U*) which occurs at position 1915, within the highly modified oligonucleotide sequence 1911-ΨAACU*AΨ-1917, was characterized as 3-methylpseudouridine ($m^3\Psi$). The assignment was confirmed by chemical synthesis of $m^3\Psi$ and comparison with the natural nucleoside by liquid chromatography–mass spectrometry. 3-Methylpseudouridine is previously unknown in nature and is the only known derivative of the common modified nucleoside pseudouridine thus far found in bacterial rRNA.

INTRODUCTION

Knowledge of the structures and functions of post-transcriptional modifications in ribosomal RNA is, in a phylogenetic sense, very limited. Twenty eight different modified nucleosides are presently known in rRNA (1), with most detailed work having been carried out on 16S and 23S rRNA from *Escherichia coli* (citations summarized in 2–4) and 28S rRNA from yeast and man (5). In contrast, 79 modifications are known in tRNA from a wider range of organisms, for which functional roles have been dealt with in much greater detail (6–9). In the case of rRNA the lower level of current structural information is due in part to experimental difficulties associated with assignment of correct identities of modification (as opposed to sites of modification) in large RNAs. The concept that post-transcriptionally modified nucleosides in RNA are functionally important (10) is based, in part, on their location in nucleotide sequences that are very highly conserved, single stranded and surface exposed. In 23S rRNA in particular the importance of accurate structural characterization of modifications as a prerequisite to the ultimate understanding of the higher order structure of rRNA and its function in translation (11) is especially strong for domains IV (nt 1656–2004) and V (nt 2043–2625). Phylogenetic evidence, based on comparative sequence analysis, indicates that domain IV (and domain II) interacts with domain V in forming the three-dimensional structure of the peptidyl transferase center within the ribosome (12). This interaction is supported by chemical footprinting experiments, which have shown that specific bases in domain IV are protected from chemical modification by both A-site (C-1941) and P-site (A-1916 and A-1918) bound tRNA (13). Cross-linking experiments have revealed tertiary interactions within single-stranded regions of domain IV (nt 1911–1921 and 1964) (14) and between single-stranded regions of domains IV (nt 1777–1792) and V (nt 2584–2588) (15). Furthermore, inter-RNA cross-linking experiments identify the same region of domain IV (nt 1912–1920) with two regions of 16S rRNA (nt 1408–1411 and 1518–1520; 16), implying that these regions are exposed at the interface between ribosomal subunits.

General methods based on mass spectrometry (2,4) that facilitate the characterization of post-transcriptionally-modified nucleosides at the primary structure level have been developed and recently applied to the identification of modifications in the central loop of domain V from *E.coli* 23S rRNA (4). Here we describe the application of these techniques to the characterization of post-transcriptionally-modified nucleosides found in the1900–1974 nt region of domain IV in 23S rRNA, resulting in identification at position 1915 of 3-methylpseudouridine (Fig. 1), $m^3\Psi$ (symbols and nomenclature used for modified nucleosides are taken from 1), a nucleoside not previously known in nature.

MATERIALS AND METHODS

Authentic 2′-*O*-methylpseudouridine (Ψm) was a gift from Prof. M.J.Robins (Brigham Young University, Provo, UT).

Synthesis of 3-methylpseudouridine (3-methyl-5-β-D-ribofuranosyluracil)

Pseudouridine (Ψ, 2.44 mg, 10 μmol; Sigma) was dissolved in 2 ml anhydrous methanol. Trimethylsilyldiazomethane (2 M solution in a mixture of hexanes; Aldrich) was added in 25 μl

* To whom correspondence should be addressed at: 311A Skaggs Hall, University of Utah, Salt Lake City, UT 84112, USA

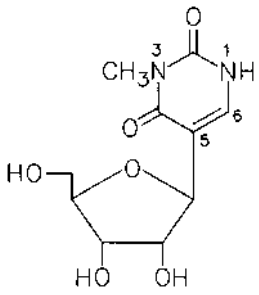

Figure 1. Structure of 3-methylpseudouridine.

aliquots five times (125 µl total) over a period of 3 h, while stirring at room temperature (17,18). The resulting clear colorless solution was analyzed by reversed phase HPLC–mass spectrometry (LC/MS) (19), showing a product ~95% pure: HPLC t_R, 11.9 min without internal reference; A_{254}/A_{280} ratio by HPLC, 2.34; λ_{max} (H_2O, pH ~6), 261 nm, λ_{max} (pH 13), 283 nm; molecular mass, 258 (MH^+, *m/z* 259), corresponding to an isomer of monomethylated uridine. The main product was collected and dried using a SpeedVac vacuum centrifuge. The reaction yield of $m^3\Psi$ was estimated by UV absorbance measurement to be 40%. The preparation of $m^3\Psi$ has been previously reported (20) as one of several products formed by treatment of pseudouridine with diazomethane, but due to the stated (20) tentative nature of the structure assignment, care was taken in the present study to ensure placement of the methyl group in the synthetic product at N-3, rather than N-1 or in ribose, by mass spectrometry. The collision-induced dissociation mass spectrum of the protonated synthetic molecule (MH^+, 259) produced by fast atom bombardment ionization of the synthetic product was determined using a VG-70SEQ tandem mass spectrometer. Dissociation of the uracil ring occurs by a retro-Diels Alder type reaction in which N(3), C(4) and O^4 are expelled as HNCO (N-3-unsubstituted) or CH_3NCO (N-3 methylated) (21). The common fragment ion (base + CH_2)$^+$, consisting of the base moiety plus C-1′ (22), was used to monitor the N(3),C(4),O^4 loss reaction, which occurs in Ψ as $125^+ \rightarrow 82^+$. In the mass spectrum of synthetic $m^3\Psi$ this process was observed as loss of 57 mass units (CH_3NCO), 139^+ (65% rel. int.)$\rightarrow 82^+$ (72% rel. int.), confirming methylation of Ψ at N-3. An ion corresponding to loss of HNCO was observed (*m/z* 96, 11% rel. int.), in close accord with studies of the dissociation of isotopically labeled uracil, in which N(1),C(2),O^2 is lost in an alternate minor pathway (21).

Isolation of rRNA

50S ribosomal subunits were prepared from frozen *E.coli* MRE 600 cells (1/2 log phase; Grain Processing Corp., Muscatine, IA) (23). Ribosomal RNA (23S and 5S) was isolated from 50S subunits by extraction with phenol and chloroform (23). 23S rRNA was separated from 5S rRNA by precipitation of the former in the presence of 2 M LiCl. Purity of the 23S rRNA isolate was assessed by gel electrophoresis (1% agarose, 3.5× 50 mm tube) using an Applied Biosystems Model 230A Micro-Preparative Electrophoresis System.

Synthesis of complementary DNA and preparation of rRNA fragments by nuclease protection of an RNA:DNA heteroduplex

A deoxyoligonucleotide complementary to the *E.coli* 23S rRNA sequence from positions 1900 to 1974 (75mer) was synthesized on a 1.0 µmol scale using an Applied Biosystems Model 380B DNA Synthesizer at the University of Utah Protein/DNA Core Facility. The DNA 75mer was purified by a DEAE anion exchange HPLC method, as described (4). Following protection of 10 nmol rRNA (residues 1900–1974) by the DNA 75mer unprotected RNA was digested with mung bean nuclease and the RNA 75mer was isolated as previously detailed (4).

Enzymatic hydrolysis of rRNA

An aliquot of the RNA 75mer (0.3 nmol) was completely hydrolyzed to nucleosides using nuclease P1 (Gibco BRL), venom phosphodiesterase (Sigma) and alkaline phosphatase (Calbiochem) (24). The remaining portion of the RNA 75mer (2.7 nmol) was digested using RNase T_1 (2000 U; Ambion) for 30 min at 37°C.

High performance liquid chromatography

Anion exchange chromatography (DEAE) as previously described (4) was used for purification of the synthetic DNA 75mer, the resulting RNA 75mer and the oligonucleotides resulting from complete RNase T_1 hydrolysis of the RNA 75mer. The RNA oligonucleotide fractions were further purified prior to mass spectrometry by reversed phase HPLC with a Supelcosil LC-18S column (4.6 × 250 mm) and pre-column (4.6 × 20 mm) using gradient conditions as described (2).

Directly combined HPLC–mass spectrometry

Analysis of nucleosides in enzymatic digests was carried out using a combined HPLC–mass spectrometer in which nucleosides separated by HPLC were passed through a UV detector and into a mass spectrometer, as earlier detailed (19,25). The mass spectrometer consists of a non-commercial quadrupole mass analyzer with thermospray interface (Vestec Corp, Houston, TX), controlled by a Vector/One data system (Teknivent Corp., St Louis, MO). Reversed phase HPLC separation of nucleosides was carried out using a gradient elution system based on buffered NH_4OAc (19). Thermospray ionization mass spectra were acquired by scanning the mass range 110–350 every 1.7 s during the 35 min chromatographic separation. Identification of nucleosides was based principally on: (i) relative retention time, compared with values obtained from reference nucleosides acquired under standardized operating conditions (19); (ii) characteristic mass values for the protonated base and protonated molecule (MH^+). These values (19) for the nucleosides of interest in the present study are as follows [retention time in min (mass protonated base/mass MH^+)]: Ψ, 3.5 (base ion absent/245); $m^1\Psi$, 8.6 (base ion absent/259); Ψm, 11.9 (base ion absent/259); m^5C, 12.4 (126/258); m^5U, 15.4 (127/259); Um, 17.0 (113/259); m^3U, 17.1 (127/259). The procedures for interpretation of data for characterization of nucleosides in RNA hydrolysates have been described (19,25).

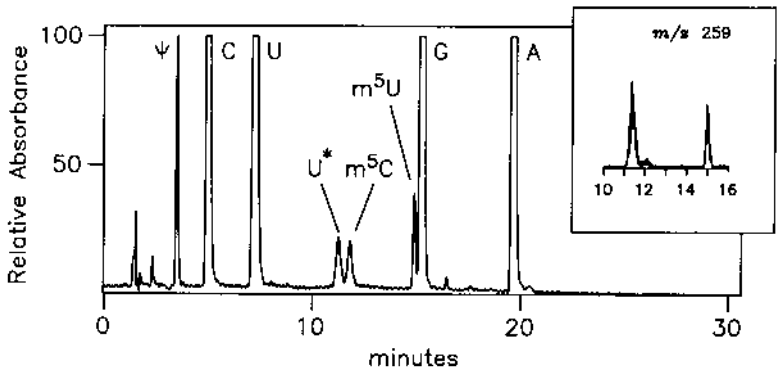

Figure 2. Chromatogram from LC/MS analysis of nucleosides from an enzymatic digest of 0.3 nmol 75mer fragment (nt 1900–1974) from *E.coli* 23S rRNA, showing unknown nucleoside U* eluting at 11.3 min. (Inset) Reconstructed ion chromatogram from the 10–16 min region, using mass 259 for selective detection of methyluridine isomers.

Electrospray mass spectrometry of isolated oligonucleotides

Oligonucleotides from RNase T_1 hydrolysis of the 75mer fragment from *E.coli* 23S rRNA were dissolved in H_2O and diluted with CH_3OH to 1:9 (v/v) to a final sample concentration of ~2.7 pmol/µl. This concentration is calculated assuming quantitative recovery from two chromatographies, which is unlikely; the value therefore represents an upper limit to the amount analyzed. Samples were continuously infused at a flow rate of 2.0 µl/min, using a Harvard model 22 syringe pump, into the electrospray ion source of a Sciex API III+ mass spectrometer (Thornhill, Ontario, Canada). Negative ion mass spectra were acquired, with the instrument operated with mass analyzer-3 in r.f.-only mode. Calibration of the mass scale was accomplished using synthetic oligodeoxynucleotides as mass reference standards (2).

RESULTS

An analysis of post-transcriptionally-modified nucleosides present in a total hydrolysate of *E.coli* 23S rRNA using combined LC/MS has recently been reported (4). During the course of this LC/MS analysis the presence of a previously unidentified ribonucleoside species of *m*/*z* 259 was detected both as a nucleoside and as a component of an adenosine-containing dinucleotide (4). We now describe the identification of this novel ribonucleoside, including confirmation by chemical synthesis, and demonstrate the sequence location of this nucleoside using a nuclease protection strategy (4) in combination with mass spectrometric methodologies developed in our laboratory (2).

Total nucleoside analysis of oligonucleotide fragment 1900–1974 from *E.coli* 23S rRNA

An RNA 75mer constituting nt 1900–1974 was prepared as described in Materials and Methods. The yield of the RNA 75mer (3.0 nmol) was determined by UV absorbance at 260 nm. An aliquot (10% of the sample) was hydrolyzed to its nucleoside substituents and the resultant hydrolysate was analyzed by LC/MS to give the chromatogram shown in Figure 2. In addition to the four major ribonucleosides, three post-transcriptionally-modified species of known structure were found: pseudouridine (Ψ, retention time t_R, 3.55 min), 5-methylcytidine (m^5C, t_R, 11.85 min) and 5-methyluridine (m^5U, t_R, 14.95 min). The detection of these modified nucleosides is consistent with the modifications known to occur in this region: Ψ-1911 and Ψ-1917 (26,27), m^5U-1939 (26,27) and m^5C-1962 (26,28). Their identification by LC/MS provides rigorous identification of the structure of each of the three nucleoside species. Interestingly, a fourth post-transcriptionally-modified species was detected, eluting 0.7 min before m^5C (see Fig. 2) and corresponding to a t_R of 11.7 min, referenced internally to the elution time of adenosine (19.9 min; 19). The mass spectrum recorded at this elution time consisted principally of an ion of *m*/*z* 259 (data not shown), which was interpreted as the protonated molecular ion (MH^+) of a monomethylated uridine derivative (M_r 258), corresponding to a nucleoside previously detected by LC/MS in a total digest of 23S rRNA (4). The absence of a corresponding base fragment ion in the mass spectrum is characteristic of C-nucleosides, e.g. Ψ and its derivatives (19), and suggests that the unidentified nucleoside is a methylated pseudouridine. The reconstructed ion chromatogram (RIC) shown (inset of Fig. 2) confirms that the ion of *m*/*z* 259 corresponds to the unknown nucleoside (after minor allowance for a 6 s offset due to the transit time between the UV detector and mass spectrometer). Other ions of *m*/*z* 259 shown in the RIC correspond to the ^{13}C isotope peak of m^5C (MH^+ 258) and MH^+ of m^5U at their characteristic retention times (19). Based on their chromatographic retention times (19), the following known methylated uridine derivatives were excluded as possible candidates; 1-methylpseudouridine ($m^1Ψ$), 3-methyluridine (m^3U), m^5U and 2′-*O*-methyluridine (Um). The latter three compounds had also been excluded as the identity of U*-1915 on the basis of HPLC data by Smith *et al.* (28).

Determination of base composition by electrospray mass spectrometry of RNase T_1 hydrolysis products from the region 1900–1974

The RNA 75mer (2.7 nmol) was enzymatically hydrolyzed with RNase T_1 and the resultant oligonucleotide mixture was fractionated using DEAE anion exchange HPLC (data not shown). The oligonucleotide fractions were dried in a vacuum centrifuge, dissolved in H_2O and re-chromatographed by reversed phase HPLC (data not shown) in order to facilitate subsequent analysis by electrospray mass spectrometry. Three modification-contain-

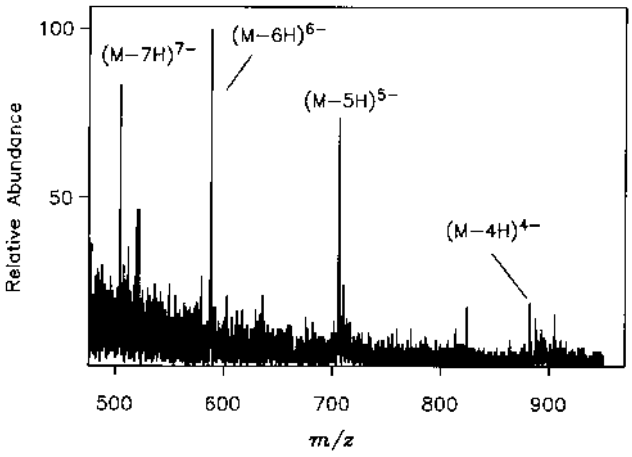

Figure 3. Electrospray mass spectrum of the oligoribonucleotide 11mer (nt 1911–1921) produced by RNase T_1 hydrolysis of the 75mer fragment (nt 1900–1974).

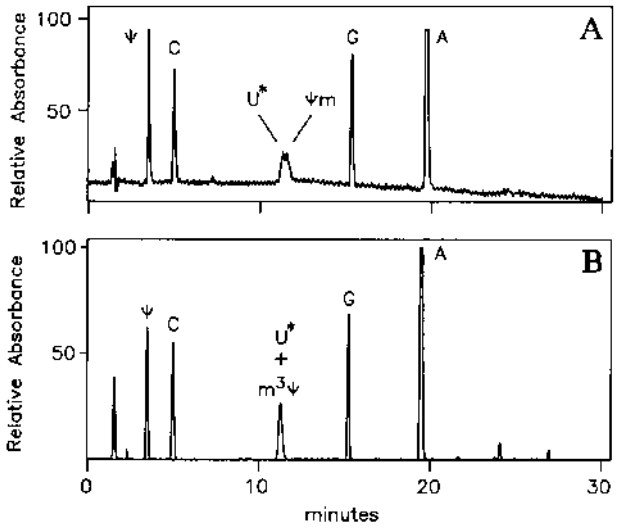

Figure 4. Chromatograms from LC/MS analysis of nucleosides from enzymatic digests of 0.27 nmol oligoribonucleotide 11mer (nt 1911–1921) co-injected with (**A**) authentic 2′-*O*-methylpseudouridine or (**B**) 3-methylpseudouridine.

ing oligonucleotides (listed in Table 1) were identified based (29) on accurate molecular mass measurement. The presence of post-transcriptional modification is revealed by incremental increases in mass (e.g. net increase of +14 Da for CH_3) not predicted by the rDNA sequence (2). For example, the mass spectrum presented in Figure 3 shows four multiply-charged ions (at *m/z* 504.0, 588.1, 705.9 and 882.6), corresponding to the loss of seven to four protons from the neutral oligonucleotide, and from which the M_r value was derived as 3534.67. Based on the experimentally determined molecular mass of this oligonucleotide, the base composition was determined (29) to be $C_2U_3A_5G{>}p + CH_2$ (>p denotes a 2′,3′-cyclic phosphate) (calculated M_r 3534.16). Similarly, solely from accurate molecular weight measurement, the base composition and net modification of the two other oligonucleotides (see Table 1) were determined to be $C_2UAGp + CH_2$ and $C_2U_4A_3Gp + CH_2$ respectively.

Table 1. Compositions of modification-containing oligonucleotides from RNase T_1 hydrolysis of a 75mer (nt 1900–1974) from *E.coli* 23S rRNA

Nucleotides	M_r	Composition
1960–1964	Measured 1622.70	$C_2UAGp + CH_2$
	Calculated 1622.99	
1911–1921	Measured 3534.67	$C_2U_3A_5G{>}p + CH_2$
	Calculated 3534.16	
1936–1945	Measured 3199.95	$C_2U_4A_3Gp + CH_2$
	Calculated 3199.92	

Total nucleoside analysis of oligonucleotide M_r 3534.67

An aliquot (0.27 nmol) of the 11mer ($C_2U_3A_5G{>}p + CH_2$) was enzymatically hydrolyzed to its nucleoside substituents and analyzed by LC/MS. The resultant chromatogram (data not shown) revealed that this oligonucleotide was composed of Ψ, C, G, A and the unidentified methylated uridine derivative, eluting at the same position shown for U* in Figure 2. Enzymatic conversion of U→Ψ is the only post-transcriptional modification not manifested by a concomitant change in molecular mass and so the presence of Ψ in the 11mer is not reflected in the composition determined by oligonucleotide mass measurement (Table 1). Therefore, the U_3 component of the 11mer established by molecular mass measurement is shown by LC/MS to be composed of Ψ and U*, with no unmodified U.

Two additional aliquots (0.27 nmol each) of the oligonucleotide 11mer were enzymatically hydrolyzed for further LC/MS analysis. In consecutive analyses one aliquot was co-injected with authentic Ψm while the other aliquot was co-injected with authentic $m^3Ψ$. The resultant chromatograms are presented in Figure 4. Partial chromatographic resolution of U* from authentic Ψm demonstrates only that the modified residue is not Ψm (Fig. 4A). The elution time of the isomer $m^1Ψ$ is significantly earlier when using the same gradient system, 8.6 min (19). Retention times of other monomethyl uridines are: m^3U, 17.1 min; m^5U, 15.4 min; Um, 17.0 min (19). Co-elution of U* with authentic $m^3Ψ$ identifies the modified nucleoside as $m^3Ψ$ (Fig. 4B). A_{254}/A_{280} absorbance ratios by HPLC: U*, 2.2 ± 0.2; synthetic $m^3Ψ$, 2.34.

Total nucleoside analysis of oligonucleotide M_r 3199.95

An aliquot (0.27 nmol) of the 10mer ($C_2U_4A_3Gp + CH_2$) was enzymatically hydrolyzed to its nucleoside substituents and analyzed by LC/MS to verify the base composition determined directly from molecular mass measurement. The resultant chromatogram (shown in Fig. 5) revealed that the modified residue was m^5U. The composition of this oligonucleotide species was found to be $C_{2.04}U_{2.77}G_{1.00}A_{2.84} + m^5U$, based on comparison of molar response ratios from UV detection compared with authentic standards (except for m^5U). These results are consistent with earlier work in which the nucleotide sequence was deduced as $AAAm^5UUCCUUG$ (27,30), confirmed by the

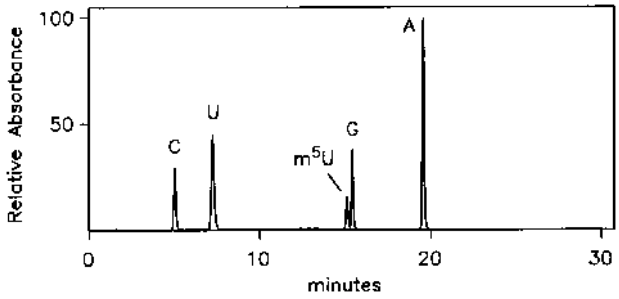

Figure 5. Chromatogram from LC/MS analysis of nucleosides from an enzymatic digest of 0.27 nmol oligoribonucleotide 10mer (nt 1936–1945).

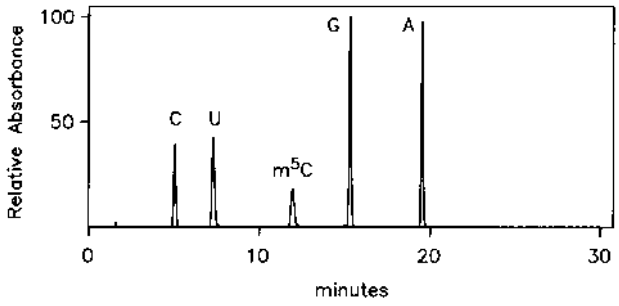

Figure 6. Chromatogram from LC/MS analysis of nucleosides of an enzymatic digest of 0.27 nmol oligoribonucleotide 5mer (nt 1960–1964).

corresponding gene sequence (31) to represent 23S rRNA nt 1936–1945.

Total nucleoside analysis of oligonucleotide M_r 1622.70

An aliquot (0.27 nmol) of the 5mer (C_2UAGp + CH_2) was enzymatically hydrolyzed to its nucleoside substituents and analyzed by LC/MS to verify the base composition determined directly from molecular mass measurement. The resultant chromatogram (shown in Fig. 6) revealed that the modified residue was m^5C. The composition of this oligonucleotide species was measured as $C_{1.06}U_{0.99}G_{1.00}A_{1.04}$ + m^5C based on comparison of molar response ratios from UV detection compared with authentic standards (except for m^5C). These data, with the corresponding gene sequence (31), corroborate the structure of the oligonucleotide as 1960-ACm^5CUG-1964, reflecting identification of position 1962 as m^5C (28) and correction (28) of earlier incorrect sequences (26,30) which had been determined prior to availability of the gene sequence.

DISCUSSION

The case for establishment of a complete modification map of 23S rRNA is 3-fold: first, accumulating evidence which demonstrates a role for 23S rRNA in translation (11,32); second, conceptual arguments that modified nucleosides are functionally important (10), principally as borne out by studies of tRNA (6,8); third, the striking fact that nearly all modified nucleosides in 16S and 23S rRNA are concentrated near the functional center of the ribosome (33,34). Correct assignment of positions and, especially, identities of post-transcriptional modifications in rRNA has been problematical (discussed in 1). As a consequence, in spite of the availability of a large number of rRNA gene sequences (see for example 35), not one fully processed 23S RNA sequence is known at the RNA level and only one 16S structure is probably complete, with the recent placement of Ψ-516 in *E.coli* (3).

Structural characterization of modified nucleosides is particularly difficult if, as in the present case, the nucleoside is previously unknown. Mass spectrometry has evolved as a particularly important approach in such work (36), because of its relatively high sensitivity to subtle structural features and the advantages that accrue from direct coupling to HPLC. The fact that U-1915 was probably a methylated uridine derivative was known from earlier work demonstrating incorporation of [^{14}C]methyl groups (26,27; cf. discussion in 28). Furthermore, treatment of synthetic 23S RNA transcripts with a partially purified enzyme fraction from *E.coli* resulted in Ψ-1915 formation (footnote 2 in 37). Thus, from earlier work there was reason to believe that U*-1915 was a modified Ψ which contained a post-transcriptionally added carbon atom derived from a methyl group. Reverse transcription was strongly inhibited at position 1915 (34), indicative of a uracil substitution at a position inhibitory to normal base pairing. In principle, the N(1)-O^2 group would still be available for hydrogen bond pairing. However, the observed reverse transcriptase stop is consistent with recent NMR results (38) showing that N(1)-H of Ψ is involved in hydrogen bonding to the phosphate backbone. Thus the normal *anti* conformation of in a Ψ–A base pair would be unable to switch to the required *syn* conformation. Interestingly, inhibition of reverse transcription serves to distinguish $m^3Ψ$ from the ribose methylated isomer Ψm, a pair which is otherwise differentiated by ~0.25 min difference in HPLC retention time (Fig. 4A). As in the present case, mass spectrometry distinguishes derivatives of U and Ψ by the clear absence of glycosidic bond cleavage in the latter, due to greater strength of the C–C (versus C–N) bond (19,25).

Although pseudouridine itself is widely distributed in rRNA (39), 3-methylpseudouridine is the only derivative of Ψ thus far found in bacterial rRNA (1). Eukaryotic rRNA additionally possesses $m^1Ψ$ (40), Ψm (41,42) and the hypermodified derivative $m^1acp^3Ψ$ (43,44). It is worth noting that *Bacillus subtilis* and *Zea mays* chloroplast LSU rRNA show a strong reverse transcriptase stop at the position equivalent to $m^3Ψ$-1915 (39).

The functional role of $m^3Ψ$ is unknown, even though it occurs in a very highly conserved section of domain IV which is structurally remarkable in containing two pseudouridines, Ψ-1911 and Ψ-1917 (26,27), as shown in Figure 7. Pseudouridine has been speculated to play a role in the peptidyl transferase reaction (45,46) and more recently has been demonstrated by NMR to confer significantly increased stabilization to A-form helical structure through enhanced base stacking and an increased population of the C3′-endo conformer (compared with U) (38). As pointed out (38), these modifications also provide a means for control of regional hydrophobicity in RNA loops, by a decrease (U→Ψ) or increase (Ψ→$m^3Ψ$ or $m^1Ψ$) in hydrophobic character of the base.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health grant GM29812. The University of Utah Protein/DNA Core Facility is supported by grant 5P30CA42014 from the National Cancer

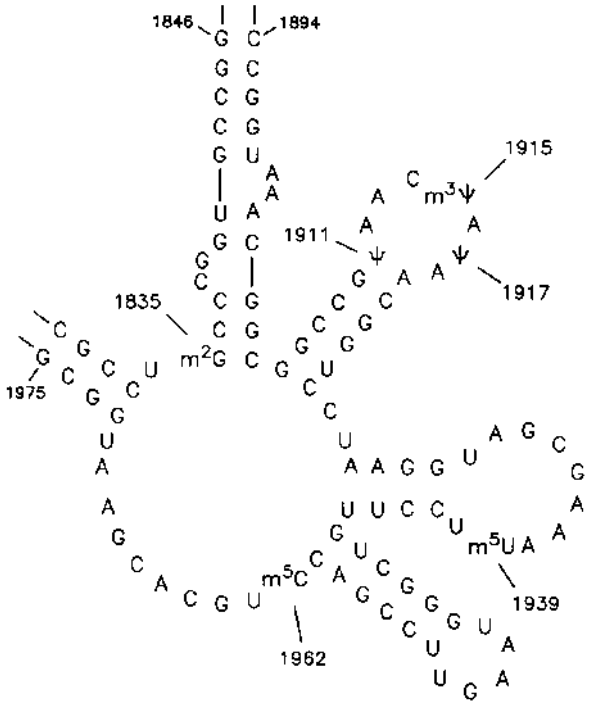

Figure 7. Secondary structure model of the highly conserved central loop region of domain IV of *E.coli* 23S rRNA, showing $m^3\Psi$-1915 (see text) and other known sites of post-transcriptional modification: m^2G-1825 (26), Ψ-1911 and Ψ-1917 (26,27), m^5U (26,27) and m^5C-1962 (26,28).

Institute. The authors are indebted to Robin Gutell for helpful discussions and to Morris J.Robins for a generous gift of 2′-*O*-methylpseudouridine.

REFERENCES

1 Limbach,P.A., Crain,P.F. and McCloskey,J.A. (1994) *Nucleic Acids Res.*, **22**, 2183–2196.
2 Kowalak,J.A., Pomerantz,S.C., Crain,P.F. and McCloskey,J.A. (1993) *Nucleic Acids Res.*, **21**, 4577–4585.
3 Bakin,A., Kowalak,J.A., McCloskey,J.A. and Ofengand,J. (1994) *Nucleic Acids Res.*, **22**, 3681–3684.
4 Kowalak,J.A., Bruenger,E. and McCloskey,J.A. (1995) *J. Biol. Chem.*, **270**, 17758–17764.
5 Maden,B.E.H. (1990) *Prog. Nucleic Acids Res. Mol. Biol.*, **39**, 241–303.
6 Björk,G., Ericson,J.U., Gustafsson,C.E.D., Hagervall,T.G., Jönsson,Y.H. and Wikström,P.M. (1987) *Annu. Rev. Biochem.*, **56**, 263–287.
7 Björk,G.R. (1992) In Hatfield,D.L., Lee,B.J. and Pirtle,R.M. (eds), *Transfer RNA in Protein Synthesis.* CRC Press, Boca Raton, FL, pp. 23–85.
8 Björk,G.R. (1995) In Söll,D. and RajBhandary,U.L. (eds), *tRNA: Structure, Biosynthesis, and Function.* American Society for Microbiology, Washington, DC, pp. 165–205.
9 Yokoyama,S. and Nishimura,S. (1995) In Söll,D. and RajBhandary,U.L. (eds), *tRNA: Structure, Biosynthesis, and Function.* American Society for Microbiology, Washington, DC, pp. 207–223.
10 Noller,H.F. and Woese,C.R. (1981) *Science*, **212**, 403–411.
11 Noller,H.F. (1991) *Annu. Rev. Biochem.*, **60**, 191–227.
12 Gutell,R.R., Larsen,N. and Woese,C.R. (1994) *Microbiol. Rev.*, **58**, 10–26.
13 Moazed,D. and Noller,H.F. (1989) *Cell*, **57**, 585–597.
14 Döring,T., Greuer,B. and Brimacombe,R. (1990) *Nucleic Acids Res.*, **19**, 3517–3524.
15 Stiege,W., Glotz,C. and Brimacombe,R. (1983) *Nucleic Acids Res.*, **11**, 1687–1706.
16 Mitchell,P., Osswald,M. and Brimacombe,R. (1992) *Biochemistry*, **31**, 3004–3011.
17 Aoyama,T. and Shioiri,T. (1990) *Tetrahedron Lett.*, **31**, 5507–5580.
18 Hashizume,T. and McCloskey,J.A. (1992) *Abstracts for the 10th International Roundtable Nucleosides, Nucleotides and their Biological Applications*, Park City, UT, p. 33.
19 Pomerantz,S.C. and McCloskey,J.A. (1990) *Methods Enzymol.*, **193**, 796–824.
20 Cohn,W.E. (1960) *J. Biol. Chem.*, **235**, 1488–1498.
21 Nelson,C.C. and McCloskey,J.A. (1994) *J. Am. Soc. Mass Spectrom.*, **4**, 339–349.
22 Shaw,S.J., Desiderio,D.M., Tsuboyama,K. and McCloskey,J.A. (1970) *J. Am. Chem. Soc.*, **92**, 2510–2522.
23 Moazad,D., Van Stolk,B.J., Douthwaite,S. and Noller,H.F. (1986) *J. Mol. Biol.*, **191**, 483–493.
24 Crain,P.F. (1990) *Methods Enzymol.*, **193**, 782–790.
25 Edmonds.C.G., Vestal,M.L. and McCloskey,J.A. (1985) *Nucleic Acids Res.*, **13**, 8197–8206.
26 Fellner,P. and Sanger,F. (1968) *Nature*, **219**, 236–238.
27 Branlant,C., Sri Widada,J., Krol,A., Fellner,P. and Ebel,J.P. (1975) *Biochimie*, **57**, 175–225.
28 Smith,J.E., Cooperman,B.S. and Mitchell,P. (1992) *Biochemistry*, **31**, 10825–10834.
29 Pomerantz,S.C., Kowalak,J.A. and McCloskey,J.A. (1993) *J. Am. Soc. Mass Spectrom.*, **4**, 204–209.
30 Sri Wadada,J., Branlant,C. and Ebel,J.-P. (1979) *Biochimie*, **61**, 869–876.
31 Brosius,J., Dull,T.J. and Noller,H.F. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 201–204.
32 Samaha,R.R., Green,R. and Noller,H.F. (1995) *Nature*, **377**, 309–314.
33 Brimacombe,R., Mitchell,P., Osswald,M., Stade,K. and Bochkariov,D. (1993) *FASEB J.*, **7**, 161–167.
34 Bakin,A. and Ofengand,J. (1993) *Biochemistry*, **32**, 9754–9762.
35 Maidak,B.L., Larsen,N., McCaughey,J., Overbeck,R., Olsen,G.J., Fogel,K., Blandy,J. and Woese,C.R. (1994) *Nucleic Acids Res.*, **22**, 3485–3487.
36 Crain,P.F. and McCloskey,J.A. (1994) In Matsuo,T., Caprioli,R.M, Gross,M.L. and Seyama,Y. (eds), *Biological Mass Spectrometry: Present and Future*. John Wiley, New York, NY, pp. 509–537.
37 Bakin,A., Lane,B.G. and Ofengand,J. (1994) *Biochemisty*, **33**, 13475–13483.
38 Davis,D.R. (1995) *Nucleic Acids Res.*, **23**, 5020–5026.
39 Ofengand,J., Bakin,A., Wrzesinski,J., Nurse,K. and Lane,B.G. (1995) *Biochem. Cell Biol.*, **73**, in press.
40 Brand,R.C., Klootwijk,J., Planta,R.J. and Maden,B.E.H. (1978) *Biochem. J.*, **169**, 71–77.
41 Gray,M.W. and Keddy,G.M. (1974) *Biochemistry*, **13**, 5453–5463.
42 Maden,B.E.H. and Salim,M. (1974) *J. Mol. Biol.*, **88**, 133–164.
43 Saponara,A.G. and Enger,M.D. (1974) *Biochim. Biophys. Acta*, **349**, 61–77.
44 Maden,B.E.H., Forbes,J., de Jonge,P. and Klootwijk,J. (1975) *FEBS Lett.*, **59**, 60–63.
45 Lane,B.G., Ofengand,J. and Gray,M.W. (1992) *FEBS Lett.*, **302**, 1–4.
46 Lane,B.G., Ofengand,J. and Gray,M.W. (1995) *Biochimie*, **77**, 7–15.