Number, position, and significance of the pseudouridines in the large subunit ribosomal RNA of *Haloarcula marismortui* and *Deinococcus radiodurans*

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ABSTRACT

The number and position of the pseudouridines of *Haloarcula marismortui* and *Deinococcus radiodurans* large subunit RNA have been determined by a combination of total nucleoside analysis by HPLC-mass spectrometry and pseudouridine sequencing by the reverse transcriptase method and by LC/MS/MS. Three pseudouridines were found in *H. marismortui*, located at positions 1956, 1958, and 2621 corresponding to *Escherichia coli* positions 1915, 1917, and 2586, respectively. The three pseudouridines are all in locations found in other organisms. Previous reports of a larger number of pseudouridines in this organism were incorrect. Three pseudouridines and one 3-methyl pseudouridine ($m^3\Psi$) were found in *D. radiodurans* 23S RNA at positions 1894, 1898 ($m^3\Psi$), 1900, and 2584, the $m^3\Psi$ site being determined by a novel application of mass spectrometry. These positions correspond to *E. coli* positions 1911, 1915, 1917, and 2605, which are also pseudouridines in *E. coli* (1915 is $m^3\Psi$). The pseudouridines in the helix 69 loop, residues 1911, 1915, and 1917, are in positions highly conserved among all phyla. Pseudouridine 2584 in *D. radiodurans* is conserved in eubacteria and a chloroplast but is not found in archaea or eukaryotes, whereas pseudouridine 2621 in *H. marismortui* is more conserved in eukaryotes and is not found in eubacteria. All the pseudouridines are near, but not exactly at, nucleotides directly involved in various aspects of ribosome function. In addition, two *D. radiodurans* Ψ synthases responsible for the four Ψ were identified.

Keywords: pseudouridine sequencing; *H. marismortui*; *D. radiodurans*; mass spectrometry sequencing; pseudouridine synthases

INTRODUCTION

Pseudouridine (Ψ) (Cohn 1960) is the most common modified nucleoside found in RNA (Rozenski et al. 1999). Although it was first identified in a precise position in the T Ψ CG sequence in tRNA (Zamir et al. 1965) and later at other sites (Sprinzl and Vassilenko 2002), many more Ψ residues are to be found in ribosomal RNA, both in the large and small rRNAs (Maden 1990, Ofengand and Bakin 1997; Ofengand et al. 2001b; Ofengand 2002). The determination of the sites of the Ψ residues in large ribosomal subunit (LSU) RNAs of a number of representative species

has revealed a remarkable conservation of location, although not necessarily of exact position. Ψ in small ribosomal subunits do not appear to specifically localize to known functional sites except for *Escherichia coli*, whose single Ψ is near the decoding site (Ofengand et al. 2001b; Ofengand 2002). In *E. coli*, the LSU Ψ cluster in and around the peptidyl transferase center (PTC), here loosely defined as that region that binds the amino acyl ends of tRNAs in the A, P, and E sites. There is also a set of three in the terminal loop of helix 69. This stem-loop is thought to interact with A and P site bound tRNAs (Yusupov et al. 2001). Despite this juxtaposition to functional regions of the LSU, the significance of the locations of Ψ has remained obscure.

Localization of the Ψ residues in a known three-dimensional ribosome structure could be expected to help define Ψ function. Unfortunately, those organisms whose ribosomal Ψ have been mapped (Ofengand and Bakin 1997) have not had their ribosome structures determined, and the

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two organisms whose LSU structure is known, *Haloarcula marismortui* (Ban et al. 2000) and *Deinococcus radiodurans* (Harms et al. 2001), have not had their rRNA mapped for Ψ . This study was initiated to remedy this lack of knowledge. Here we report the Ψ content and sites for the 23S RNA of *H. marismortui* and *D. radiodurans*.

RESULTS

Determination of the total number of Ψ

As a prelude to, and a reality check on, mapping the Ψ sites by sequencing, the total number of Ψ in the ribosomal RNAs of *D. radiodurans* and *H. marismortui* were determined (Table 1). The methodology was checked by analysis of *E. coli* rRNA, whose number of Ψ sites is known from complete sequence analysis (Ofengand 2002). As shown in the table, values close to expected were obtained. Earlier analysis of the accuracy of the total digestion method (Gehrke and Kuo 1990) in our hands suggested errors of $\pm 15\%$ of the number of modified residues measured (Noon et al. 1998). The slight discrepancy in the 23S RNA value could thus be due to measurement error and/or $<100\%$ modification at one or more of the known sites. There was no detectable Ψ in *H. marismortui* 16S RNA but three Ψ were found in the 23S RNA. This value agrees with the sequence analysis (see Fig. 1), indicating that virtually complete modification had occurred at each site. There was no detectable Ψ in *D. radiodurans* 16S RNA whereas the 23S RNA had a measured net value of 2.0 residues. Since three Ψ sites were clearly identified by sequencing (see below), it appears that in this case the average modification level of the Ψ sites was only 67%.

TABLE 1. Total number of pseudouridines in *H. marismortui* and *D. radiodurans* ribosomal RNA

	16S RNA	23S RNA
<i>E. coli</i>	1.1 (1)	8.1 (9 ^a)
<i>H. marismortui</i>	<0.1	3.1
<i>D. radiodurans</i>	<0.1	2.0

Analysis was done as described in Materials and Methods. Values in parentheses are those expected for *E. coli* based on sequencing analysis (Ofengand 2002).

^aNot including 3-methyl Ψ .

FIGURE 1. Ψ sequencing analysis of 23S RNA from *D. radiodurans* (A) and *H. marismortui* (B). RNA was treated with (+) or without (−) CMC followed by treatment with alkali (hr OH[−]) for 4 or 6 h, and then used as template for primer extension with reverse transcriptase (Ofengand et al. 2001a). In this assay, a CMC-dependent reverse transcription stop one base 3' of a uridine (indicated by arrows) identifies a Ψ . The position of each Ψ is numbered and *E. coli* equivalents are given in parentheses. RNA sequence is shown in A, C, G, and U lanes. (*) Putative m³ Ψ . The autoradiographs shown are the only Ψ found in two comprehensive analyses covering residues 1–2852 (99%) of *D. radiodurans* and residues 1–595, 633–2887 (98%) of *H. marismortui* 23S RNA. The stop in all lanes in B corresponding to m³U2619 is indicated.

Mapping the location of Ψ in *D. radiodurans* and *H. marismortui* 23S RNA

To precisely locate the positions of 23S RNA Ψ , total RNA from *D. radiodurans* and *H. marismortui* was isolated and subjected to Ψ sequencing. Comprehensive analysis of 99% of the *D. radiodurans* 23S RNA sequence located three Ψ at positions U1894, U1900, and U2584 (Fig. 1A); the CMC-independent stop at U1898 will be addressed below. Similarly, three Ψ were located in *H. marismortui* 23S RNA at positions U1956, U1958, and U2621 (Fig. 1B). This total number of Ψ sites is consistent with the amount obtained by RNase digestion and HPLC analysis (Table 1).

Other modifications

Methylation or other modification that blocks base-pair formation has the effect of resulting in a pause or stop of reverse transcription, resulting in a band across all lanes of a sequencing gel. We could confirm the finding by Hansen et al. (2002) of a strong reverse transcriptase stop at U2619 (2584) as shown in Figure 1B. We also observed a very strong stop in several independent preparations of *H. marismortui* 23S RNA at A629 with stuttering at A628 (data not shown). These results are consistent with the finding of m³U2619 and m¹A628 by X-ray crystallography (Klein et al. 2004). The RNAs from both *D. radiodurans* and *H. marismortui* were more fragmented than typically found in other organisms (Ofengand and Bakin 1997). Therefore, it was not possible to assign the additional bands that occurred

across all lanes as sites of putative modification with any degree of certainty.

Identification and location of 3-methylpseudouridine ($m^3\Psi$) in *D. radiodurans* 23S RNA

Any Ψ with an additional modification (such as a methyl group) that interferes with base pairing would produce a CMC-independent stop of reverse transcriptase and thus could not be confirmed as Ψ since the assay is based on the CMC dependence of reverse transcriptase blockade. This is the case for Ψ 1915 in *E. coli*, which was shown to be $m^3\Psi$ (Kowalak et al. 1996). In that case, a CMC-independent stop for Ψ 1915 appeared between the CMC-dependent stops for Ψ 1911 and Ψ 1917. However, Ψ 1915 was produced upon reaction of a 23S RNA transcript with its purified Ψ synthase in vitro (Raychaudhuri et al. 1998). An identical pattern was observed for the equivalent residues in *D. radiodurans* 23S RNA. There is a single CMC-independent stop for U1898 between the CMC-dependent stops for Ψ 1894 and Ψ 1900 (Fig. 1A). This observation implied that U1898 might also be $m^3\Psi$. This was established by LC/MS analysis of a total rRNA nucleoside digest, after first determining the complete absence of this nucleoside in the 16S portion of the RNA. The diagnostic mass spectral peaks and exact HPLC elution times were directly compared in back-to-back analyses against *E. coli* 23S rRNA that contains $m^3\Psi$ (Kowalak et al. 1996). In both experiments the elution time of the putative $m^3\Psi$ component (11.2 min) was accurately matched against that of m^5C as an internal retention time marker, using m/z 259 (MH^+ for $m^3\Psi$ and the first isotope peak from MH^+ of m^5C) and m/z 223, the characteristic MH^+-2H_2O ion of any methylpseudouridine. Peaks for m/z 259 and m/z 223 from reconstructed ion chromatograms were co-incident in time as required for the $m^3\Psi$ nucleoside, and followed the m/z 259 peak for m^5C by 3.6 sec in both rRNA analyses (data not shown). This result confirmed the presence of $m^3\Psi$ in *D. radiodurans* 23S rRNA.

To determine if $m^3\Psi$ is present in *D. radiodurans* 23S RNA specifically at position 1898, an aliquot of the mixed 16S and 23S RNA was digested with RNase T1 and subjected to LC/MS/MS analysis in the negative ion mode for de novo sequencing (Ni et al. 1996) of the predicted oligonucleotide 1894- Ψ AAC $m^3\Psi$ A Ψ AACGp (Fig. 2). Ions were observed in the low mass region of the CID spectrum that are consistent with the presence of a methylpseudouridine residue. These included ions of m/z 417, 337, and 319, corresponding in mass to a methyluridine diphosphate, monophosphate, and its dehydration product, respectively (not annotated in Fig. 2). The presence of methylpseudouridine, and not a uridine methylated in the base or ribose, was deduced by the observation of an ion at m/z 221 (not annotated; the negative ion counterpart of 223^+ discussed above), which is consistent with the interpretation of $m\Psi-2H_2O$, the facile loss of water being a hallmark of pseudo-

FIGURE 2. Sequencing mass spectrum of RNase T1 fragment M_r 3550.5 from *D. radiodurans* LSU RNA showing $m^3\Psi$ at position 1898. The spectrum is represented in maximum entropy deconvolution (MaxEnt3) format.

uridine mass spectra (e.g., Felden et al. 1998). No ions characteristic of a base-methylated uridine (m/z 125), which would have been indicative of a normal N-C glycosidic bond, were observed. The methyl group was localized to position 1898 by the nearly complete series of w and y series sequencing ions listed in Table 2 (for sequence ion nomenclature see McLuckey et al. 1992). These ions are indicative of the sequence from the 3' terminus, whose interpretation necessitated the presence of a methyl group in the fifth nucleotide (corresponding to rRNA position 1898). The mass difference of 320 units between ions w_6 and w_7 (Table

TABLE 2. Sequence ions from the mass spectrum of *D. radiodurans* RNase T1 product M_r 3550.5^a (Fig. 2)

Assignment	Mass, calc.	Mass, found
y_1	362.050	362.061
y_2	667.091	667.105
y_3	996.144	996.174
y_4	1325.196	1325.222
y_5	1631.222	1631.217
y_6	1960.274	1960.292
y_7	2280.319	2280.343
y_8	2585.360	2585.466
y_9	2914.413	2914.712 ^b
y_{10}	3243.465	3243.473
w_1	442.016	442.099
w_2	747.058	747.066
w_3	1076.110	1076.135
w_4	1405.163	1405.186
w_5	1711.188	1711.247
w_6	2040.240	2040.407
w_7	2360.286	2360.286
w_8	2665.327	2665.341
w_9	2994.379	2994.102 ^b
w_{10}	3323.432	3323.408

^aPrecursor ion selected for dissociation was $(M-6H)^{6-}$, m/z 590.8.

^bAssignment compromised by poor signal-to-noise ratio.

2) corresponds to methyl-U/ Ψ . This was corroborated by a nearly complete pair of ion series (**a**-Base and **d**-H₂O; not annotated) (McLuckey et al. 1992) representing the sequence from the 5' terminus of the T1 fragment. Although a relatively abundant **y**₆ ion was seen, the expected enhancement of the complementary **a**₅ and **w**₆ ion pair was not observed. This may be a consequence of the difficulty in retaining two and three adenine residues, respectively, on the first-generation dissociation products (McLuckey and Habibi-Goudarzi 1993). Other less abundant ions that arise from multiple dissociation events resulting in scission through the ribose-phosphate backbone and that can be used in further support of the presence of a methyl group at position 1898 are listed in Table 3.

Sequence placement of Ψ at positions 1894 and 1900 was determined by the characteristically elevated responses of **w** and **y** series ions immediately 3' of the sites of pseudouridylation (S.C. Pomerantz and J.A. McCloskey, in prep.). The effect is particularly notable in the case of Ψ at the 5' terminus of the oligonucleotide (ions **w**₁₀ and **y**₁₀). The significant abundance of ion *m/z* 165 reflects the prominence of the dissociation reaction *m/z* 225 (ion **a**₁) → 165, also a characteristic effect of Ψ at the 5' position (S.C. Pomerantz and J.A. McCloskey, in prep.). The **a**₇ ion, the 5' complement to the **w**₄ ion, did not exhibit any enhanced abundance. This may be reflective of the difficulty in keeping three adenine bases attached to the **a**₇ fragment during fragmentation, as described above.

Sequence localization of the Ψ residues in *H. marismortui* 23S rRNA

LC/MS/MS screening of an RNase T1 digest of the LSU for Ψ by the multiple reaction monitoring method (data not shown) indicated two principal characteristic responses (*m/z* 207 → 164) for oligonucleotides that contained an internal Ψ . These oligonucleotides had measured *M_r* values of 1624.95 (5-mer) and 2573.45 (8-mer). The only possible placement of the pentanucleotide within the computer-predicted RNase T1 digest was 2619-UUUAGp, and necessitated the presence of a methyl group (*M_r* calc. 1624.97). This result was consistent with the reverse transcriptase based finding of 2619-m³U, as described above. LC/MS/MS sequencing of this oligonucleotide (from the (M-3H)³⁺ pre-

cursor ion, 60 eV collision *E_{lab}*; data not shown) confirmed the CMC-detected presence of Ψ at position 2621 by the enhanced **w**₂ (64% relative abundance [RA]) and **y**₂ (100% RA) ions, and the complementary **a**₃ ion present at 50% RA.

The only plausible sequence possibility for the 8-mer in the LSU RNA was 1952-UAACUAUGp (*M_r* calc. 2573.56), given the constraints (3' G terminus, due to use of RNase T1) and mass measurement accuracy of the experiment. This assignment placed the Ψ residue(s) within the structurally conserved helix 69 terminal loop. The LC/MS/MS sequencing spectrum (data not shown) of this oligonucleotide from the (M-4H)⁴⁺ precursor (80 eV, *E_{lab}*) did not reveal strong responses at *m/z* 225 and 165, nor was there an intense **w**₇ ion. These findings precluded the placement of a Ψ at the 5' terminus (position 1952). Relatively intense responses were observed for the **a**₅ (34% RA) and **a**₇ (35% RA) fragment, and their respective complementary ions **w**₃ (32% RA) and **w**₁ (72% RA). The **w**₁ ion was significantly elevated over the response observed in the *D. radiodurans* loop 69 oligonucleotide (6% RA). In toto, these data support the RT-based finding of Ψ at positions 1956 and 1958.

Two-dimensional location of *D. radiodurans* and *H. marismortui* Ψ

The Ψ positions in *D. radiodurans* and *H. marismortui* are indicated on secondary structure backbone diagrams of the 23S RNA of the two organisms in Figure 3, panels A and B, respectively. The sites identified in other organisms within the sequences shown are also indicated in the figure. Consistent with all bacteria surveyed so far except *E. coli*, Ψ in both organisms is only found in the 3' half of the RNA. As indicated in the figure, none of these Ψ positions are novel. *D. radiodurans* Ψ 1894, 1898, and 1900, located in helix 69, are equivalent to *E. coli* positions 1911, 1915, and 1917 and are the most conserved Ψ positions among all organisms' cytoplasmic ribosomes from all three phylogenetic kingdoms (Ofengand et al. 2001b). Ψ at position 2584 is equivalent to *E. coli* position 2605, which is also Ψ in *E. coli*, *Bacillus subtilis*, and *Zea mays* chloroplasts. This single Ψ is made by the Ψ synthase RluB in both *E. coli* (Del Campo et al. 2001) and *B. subtilis* (Niu et al. 1999). The Ψ of *H. marismortui* are also not novel. Ψ 1956 and 1958 are the aforementioned "most conserved Ψ " that appear in helix 69 and are equivalent to *E. coli* Ψ 1915 and 1917 except that Ψ 1956 is not methylated as it is in *E. coli*. The Ψ at 2621 is a position found in another archaeon, *Halobacter halobium*, as well as in *Drosophila melanogaster* and *Mus musculus* (Ofengand et al. 2001b).

Identification of RluB and RluD homologs in *D. radiodurans*

A total of seven Ψ synthase homologs have previously been identified in the genome of *D. radiodurans* (Ofengand et al.

TABLE 3. Sequence assignments for ions observed in support of 1898-m³ Ψ

<i>m/z</i>	Assignment
953.2	1896-ACm ³ Ψ > p or 1897-Cm ³ Ψ A > p
1221.2	1898-m ³ Ψ A Ψ A-OH
1282.2	1895-AACm ³ Ψ > p or 1896-ACm ³ Ψ A > p
1526.2	1894- Ψ AACm ³ Ψ or 1897-Cm ³ Ψ A Ψ A
1710.3	1898-pm ³ Ψ A Ψ AAp

FIGURE 3. Location of Ψ on secondary structures of 23S RNA from *D. radiodurans* (A) and *H. marismortui* (B). In each panel, the secondary structure of the 3' half of 23S RNA (adapted from the Comparative RNA site, <http://www.rna.icmb.utexas.edu>) is shown. The regions of the molecule that contain Ψ (bold backbone tracing) are shown in more detail to the right. (*) $m^3\Psi$. Numbering as in Figure 1. The locations of Ψ in other organisms are as indicated. (E) *E. coli*; (B) *B. subtilis*; (Z) *Z. mays* chloroplasts; (A) *H. halobium*; (S) *S. acidocaldarius*; (Y) *Saccharomyces cerevisiae*; (G) *Euglena gracilis*; (D) *D. melanogaster*; (M) *M. musculus*; (H) *H. sapiens* (Ofengand 2002; Russell et al. 2004; M.W. Gray, pers. commun.).

2001b; Kaya and Ofengand 2003), but only four belong to the families (RsuA and RluA) known to modify eubacterial rRNA. Among these four, it was expected that *D. radiodurans* had homologs to *E. coli* RluD and RluB since all four Ψ found in *D. radiodurans* 23S RNA are equivalent to *E. coli* Ψ made by these two Ψ synthases. To determine which *E. coli* synthases were most similar to the four *D. radiodurans* homologs, each *E. coli* synthase from the RsuA and RluA family was used in a BlastP search against the complete genomic sequence of *D. radiodurans* (Table 4). Consistent with previous analyses, only four *D. radiodurans* homologs were identified in this search: one RsuA family member and three RluA family members. The RsuA family member was

closest to *E. coli* RluB and RluF. This is not surprising because RluB and RluF are closely related to each other and target adjacent U residues (Del Campo et al. 2001). Hence, the putative protein gi|15805921| is an RluB homolog. Out of the three RluA family members, the putative protein gi|15806790| is clearly the most similar to *E. coli* RluD and is, therefore, an RluD homolog. For the last two RluA family members, the assignment is unclear. Putative protein gi|15806687| appears to be closest to *E. coli* RluA, RluC, and RluD, and putative protein gi|15805985| is closest to RluC, RluD, and TruC. The putative protein gi|15806687| likely modifies rRNA because it has an N-terminal S4 domain, a domain thus far found in Ψ synthases that modify rRNA (Del Campo et al. 2004). However, this protein does not appear to be an active synthase since it has a Gly (Gly152; GGG codon) in place of the conserved Asp (GAT or GAC codon) required by Ψ synthases for catalytic activity. Putative protein gi|15805985| has a conserved Asp and is likely to make Ψ in *D. radiodurans* tRNA because all of the rRNA Ψ s are accounted for by other putative synthase genes.

D. radiodurans has a bona fide RluB and RluD

After mapping the positions of *D. radiodurans* Ψ and identifying Ψ synthase homologs predicted to form them, the next logical step was to determine whether these Ψ synthase homologs were bona fide Ψ synthases and, if so, which Ψ they made. The most straightforward approach would have been to make gene deletions for the RluB and RluD homologs and to use Ψ sequencing to assess the disappearance of Ψ , as was done for *E. coli*. Despite the fact that many genetic tools are becoming available for *D. radiodurans*, making clean gene deletions is not as straightforward and routine as it is in *E. coli*. Thus, it seemed more practical to use an approach that took advantage of the *E. coli* Ψ synthase mutants already in our possession.

If the putative *D. radiodurans* RluB makes Ψ 2584 (*E. coli* Ψ 2605), then it might make Ψ 2605 when placed in an *E. coli* strain lacking RluB (Δ rluB). Similarly, if the putative *D. radiodurans* RluD makes Ψ 1894, 1898, and 1900, then *D. radiodurans* RluD might make Ψ 1911, 1915, and 1917 when placed in an *E. coli* strain lacking RluD (Δ rluD::kan). The *D. radiodurans* rluB and rluD homologs were each cloned into expression vector pTrc99A, transformed into the *E. coli* Δ rluB and Δ rluD::kan strains, respectively, and grown without induction of the cloned gene. Figure 4A shows that *D. radiodurans* RluD is a Ψ synthase able to form Ψ 1911 and 1917 in *E. coli* (the nature of 1915 was discussed above), and, therefore, should be the Ψ synthase responsible for Ψ 1894, 1898, and 1900 in *D. radiodurans*. Figure 4B shows that *D. radiodurans* RluB is a Ψ synthase able to form Ψ 2605 in *E. coli* and, therefore, should be the Ψ synthase responsible for Ψ 2584 in *D. radiodurans*.

TABLE 4. *D. radiodurans* homologs of *E. coli* RsuA and RluA family Ψ synthases

<i>E. coli</i> Ψ synthase ^a	<i>D. radiodurans</i> putative proteins			
	gil158059211 ^b	gil158067901	gil158066871	gil158059851
RsuA family				
RsuA	5c ^{-19c}	0.66	—	—
RluB	2e ⁻³¹	4.5	—	—
RluE	3e ⁻²³	2.3	2.3	—
RluF	1e ⁻²⁹	0.003	—	—
RluA family				
RluA	0.12	4e ⁻²⁶	3e ⁻²²	1e ⁻¹⁴
RluC	2.9	1e ⁻²⁸	5e ⁻²²	1e ⁻¹⁸
RluD	5.2	6e ⁻⁴⁹	7e ⁻²³	1e ⁻¹⁷
TruC	0.031	2e ⁻¹⁹	7e ⁻¹³	5e ⁻¹⁸

^aEach *E. coli* Ψ synthase from the RsuA and RluA families was used as a query in a BlastP search (low-complexity filtering = off; expect value ≤ 10) against only the *D. radiodurans* genomic database (<http://www.ncbi.nlm.nih.gov/BLAST>).

^bGenbank identification numbers.

^cExpect values; — indicates >10 .

DISCUSSION

Sequencing results

It was previously reported that there were at least 11 (Ofengand et al. 2001b) or 19 (Ofengand 2002) Ψ located at specific sites in *H. marismortui* 23S RNA. These reports were incorrect. We inadvertently failed to completely strip the CMC group from some of the U residues in these experiments, leading us to score those U sites as Ψ . We then compounded our error by failing to compare the stop intensity of those Ψ sites with other ones such as the Ψ 1915 and Ψ 1917 stops. Had we done so, we would have realized that the stops seen were very weak in comparison. We apologize if anyone was misled by these erroneous claims. The error became apparent when the number of Ψ obtained by sequencing was compared with the number from direct nucleoside analysis (Table 1). As a result of this experience, we advise that Ψ sequencing by the CMC-reverse transcriptase method (Ofengand et al. 2001a) always be checked by total nucleoside analysis.

The identification and location of the m³ Ψ residue in *D. radiodurans* 23S RNA was carried out by mass spectrometry. Nucleosides from a total nucleoside digest of 16S RNA were analyzed by LC/MS, and no m³ Ψ was detected. A component in the total RNA (16S + 23S) digest having the correct molecular weight (258), fragmentation behavior, and accurate HPLC retention time for m³ Ψ was observed, which exhibited characteristics identical to m³ Ψ , compared in a back-to-back analysis of *E. coli* 23S RNA. The sequence location was independently confirmed by gas phase sequencing by LC/MS/MS of the expected RNase T1 fragment of composition U₃C₂A₅Gp + CH₂ (*M_r* 3550.49) in the RNA (Ψ and U being indistinguishable in mass). Placements of Ψ 1894, m³ Ψ 1898 and Ψ 1900 were based on masses of sequence ions, and abundance changes that are characteristic

for pseudouridine and its derivatives (S.C. Pomerantz and J.A. McCloskey, in prep.).

Conservation of the Ψ sites

Finding three and two Ψ in the helix 69 loop of *D. radiodurans* and *H. marismortui*, respectively, was not surprising given the high conservation of these modification sites. As shown in Table 5, position 1917 is almost always Ψ , at least in the 12 organisms so far sequenced in this area, and excluding known mitochondrial ribosomes that have no Ψ in this stem-loop. Position 1915 is also Ψ or m³ Ψ in all the organisms except *Sulfolobus acidocaldarius* despite the presence of U1915 in that organism. As archaea likely make their ribosomal Ψ using guide RNAs for specifying the sites rather than an RluD-like enzyme, it appears that the needed guide RNA is absent in *S. acidocaldarius*. Position 1911 is more variable, being Ψ in all four of the eubacteria and eubacteria-derived organelles, U in the three archaea, and either U or Ψ in the eukaryotes. Thus, one

FIGURE 4. Ψ sequencing of 23S RNA from *E. coli* Ψ synthase mutants complemented with putative Ψ synthases from *D. radiodurans*. (A) The *E. coli* Ψ synthase mutant $\Delta rluD$ transformed with plasmid pTrc99A carrying no insert (p) or RluD from *E. coli* (pDE) or *D. radiodurans* (pDD). (B) The *E. coli* Ψ synthase mutant $\Delta rluB::kan$ transformed with plasmid pTrc99A carrying no insert (p) or RluB from *E. coli* (pBE) or *D. radiodurans* (pBD). In both panels, Ψ are indicated by *E. coli* numbering and RNA sequence is shown in A, C, G, and U lanes. Transformation, growth of uninduced transformed cells, and isolation of total RNA was done as described (Del Campo et al. 2001).

striking finding of this work is that the conservation of Ψ sites in the loop of helix 69 persists in the two diverse organisms, *D. radiodurans* and *H. marismortui*.

We did not expect to find that, with the exception of helix 69, there would be only a single Ψ in the entire 23S RNA of both organisms because analysis of other bacteria has generally found more (Ofengand et al. 2001b). Our original hope had been that enough Ψ would be found in sufficiently diverse locations that knowing their three-dimensional position in the ribosome would enable some assessment of their functional and/or structural role. On the other hand, the fact that there is only one additional Ψ in each organism, albeit a different one in the two cases, implies that there is some specific importance to these two sites. At the 2586 site, the eubacteria lack Ψ even though *E. coli* has a U2586. *H. marismortui* and *H. halobium* as well as three eukaryotes have Ψ 2586 (2585 in *Homo sapiens*). Curiously, yeast does not have Ψ there despite having U2586. The *D. radiodurans* Ψ 2605 is also present in the other eubacteria but is absent from *H. marismortui*, *H. halobium*, and the eukaryotes despite the presence of the precursor U at that site. Instead, the eukaryotes have Ψ 2606, which is not possible in the archaea or eubacteria because the position is occupied by a C.

The first conclusion to be made from this analysis is that Ψ at the apex of helix 69 is necessary, although the exact number is subject to some variation. The second conclusion is that there is some ill-defined connection between Ψ 2586 and Ψ 2605–2606. Since U2605 is available in all the eukaryotes studied but is not modified, Ψ 2606 is likely to be the preferred site of modification. It is not available in the eubacteria and archaea that were sequenced for Ψ , and in fact this residue is C in >80% of known archaeal and eubacterial 23S RNA sequences (Comparative RNA site, <http://www.rna.icmb.utexas.edu>).

Eubacteria appear to use the adjacent residue, Ψ 2605, as a substitute (*E. coli* has Ψ 2604 in addition) whereas in the two archaeons studied, U2605 is not used but instead U2586 is converted to Ψ . Interestingly, eukaryotes with the exception of yeast make Ψ at both U2606 and U2585, perhaps to achieve a stronger effect of whatever it is that Ψ does.

Potential roles for the Ψ residues identified in this work

The most important question about Ψ in rRNA is what they do that U cannot. This is still an unanswered question. Nevertheless, there are intriguing correlations between the locations of the Ψ residues and nucleosides known to be important for ribosome function. In the 70S ribosome-tRNA structure (Yusupov et al. 2001), the loop of helix 69 containing the Ψ supports the anticodon arm of A-site tRNA near its juncture with the amino acid arm. The middle of helix 69 does the same thing for P-site tRNA. Unfortunately, the resolution in this work was not sufficient to reveal any specific roles for the Ψ residues. On the other hand, a recent study of the location of the ribosome release factor RRF on the 70S ribosome has shown that $m^3\Psi$ 1915 makes a direct contact with amino acid residues E122 and V126 of the ribosome release factor RRF (Agrawal et al. 2004). It may therefore turn out that the primary protein synthesis functional role for Ψ in helix 69 is in ribosome release rather than in the more conventional aspects of protein synthesis.

To see if the putative connection between Ψ 2605 and Ψ 2586 (*E. coli* numbering) based on conservation (Table 5) has any structural basis, the environment around *D. radiodurans* (PDB no. 1NKG) Ψ 2584 and C2565 (*E. coli* 2605

TABLE 5. Species conservation of Ψ at or near the sites found in *H. marismortui* and *D. radiodurans*

Class	Organism	Position (<i>Escherichia coli</i> numbering)							
		1911	1915	1917	2585	2586	2604	2605	2606
Eubacteria, chloroplasts	<i>Escherichia coli</i>	Ψ	$m^3\Psi$	Ψ	U	U	Ψ	Ψ	C
	<i>Bacillus subtilis</i>	Ψ	Ψ^*	Ψ	U	C	U	Ψ	C
	<i>Deinococcus radiodurans</i>	Ψ	$m^3\Psi$	Ψ	U	C	U	Ψ	C
	<i>Zea mays</i> chloroplasts	Ψ	Ψ^*	Ψ	U	C	U	Ψ	C
Archaea	<i>Halobacter halobium</i>	U	Ψ	Ψ	U	Ψ	G	U	C
	<i>Haloarcula marismortui</i>	U	Ψ	Ψ	U	Ψ	G	U	C
	<i>Sulfolobus acidocaldarius</i>	U	U	Ψ	U ^a	U ^a	G	U ^a	C
Eukarya	<i>Saccharomyces cerevisiae</i>	U	Ψ	Ψ	U	U	G	U	Ψ
	<i>Euglena gracilis</i>	U	Ψ	U	U ^a	U ^a	G	U ^a	U ^a
	<i>Drosophila melanogaster</i>	Ψ	Ψ	Ψ	U	Ψ	G	U	Ψ
	<i>Mus musculus</i>	Ψ	Ψ	Ψ	U	Ψ	G	U	Ψ
	<i>Homo sapiens</i>	Ψ	Ψ	Ψ	Ψ	U	G	U	Ψ

Data from sources cited in Figure 3.

(Ψ^*) Probably $m^3\Psi$ (Ofengand and Bakin 1997).

^aU in gene sequence, not known if U or Ψ in the RNA.

and 2586, respectively) and around *H. marismortui* (PDB no. 1S72) Ψ 2621 and U2640 (*E. coli* 2586 and 2605, respectively) was examined. Although the N_1 position of the equivalent residues are only about 14 Å apart when both Ψ are placed in either structure, the N_1 -H bonds do not point in a common direction nor do they interact with any shared in common component of the ribosome or its bound tRNA analogs at A, P, or E sites (PDB nos. 1S72, 1FFZ, 1KQS, 1M9O, 1QVF, 1NJM, 1NJO, and 1NJP were examined). *H. marismortui* Ψ 2621 is at the tunnel entrance but in *D. radiodurans* Ψ 2584 is not, and there are no apparent ribosomal components in position to interact with either Ψ . There is, on the other hand, a certain “always a bridesmaid never a bride” quality to these two Ψ , a property noted also in other Ψ in rRNA (Ofengand et al. 2001b; Ofengand 2002). For example, *H. marismortui* Ψ 2621 is adjacent to the universally conserved U2620, which makes contact with tRNA analogs in the 50S structure (PDB no. 1KQS). Likewise, *D. radiodurans* Ψ 2584 is only three residues removed from A2581, a virtually universal residue that is bulged out from the tunnel wall, and is thought to provide half of the double anchor for the A- to P-site spiral motion proposed by the group of Yonath (Agmon et al. 2004). Thus Ψ residues continue to be near, but not exactly at, nucleotides directly involved in ribosome function.

Ψ synthases in *D. radiodurans*

We identified two Ψ synthases, RluB and RluD, most likely responsible for Ψ s 2584 and 1894, 1898, and 1900, respectively, in *D. radiodurans* 23S RNA by combining our knowledge of *E. coli* Ψ sites and synthases with bioinformatics tools. While definitive assignment can only be done by performing the genetic analysis in *D. radiodurans*, our complementation studies of *E. coli* mutants clearly show that *D. radiodurans* RluB and RluD have Ψ synthase activity with site capability identical to *E. coli* RluB and RluD, respectively. Of the two remaining *D. radiodurans* homologs that could also make these rRNA Ψ s if one assumes overlapping site specificity, one (gi|15806687|) appears to be an inactive Ψ synthase lacking the conserved catalytic Asp. It would take two base changes to obtain the conserved Asp from the Gly codon (GGG) at the active site, so this is not likely a DNA sequencing error. Perhaps this synthase has lost its capacity to make Ψ but has another function in *D. radiodurans*. The other homolog (gi|15805985|) might share site specificity with either RluB or RluD, but it is more likely that this Ψ synthase is specific for tRNA since bacterial Ψ synthases do not appear to have overlapping specificity.

MATERIALS AND METHODS

Strains and growth conditions

D. radiodurans R1 (a gift from Michael J. Daly, Uniformed Services Univ. of the Health Sciences) was grown in TGY medium (0.5% Bacto-tryptone, 0.1% glucose, 0.3% Bacto-yeast extract) at

30°C. *H. marismortui* (a gift from Peter Moore, Yale University, who also supplied an initial sample of ribosomes) was grown in a modified ATCC medium 1230 at 37°C. The medium contained 25 mM KCl, 76 mM MgSO₄, 11 mM Na citrate, 4 M NaCl, 1.7 mM CaCl₂, 5 g/L yeast extract (Difco), 47 mM Tris-HCl (pH 7.2), 4 g/L glucose, 0.01 mM MnCl₂, and 1.7 mM FeCl₃ adjusted to pH 7.2–7.4. The following *E. coli* strains were used: MG1655 (wild-type *E. coli* K12), MG1655 Δ rluB (Del Campo et al. 2001), and MG1655 Δ rluD::kan (N. Gutgsell and J. Ofengand, unpubl. results). *E. coli* strains were grown at 37°C in LB medium (1% Bacto-tryptone, 1% NaCl, 0.5% Bacto-yeast extract) supplemented with carbenicillin (100 µg/mL) for plasmid selection. In addition, kanamycin (30 µg/mL) was used when growing the Δ rluD::kan strain.

RNA isolation and Ψ sequencing

E. coli and *H. marismortui* total RNA were isolated from cells grown to an A₆₀₀ of 1–1.5 at 37°C, following the procedure described by Ofengand et al. (2001a). Total *D. radiodurans* rRNA was obtained from cells grown to an A₆₀₀ of 0.9, and isolated using the RNeasy kit (Qiagen) according to the manufacturer's instructions. This latter procedure removes small MW RNAs such as 5S RNA but does not separate the 16S and 23S RNAs. *E. coli* and *H. marismortui* total RNA were fractionated into 16S and 23S RNAs by sucrose gradient centrifugation. The RNA, dissolved in 20 mM HEPES (pH 7.5), 100 mM LiCl, 2 mM EDTA, 0.1% SDS, was run on 10%–25% sucrose gradients in the same buffer at 22,000 rpm for 30 h at 17°C in a Beckman SW28 rotor. Fractions were collected and the size and purity examined by glyoxal-Me₂SO denaturing gel electrophoresis (Denman et al. 1989). *D. radiodurans* 16S and 23S RNA was obtained by first isolating 30S and 50S ribosomal subunits on sucrose gradients and then extracting the subunits with phenol/chloroform to obtain the RNA. This procedure was followed because the 23S RNA in 50S subunits has cleavages that would result in contamination of the 16S RNA if it were isolated by the same procedure used for the other rRNAs. *D. radiodurans* ribosomal subunits were obtained as follows. Cells were washed in buffer A (10 mM HEPES at pH 7.8, 4 mM mercaptoethanol, 150 mM NH₄Cl, 30 mM Mg(OAc)₂) and then suspended in buffer A at 125 mg/mL and treated with 0.25 µg/mL lysozyme for 20 min at 0°C with shaking. The mixture was then passed through a French press at 600–650 bars, RNase-free DNase was added at 0.1 µg/mL, and the mixture incubated at 0°C for 30 min. Cell debris was removed by centrifugation for 20 min at 13,000 rpm in a Sorvall SS-1 rotor. Per volume of supernatant, 0.2 volumes of 1.1 M sucrose in buffer A was added and centrifuged 3 h at 19,000 rpm in a Beckman 45Ti rotor. This supernatant was layered over a cushion of 1.1 M sucrose in buffer A and centrifuged (60Ti rotor, 42,000 rpm, 22 h). The pellet was resuspended in buffer A and layered on a sucrose gradient in 10 mM HEPES (pH 7.8), 4 mM mercaptoethanol, 100 mM NH₄Cl, 5–6 mM Mg(OAc)₂ and centrifuged to obtain the separated subunits. All RNAs were sequenced for Ψ as previously described (Ofengand et al. 2001a) except that the alkaline treatment was done for 4 and 6 h to better assess CMC removal from U and G residues.

Cloning *D. radiodurans* RluB and RluD homologs

The *D. radiodurans* genes *rluB* (gi|15805921|) and *rluD* (gi|15806790|), whose gene products are homologs of *E. coli* RluB

and RluD, respectively, were PCR amplified from *D. radiodurans* R1 genomic DNA, subcloned into the pGEM-T vector (Promega), and then cloned into the NcoI and HindIII sites of the pTrc99A expression vector. For the 774 bp *rluB*, the N-terminal primer (5'-CTAGAAGACTTCATGTCCTCCGCTGAGCGCTTGCA-3') incorporated a BbsI site (italics) at the fourth nucleotide preceding the AUG start codon (underlined) and the C-terminal primer (5'-AATGAAGCTTGCGAGAAGGCGCTGTGAGGC-3') incorporated a HindIII site (italics) at the 26th nucleotide following the TAA stop codon. For the 1017 bp *rluD*, the N-terminal primer (5'-AATAGGTCTCACATGCCACGTGGCCGGAACAG-3') incorporated a BsaI site (italics) at the third nucleotide preceding the AUG start codon (underlined) and the C-terminal primer (5'-TGTCGAAGCTTCCACCAGCGGAGAGGGTCAA-3') incorporated a HindIII site (italics) at the 50th nucleotide following the TAG stop codon. Both constructs were verified by DNA sequencing.

Nucleoside analysis

Individual 16S and 23S RNAs were digested to nucleosides with RNase P1, venom phosphodiesterase, and alkaline phosphatase as described (Crain 1990). The digests were analyzed by directly combined LC/MS in positive ion mode on a Waters Quattro II mass spectrometer fitted with the Z-spray electrospray ion source coupled to an Agilent Model 1090 liquid chromatograph. Quantitation was obtained from LC peak areas, the mass spectral data being used to verify LC peak identity and purity. Components of the digest were resolved on a Phenomenex Luna C18 (Crain 1990) column (2.1 × 250 mm, 5 μ d_p) protected by a Opti-Guard pre-column (Optimize Technologies). Buffer A was 5 mM NH₄OAc (pH 5.3) and buffer B was 40% CH₃CN. A multilinear gradient was employed (Pomerantz and McCloskey 1990) and the total liquid flow of the LC (300 μ L/min) was analyzed. Typically, full scan mass spectra were acquired from *m/z* 108–408 every 0.45 sec. The presence of *m*³ Ψ was confirmed in the combined 16S and 23S RNA digest by selected ion recording (SIR) data acquisition of the *m/z* 259 (MH⁺) and 223 (MH⁺-2H₂O) channels with a 90-msec dwell time. Quantitation of Ψ was accomplished by integration of the 260-nm peak areas from the HPLC diode array data by the MassLynx data system without data smoothing. The peak areas were calculated for Ψ and C, and then corrected for the empirically determined (in the McCloskey laboratory) relative molar response ratio at 260 nm. Since the number of C residues is known (from the gene sequence), simple multiplication allowed calculation of the number of Ψ residues.

Oligonucleotide analysis

RNAs were digested to oligonucleotides with 10 units of RNase T1 (Ambion) per pmol of RNA in Tris-EDTA buffer (pH 7.0) (Crain 1990). Oligonucleotides were chromatographically resolved on Luna C18 (Crain 1990) columns (Phenomenex) using 400 mM hexafluoroisopropanol (HFIP) titrated to pH 7 with triethylamine and a linear gradient (1%/min) against 400 mM HFIP in 50% methanol (Apffel et al. 1997). For molecular weight determination and testing for the presence of Ψ , a 1.0 × 150 mm column at a flow rate of 60 μ L/min was used (Agilent 1090). Component resolution for de novo sequencing of Ψ -containing oligonucleotides was per-

formed on a 0.5 × 150 mm column at a flow rate of 15 μ L/min. The presence of Ψ was detected by a reaction-monitoring experiment performed on a Waters Quattro II instrument in which the *m/z* 207 ion, equivalent to the doubly dehydrated nucleoside anion, was generated by elevating the cone voltage of the ion source to 70 V, from its normal 35 V used for molecular weight determination. The *m/z* 207 ion was selected in the first quadrupole, dissociated at 14 eV at an argon gas pressure of 3 × 10⁻³ mbar in the collision cell, and the product ion at *m/z* 164 was monitored in the third quadrupole with a dwell time of 30 msec (S.C. Pomerantz and J.A. McCloskey, in prep.). De novo sequencing was accomplished with a Waters CapLC chromatograph coupled to a Micro-mass Q-ToF 2 mass spectrometer. The (M-6H)⁶⁻ ion was selected in the quadrupole, and the product ions were measured using the time-of-flight analyzer after dissociation at 90 eV (laboratory frame of reference) collision energy.

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