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REVIEW

Expanding the Nucleotide Repertoire of the Ribosome with Post-Transcriptional Modifications

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ABSTRACT In all kingdoms of life, RNAs undergo specific post-transcriptional modifications. More than 100 different analogues of the four standard RNA nucleosides have been identified. Modifications in ribosomal RNAs (rRNAs) are highly prevalent and cluster in regions of the ribosome that have functional importance, have a high level of nucleotide conservation, and typically lack proteins. Modifications also play roles in determining antibiotic resistance or sensitivity. A wide spectrum of chemical diversity from the modifications provides the ribosome with a broader range of possible interactions between rRNA regions, transfer RNA, messenger RNA, proteins, or ligands by influencing local rRNA folds and fine-tuning the translation process. The collective importance of the modified nucleosides in ribosome function has been demonstrated for a number of organisms, and further studies may reveal how the individual players regulate these functions through synergistic or cooperative effects.

In all kingdoms of life, ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nucleolar RNAs (snoRNAs), and other RNAs undergo specific post-transcriptional modification by a wide variety of enzymes (*1*). To date, >100 different modifications of the four standard RNA nucleosides, adenosine, cytidine, guanosine, and uridine, have been identified (*2*). These modifications can be organized into four main types (Figure 1) (*1*). The first involves isomerization of uridine to pseudouridine (5-ribosyluracil, Ψ), which contains a C- rather than the typical N-glycosidic linkage, as well as an additional imino group that is available for unique hydrogen-bonding interactions. The second includes alterations to the bases, such as methylation (typically on carbon, primary nitrogen, or tertiary nitrogen), deamination (*e.g.*, inosine), reduction (*e.g.*, dihydrouridine), thiolation, or alkylation (*e.g.*, isopentenylation or threonylation). The third involves methylation of the ribose 2′ hydroxyl (Nm). The fourth type includes more complex modifications, such as multiple modifications (*e.g.*, 5-methylamino-methyl-2-thiouridine; 3-(3-amino-3-carboxypropyl)uridine, acp^3U; 1-methyl-3-(3-amino-3-carboxypropyl)pseudouridine, $m^1acp^3\Psi$) or “hypermodifications” that can be incorporated by specific exchange mechanisms (*e.g.*, queuosine). The possible electronic and steric effects of the nucleoside modifications on base pairing, base stacking, and sugar pucker in RNA have been discussed in detail by Davis (*3*) and Agris (*4*), among others (*1*). The effects of modifications such as Ψ on RNA hydration and dynamics have also been considered (*4*).

Modified nucleotides in the ribosome are varied in their identity but highly localized in their positions (*5*). If the sites of modification are mapped on the secondary structures of the small and large subunit (SSU and LSU) rRNAs, they might appear to be random; however, if the same modifications are located within the ribo-

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Received for review July 20, 2007
and accepted September 5, 2007.
Published online September 21, 2007
10.1021/cb7001494 CCC: $37.00

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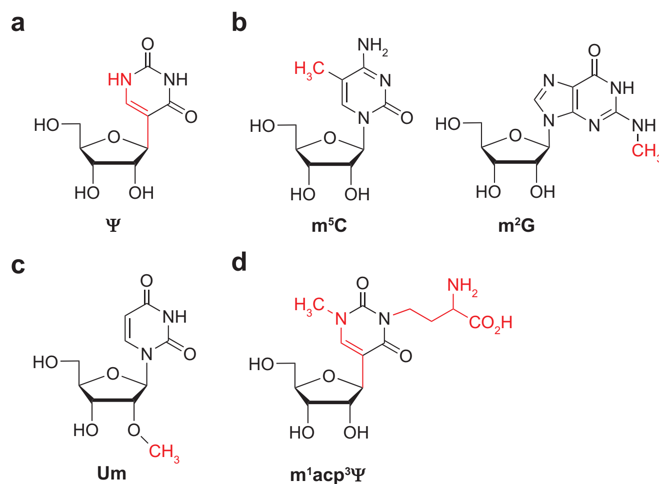

Figure 1. The four major types of rRNA modification are shown. a) Isomerization of uridine to pseudouridine (Ψ). b) Base modification of C and G to m^5C and m^2G, respectively. c) 2′-O-Methylation of uridine to 2′-*O*-methyluridine (Um). d) Multiple modifications of pseudouridine to $m^1acp^3\Psi$.

some tertiary structures from high-resolution X-ray crystal structures (*6, 7*), they occur in the most functionally important regions (Figure 2). In particular, regions dedicated to peptidyl transfer, such as the A and P sites, the polypeptide exit tunnel, and the intersubunit bridges, are highly modified. Of note, the clustering of the modified nucleosides is conserved throughout phylogeny, and their number appears to rise with increasing complexity of the organism. One example is Ψ, which occurs 10 times in the LSU rRNA of *Escherichia coli* and 55 times in the corresponding human rRNA (*8–10*). In eukaryotes, the total number of modified nucleotides ranges from 100 in yeast to ~200 in humans (*2, 10*). The availability of high-resolution X-ray crystal structures of ribosomes (*6, 7*), along with knowledge of the modified nucleotide positions, has led to the generation of modification maps that allow ribosome structure and function to be correlated (*11*).

The combined importance of the individual post-transcriptional modifications was first revealed by the greater ability of fully modified ribosomes to carry out protein synthesis compared with unmodified ribosomes (*E. coli* ribosomes reconstituted with unmodified 16S and 23S rRNAs) (*12, 13*). The global disruption of Ψ or Nm formation in ribosomes *in vivo* results in strong growth defects in yeast (*14, 15*). In contrast, most enzymes or RNA–enzyme complexes that are responsible for individual modifications in the ribosome can each be disrupted or deleted with only minimal effects on the survival or growth of the organism. Similarly, disruption or deletion of an enzyme responsible for modification at three specific sites in *E. coli* 23S rRNA (RluD) does not cause a major difference in the exponential growth rate compared with the wild-type strain; however, the mutants are out-competed by the wild-type strains, an indication that the Ψ residues confer a growth advantage to the bacteria (*16*). Thus, the hypothesis has been put forward by a number of groups that rRNA modifications are individually dispensable for survival of an organism but that together they fine-tune rRNA structure and function and ensure production of accurate and efficient ribosomes (*1, 17*).

The reason for the presence of modified nucleosides is still largely unknown. Their occurrence in functionally important regions of the ribosome is highly suggestive of a role in protein synthesis. The bacterial ribosome is a target for many antibiotics, which also

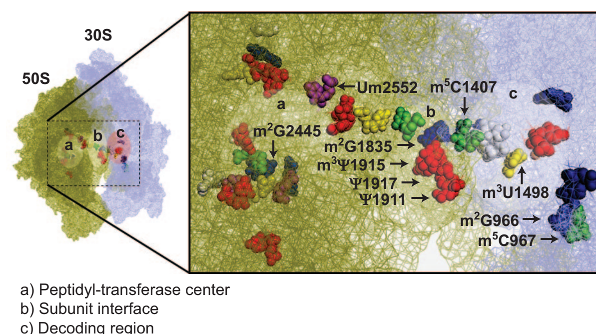

Figure 2. The distribution of the 36 naturally occurring modified nucleotides from *E. coli* rRNA is shown on the crystal structure of the 70S ribosome (Protein Data Bank IDs, 2avy for the 30S subunit and 2aw4 for the 50S subunit) (*7*). The left side shows the clustering of modified nucleotides on the LSU (50S, green) and SSU (30S, light blue). The functionally important regions are highlighted: a) the peptidyl-transferase center, b) the subunit interface, and c) the decoding region. The expanded region to the right highlights specific residues (*E. coli* numbering) that are discussed in the text. The color scheme for the 36 modified nucleotides is as follows: Ψ, $m^3\Psi$ (red); m^5U, m^3U, D (yellow); m^2G, m^7G, m^1G (blue); m^5C, s^2C (green); m^4Cm (orange); m^6A, m^2A, $m_2{}^6A$ (white); and Cm, Um, Gm (magenta) (note that not all 36 modified residues are visible from this perspective). This figure was generated with PyMOL software.

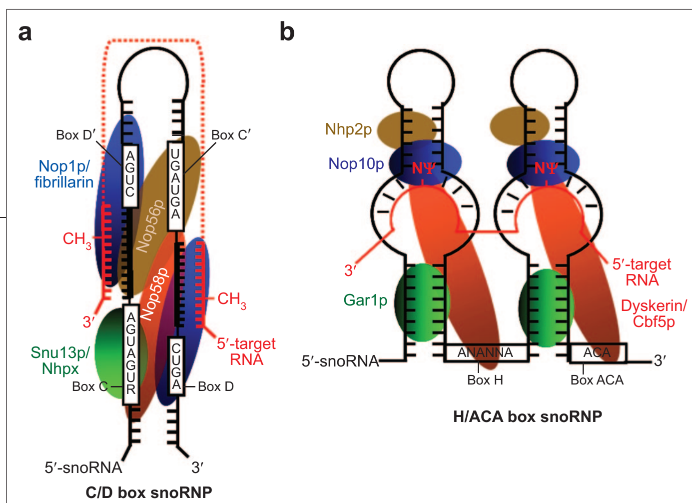

Figure 3. The eukaryotic C/D and H/ACA box snoRNPs are represented. The binding of proteins to the snoRNAs (black) and target RNAs (red) are depicted in which the C/D box class (panel a) binds to Nop1p/fibrillarin, Nop56p, Nop58p, and Snu13p/Nhpx, and the H/ACA box class (panel b) interacts with Cbf5p/dyskerin, Gar1p, Nhp2p, and Nop10p. The conserved box C, D, and H elements are highlighted, and N represents a conserved nucleotide that is located next to the target Ψ (panel b).

tend to interact with the functionally important regions. For example, the aminoglycoside antibiotics interact directly with the decoding region at the top of helix 44 in 16S rRNA (*18, 19*). Tetracycline also targets sites on the SSU rRNA, including helix 31 (*20*). Given these observed correlations between sites of nucleotide modifications, antibiotic binding, and regions of functional importance in the ribosome, perhaps it is not surprising that several cases exist in which specific rRNA modifications confer either resistance or sensitivity to the ribosome-targeting compounds (*21*). A recent example revealed that a cyclic peptide antibiotic, capreomycin, interacts across two modified regions on 16S (helix 44) and 23S (helix 69) rRNAs (*22*). Therefore, it has become increasingly apparent that knowledge about the locations of rRNA modifications, as well as their specific effects on RNA structure and antibiotic binding, will be useful for the development of new classes of antibiotics that avoid certain resistance mechanisms.

RNA Modification by MTases, Ψ Synthases, and snoRNPs. The rRNAs in bacteria and eukaryotes are modified by different pathways, even though some sites of modification are conserved throughout phylogeny. RNA modifications in bacteria are mediated by single-protein enzymes, which are generally site-specific and target unique folded structures or RNA sequences. The two major classes of RNA modifying enzymes are methyltransferases (MTases) and Ψ synthases, which catalyze the most common rRNA modifications (*23, 24*). These enzymes often employ covalent catalysis by using specific amino-acid side chains (*e.g.*, aspartic acid). MTases catalyze transfer of a methyl group from the cofactor *S*-adenosyl-L-methionine (SAM) to the target nucleotide in a highly specific fashion. With only a few exceptions, almost every site of modification in bacterial rRNAs requires a unique MTase, Ψ synthase, or other type of modifying enzyme.

Because of the presence of a much larger number of rRNA modifications in higher organisms (*i.e.*, eukaryotes) compared with bacteria, using unique modifying enzymes for each site would be metabolically inefficient. Instead, eukaryotic rRNA modifications are carried out by RNA–protein complexes. In the late 1980s, it became apparent that a large number of antisense RNAs localized to the nucleolus of eukaryotic cells contained regions of complementarity to sites of methylation in rRNA (*25–27*). These noncoding snoRNAs, which are typically 60–300 nucleotides in length, guide the site-specific modification of a target RNA. Each unique snoRNA combines with a set of common proteins to form a small nucleolar ribonucleoprotein (snoRNP) complex (*28*). The snoRNA contains a stretch of 10–20 nucleotides that are complementary to a particular region of the target rRNA. On the basis of the presence of conserved sequence motifs, the snoRNAs are divided into two major classes referred to as the C/D box and H/ACA box classes (*29*). The C/D box snoRNAs guide 2′-O-methylation, whereas the H/ACA box snoRNAs catalyze isomerization reactions of uridine to Ψ. The snoRNPs also have functions in addition to modification that will not be discussed here (*29*). In addition, C/D and H/ACA snoRNPs are universally present in eukaryotes, but they are also found in archaea (*29*).

The C/D box snoRNAs contain two conserved sequence motifs, the C box (5′-RUGAUGA-3′, where R is purine) and the D box (5′-CUGA-3′), which are located at the 5′ and 3′ termini of the snoRNA, respectively (Figure 3). Two additional motifs, the C′ and D′ boxes, lie between the C and D boxes. The C and D box motifs are in close proximity and form the stem box structure. A region of 10–21 nucleotides upstream of the D box is complementary to the methylation site and enables the snoRNA to form a duplex with the target rRNA (*30*). The residue to be modified is five nucleotides upstream of

KEYWORDS

Ribosome: Ribonucleoprotein complexes (rRNA and proteins) that manufacture proteins; they contain a small subunit (SSU) and a large subunit (LSU).

Peptidyl-transferase center: The catalytic center of the ribosome; it is located on the large ribosomal subunit and catalyzes peptide-bond formation and peptide release.

Decoding region: Located on the SSU rRNA and contains highly conserved nucleotides that are involved in interactions with transfer RNAs (tRNAs) and messenger RNA. The A site binds to aminoacyl-tRNA. The P site binds to peptidyl-tRNA.

TABLE 1. Modified nucleosides found in *E. coli* 16S and 23S rRNAs (*2*)

	G	C	A	U
16S rRNA	m^2G (966)	m^5C (967)	$m_2{}^6A$ (1518)	m^3U (1498)
	m^2G (1207)	m^5C (1407)	$m_2{}^6A$ (1519)	Ψ (516)
	m^2G (1516)	m^4Cm (1402)		
	m^7G (527)			
23S rRNA	m^1G (745)	m^5C (1962)	m^2A (2503)	Ψ (746)
	m^2G (1835)	Cm (2498)	m^6A (1618)	Ψ (955)
	m^2G (2445)	s^2C (2501)	m^6A (2030)	Ψ (1911)
	m^7G (2069)			Ψ (1917)
	Gm (2251)			Ψ (2457)
				Ψ (2504)
				Ψ (2580)
				Ψ (2604)
				Ψ (2605)
				m^3Ψ (1915)
				m^5U (747)
				m^5U (1939)
				D (2449)
				Um (2552)

the D box (*25, 26*). The proteins associated with the eukaryotic C/D box snoRNAs are Nop1p/fibrillarin (the 2′-O-methyltransferase), Nop56p, Nop58p, and Snu13p/Nhpx (*27, 28, 31*).

The H/ACA box snoRNAs contain two conserved sequence motifs, the H box (5′ANANNA3′) and the ACA trinucleotide (three nucleotides away from the 3′ end), and they have two hairpin and two single-stranded regions, referred to as the hairpin–hinge–hairpin–tail structure (*29*) (Figure 3). Both motifs are typically located in the single-stranded regions of the snoRNA secondary structure. The antisense or complementary sequences to the target rRNA are found within the internal bulge regions. To date, four proteins have been found that associate with the H/ACA snoRNAs, Cbf5p/dyskerin (the putative Ψ synthase), Gar1p, Nhp2p, and Nop10p (*27, 28, 31*).

The discovery of snoRNAs has been critical for understanding the biological roles of rRNA modifications in higher organisms. The ability to block specifically individual modifications by snoRNP mutations or knockouts allows scientists to examine the roles of each nucleotide modification in the ribosome (*32*).

Effects of rRNA Methylation on Ribosome Function. Numerous examples exist of methylated nucleotides in a variety of RNAs from different organisms (*2*). The focus here will be mainly on bacterial rRNAs (specifically *E. coli*), because most of the high-resolution structure data come from bacterial ribosomes (*6, 7*). The rRNAs in *E. coli* contain 24 methylated nucleotides, and 10 occur in the SSU (summarized in Table 1). Guanosine methylation at the N2 position (m^2G or N^2-methylguanosine) is frequent in the ribosome (*33*). Five m^2G residues have been identified in the 16S (m^2G966, m^2G1207, and m^2G1516) and 23S (m^2G1835 and m^2G2445) rRNAs of *E. coli*. Nucleotide m^2G1835 of 23S rRNA is located in a region of the ribosome with extremely high functional importance, the intersubunit bridges B2b and B2c (Figure 2) (*6, 7*). This residue resides at the center of a four-way junction involving helices 67, 68, 69, and 71, a region that contains six modifications in *E. coli*. Residue m^2G2445 of helix 74 of the 23S rRNA is also located within a cluster of modified nucleotides (including D2449 and m^7G2069) concentrated at the peptidyl-transferase center (PTC) of the ribosome (Figure 2) (*6, 7*). This methylated residue is highly

Ribose modifications may also serve to regulate ribosome function by altering ribosome stability.

conserved among the LSU rRNAs. Furthermore, helix 74 resides in a densely packed area of the ribosome that is lacking proteins, similar to other highly modified regions. Inactivation of the *ycbY* or *ygjO* genes, encoding the MTases RlmL and RlmG for G2445 and G1835, respectively, leads to growth retardation of bacteria (*34, 35*).

In the small subunit of the ribosome, modified nucleotide m^2G966 is located in the loop of helix 31 of 16S rRNA, adjacent to m^5C967 (5-methylcytidine) (Figure 2) (*36*). These residues make direct contact with the tRNA anticodon during translation (*6, 7*). In bacteria, nucleotide 966 is predominately a guanosine, but it is occupied by acp^3U in archaea (*37*) and $m^1acp^3\Psi$ in eukarya (*38*). An *E. coli* strain lacking the G966 modification reveals only a moderate growth disadvantage compared with the wild-type strain (*39*); however, recent studies have shown that specific mutations at that locus cause mistranslation (P. Cunningham, A. Saraiya, and T. Lamichhane, Wayne State University, personal communication).

Three m^5C residues are present in *E. coli* ribosomes (Table 1) at positions 967 and 1407 of 16S rRNA and residue 1962 of 23S rRNA. The m^5C967 (helix 31) modification is catalyzed by the SAM-dependent MTase RsmB (Fmu) (*36, 40*). Nucleotide C1407 is also located at a functionally active region of the ribosome in the decoding region of 16S rRNA and close to the subunit interface and P-site tRNA (Figure 2) (*6, 7*). RsmF (YebU)-directed methylation of this nucleotide appears to be conserved in bacteria. A *yebU*-knockout strain displays slower growth rates and reduced fitness in competition with wild-type cells (*41*), further evidence of the fine-tuning of ribosome function through methylation.

RsmE (YggJ) belongs to a newly discovered family of uridine MTases and converts U1498 of *E. coli* 16S rRNA to 3-methyluridine (m^3U) (*42*). This modification occurs in a highly conserved region (the P site) between residues 1492 and 1505 (Figure 2), and a *yggj*-deletion strain also shows growth defects when competing with wild-type cells. The fact that this modified residue is close to the 23S rRNA and P-site tRNA anticodon suggests that it could play a role in either intersubunit association or tRNA selection (*6, 7*).

The base-methylated residues (not all of which have been mentioned here) could be involved with specific hydrophobic interactions within the ribosome or used to prevent specific Watson–Crick or noncanonical base-pairing schemes, which may limit the range of conformations of the ribosomal helices (*43*). Specific structures that are regulated by the modified nucleotides could provide stabilization of the PTC, or stabilize specific rRNA contacts during translation. Of the methylase knockout strains that have been studied, most are still viable but have slow growth. These results all point to roles of the methylated nucleotides in fine-tuning the ribosome structure, rather than providing essential functions.

A second type of nucleotide modification involves methylation of the ribose 2′ hydroxyl (Nm). The MTase RrmJ (FtsJ) is responsible for 2′-O-methylation of the universally conserved U2552 in domain V of 23S rRNA (Table 1, Figures 1 and 2). This modification appears to be critical for ribosome stability, because the absence of RrmJ causes cellular accumulation of free 30S and 50S ribosomal subunits (*44*) and growth defects (*45*) in *E. coli*. The adjacent residue, G2553, is involved in A-site tRNA binding (the A loop) and base pairs with C75 of the tRNA. This interaction is essential for peptidyl-transferase activity in the ribosome (*46*). Furthermore, residues in the A loop are highly conserved, and mutations of residues in this loop reduce translational fidelity and efficiency of ribosome-catalyzed peptide-bond formation (*47*).

Solution NMR structures of the A loop in the presence and absence of modification (U2552 2′-O-methylation) have been solved, and they suggest that sugar methylation regulates RNA folding (*48*). The five-nucleotide A loop is closed by a noncanonical base pair between the universally conserved nucleotides C2556 and U2552. Although 2′-O-methylation has little effect on the global conformation of the A loop, the conformational properties of critical loop residues (U2552, U2555, and C2556) are influenced, and these residues mediate critical tertiary interactions within the ribosome. Thus, as with base modification, ribose modifications may also serve to regulate ribosome function by altering ribosome stability (subunit association/dissociation) or through interactions with key components of the translational machinery.

Effects of rRNA Methylation on Antibiotic Action. Nucleotide modification has been closely associated with both antibiotic sensitivity and antibiotic resistance (*21*). One well-studied example is the conversion of two adjacent adenosines (A1518 and A1519) in 16S/18S rRNA to N^6,N^6-dimethyladenosine ($m_2{}^6$A), which occurs

in all three domains of life (*49*). This is one case in which modified residues in the ribosome are almost completely conserved throughout phylogeny. Such conservation suggests that these modifications play an important role in ribosome function. These two m_2^6A residues are modified by RsmA (KsgA) in bacteria and Dim1p in yeast. In bacteria, the loss of modification leads to resistance to the aminoglycoside kasugamycin, slower growth rates, and reduced translational fidelity (*49, 50*). In yeast, a knockout mutation of the *dim1* gene that encodes the 18S rRNA dimethylase is lethal, which may be the result of misprocessed precursor rRNA (*51, 52*). Recent unexpected results revealed that kasugamycin binds to both wild-type ribosomes and those containing kasugamycin-resistance mutations (*53*). Thus, it appears that certain mutations in the ribosome can compensate for drug interactions and maintain binding affinity. It is also known that m_2^6A modifications in helix 45 destabilize the tetraloop structure, possibly preventing formation of a sheared G–A mismatch (*54*). Further evidence shows a possible role for the modification in regulation of long-range RNA–RNA interactions (*53*). This example demonstrates, however, that even for highly conserved modifications that have been studied extensively for more than two decades, their main functions are still quite elusive.

A recent study revealed that in addition to modifying regions that are far apart on secondary structure maps, a single-RNA enzyme can also modify residues on different ribosomal subunits. The TlyA MTase from *Mycobacterium tuberculosis* modifies (2′-O-methylation) C1409 in helix 44 of 16S rRNA and C1920 in helix 69 of 23S rRNA (*22*). Resistance to the cyclic peptide antibiotics capreomycin and viomycin occurs upon inactivation of the *tlyA* gene in *M. tuberculosis* (*22*). Thus, TlyA belongs to a group of MTases that confer antibiotic resistance by losing their function. The two methylated residues reside at a region of high functional significance in the ribosome, the intersubunit bridge region referred to as B2a, in which the loop of 23S rRNA helix 69 contacts helix 44 of 16S rRNA near residue 1409 (*6, 7*). The location of methylations in this functionally important rRNA region suggests that the antibiotics inhibit ribosome function through specific effects at the subunit interface (*22*). The ribosomes of many other bacteria lack *tlyA*-encoded methylations, providing a possible reason why these pathogens are less susceptible to mycobacteria-targeting drugs. The exact role of the cytidine methylations in regulating antibiotic binding is still unknown, but further biophysical and biochemical studies could reveal whether the compounds make direct contacts with the methylated residues or alter the ribosome conformation in order to regulate ligand binding.

Resistance to ribosome-targeting drugs is generally associated with the addition of methyl groups rather than their loss (*21*). In fact, an increasingly large number of 16S rRNA methylase genes that lead to aminoglycoside antibiotic resistance have been identified (*55, 56*). There are numerous other methylated residues in both the LSU and SSU rRNAs throughout phylogeny that have not been discussed. In most cases, their effects on ribosome function or antibiotic action still remain a mystery.

Effects of Pseudouridylation on Ribosome Function. The most common type of RNA modification is the conversion of uridine to pseudouridine (Ψ). This modification is challenging to identify in RNA, mainly because it is mass-neutral compared with its precursor, uridine. Several groups have reported novel approaches for identifying Ψ by the addition of mass tags, employing a combination of specific chemical reactions and mass spectrometry analysis (*57–59*).

As noted earlier, the number of Ψ modifications increases with complexity of the organism (*9*), and they are concentrated in the regions of functional importance in the ribosome, namely, the PTC. The enzyme RsuA catalyzes the synthesis of the only Ψ residue (Ψ516) in the *E. coli* SSU rRNA. In contrast, 10 Ψ's in the LSU bacterial 23S rRNA are catalyzed by six different enzymes, RluB (Ψ2605), RluE (Ψ2457), RluF (Ψ2604), RluA (Ψ746), RluC (Ψ955, Ψ2504, Ψ2580), and RluD (Ψ1911, Ψ1915, Ψ 1917) (Table 1) (*24*).

The process of Ψ formation (pseudouridylation) is different in bacteria and higher organisms (*1*). In eukaryotes, snoRNAs play a role in positioning the Ψ modifications. Pseudouridylation is guided by the H/ACA box snoRNAs (*27, 29*). In yeast, 30 Ψ's are found in the LSU rRNA, and 14 have been identified in the SSU rRNA (*8, 9, 60*). Although many

KEYWORDS

Intersubunit bridges: RNA–RNA and RNA–protein interactions at the interface formed upon association of the LSU and SSU during translation.

Pseudouridylation: The isomerization of uridines within RNA to pseudouridine (Ψ) by RNA enzymes known as Ψ synthases.

Methyltransferases: MTases catalyze highly specific transfer of a methyl group from the cofactor *S*-adenosyl-L-methionine to the -*O*, -*C*, or -*N* position of a target nucleotide.

Small nucleolar RNAs: snoRNAs are a class of small noncoding RNA molecules that guide chemical modification (*e.g.*, methylation or pseudouridylation) of eukaryotic rRNAs.

of the modifications appear to only have minor roles in regulating yeast ribosome function, translation is impaired significantly in the absence of Ψ2919 (corresponding to *E. coli* position 2554 in helix 92 of the LSU) in the conserved A loop of the LSU rRNA (*32*).

In certain sequence and structural contexts, Ψ has been shown to stabilize the RNA through a combination of base-stacking (*61–63*) and hydrogen-bonding (*64–66*) interactions. Although direct involvement of the N1-H of Ψ in hydrogen bonding with nucleotide bases has not yet been observed, water-mediated hydrogen bonds to the additional imino group have been reported. In eukaryotic U2 small nuclear RNA, the extra hydrogen bond of a highly conserved Ψ residue was shown to significantly alter the branch-site structure, which further influences the splicing activity (*67, 68*). In this case, the Ψ serves as a conformational switch in which it lowers the free energy of the system and leads to an extrahelical conformation needed for function.

Three highly conserved Ψ residues reside near the PTC of the ribosome: Ψ1911, Ψ1915, and Ψ1917 of helix 69 in domain IV of the LSU rRNA (Figure 2) (*9*). These highly conserved modified nucleotides are located at the ribosome intersubunit bridge B2a, which is also a highly conserved region of the ribosome. In bacteria, Ψ1915 is methylated to $m^3Ψ$ (*69*). These Ψ modifications have opposing effects on helix 69 structure and stability, depending on their locations (*e.g.*, stem *vs* loop regions) (*70*). A recent study suggested that the ribosome-recycling factor (RRF) contacts helix 69 through direct interactions between $m^3Ψ$ of the 23S rRNA and residue Val126 of the protein (*71*), although later studies have shown interactions with the stem region of helix 69 rather than the tip of the loop (*72*). *E. coli* strains with mutations in the rRNA at positions directly at or near the Ψ residues (*e.g.*, Ψ1915A, Ψ1917C, A1912G, A1916G, or A1919G) show severe growth defects, and their ribosomes display weakened A-site tRNA binding, inhibition of translation, low translational fidelity, or defects in 70S formation (*73, 74*). Deletion of A1916 disrupts the intersubunit bridge B2a of the 70S ribosome, promotes misreading of the genetic code, and is lethal in *E. coli* (*75*). The same mutation has been associated with resistance to the antibiotics capreomycin and viomycin in *M. tuberculosis* (*22*). Thus, although not directly associated with the Ψ residues, these antibiotics are in contact with more than one highly modified region of the ribosome.

A recent unexpected finding revealed that when helix 69 is deleted (ΔH69), the resulting ribosomes carry out accurate and full-length synthesis of a phenylalanine peptide from a poly(U) template, with elongation factor (EF)-G-dependent translocation at wild-type rates (*76*). However, some defects are observed in the ΔH69 ribosomes, such as the inability of 50S subunits to associate with 30S subunits in the absence of tRNA, a lack of release factor (RF)1-catalyzed termination, and ribosome recycling in the absence of RRF. Deletion of RluD, the pseudouridine synthase responsible for *in vivo* synthesis of Ψ1911, Ψ1915, and Ψ1917 in *E. coli,* results in loss of the expected Ψ residues in 23S rRNA and defects in subunit association, and cells lacking this enzyme have growth defects (*16, 77*). Thus, helix 69 and the Ψ that reside in this helix seem to have an important role in maintaining ribosome stability. Furthermore, a snoRNA that guides the two most conserved Ψ modifications in the LSU rRNA (2258 and 2260, corresponding to positions 1915 and 1917 in *E. coli* 23S rRNA) confers a growth advantage in yeast (*60*). Despite a strong correlation with the functional sites on the ribosome, the exact roles of these three Ψ residues, as well as the helix in which they reside (helix 69), still remain obscure. However, this is one of the few cases in which a biological function (*e.g.*, ribosome assembly or stability) can be correlated with the presence of conserved modified nucleotides.

Conclusions. rRNAs participate in all of the key interactions of the ribosome during protein synthesis. They must discriminate between functional and nonfunctional interactions during the multistep process of translation. They must also be involved with conformational switching during these processes. It seems reasonable to suggest that the four standard ribonucleotides are not enough for the ribosome to maintain translational fidelity; therefore, a wide range of modified nucleosides is employed. These post-transcriptional modifications occur in regions of the ribosome known for functional importance, a high level of nucleotide conservation, and lacking proteins. Because of the chemical diversity of the modifications (hydrogen-bonding groups, methylation, *etc.*), these nonstandard nucleotides provide the ribosome with a much broader range of possible interactions. The clustering of the modified nucleosides in the most functionally significant regions of the ribosome further supports the idea that they are important to the ribosome

for decoding, peptide-bond formation, and release of the protein products. Although their *individual* effects may be subtle, the *collective* importance of the modified nucleosides is highlighted by the superior performance of native ribosomes compared with reconstituted ribosomes containing unmodified rRNAs transcribed *in vitro*. The mechanisms of insertion for the modifications may be fundamentally different among various organisms, but the presence of highly conserved sites, such as Ψ1915 and Ψ1917 in 23S rRNA, is highly suggestive of their functional relevance. The relationship of methylation of rRNA targets to antibiotic resistance further implicates these residues in a functional role in the ribosome. The addition of a methyl group or imino group (pseudouridine) may possibly lead to changes in the local environment and influence rRNA folding, interactions with other rRNA regions, tRNA, messenger RNA, proteins, or ligands, or it may fine-tune the translation process. Further experiments on modified rRNAs will continue to lead to a better understanding of their biological functions.

Acknowledgment: We are grateful for support from the National Institutes of Health (Grants GM054632 and AI061192). We also thank P. Cunningham for helpful discussions and for sharing unpublished results.

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