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N^2-Methylguanosine is iso-energetic with guanosine in RNA duplexes and GNRA tetraloops

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ABSTRACT

Modified nucleotides are resource-intensive alternatives to the four nucleotides that constitute the bulk of natural RNAs. Yet, even in cases where modifications are highly conserved, their functions are difficult to identify. One possible function might be to modulate the stability of RNA structures. To investigate this possibility for N^2-methylguanosine (m^2G), which is present in a wide variety of RNAs, we have determined the thermodynamic consequences of substituting m^2G for G in G-C Watson–Crick pairs and G·U wobble pairs within RNA duplexes. The m^2G substitution is iso-energetic with G in all cases, except for an internal m^2G·U pair, where it has a modest (0.3 kcal/mol) stabilizing effect. We have also examined the consequences of replacing G by m^2G, and A by N^6,N^6-dimethyladenosine (m_2^6A) in the helix 45 tetraloop of 16S rRNA, which would otherwise be a standard GNRA tetraloop. This loop is a conserved, hypermethylated region of the ribosome where methylation appears to modulate activity. $m^2{}_6A$ substitution destabilizes the tetraloop, presumably because it prevents the formation of the G·A sheared pair it would otherwise contain. m^2G substitution has no effect on tetraloop stability. Together, these results suggest that m^2G is equally stable as either the *s-cis* or *s-trans* rotamer. The lack of a significant effect on secondary structural stability in these systems suggests that m^2G is introduced into naturally occurring RNAs for reasons other than modulation of duplex stability.

INTRODUCTION

Post-transcriptional modifications increase the chemical diversity of the nucleotides found in RNAs. tRNAs are densest in modified bases, but rRNA, snRNA and mRNA are also significantly modified (1). The metabolic cost of modification is very high, yet, with few exceptions, the purposes served are unknown. Among more than 100 naturally occurring nucleotide modifications, the simplest and most common are methylations of nucleotide base or ribose moieties (1). One such example is methylation of the exocyclic amine of G which occurs within tRNA, rRNA and snRNA (1). N^2-methylguanine (m^2G) is found within both helical and looped regions of RNA secondary structure (2,3), and it can exist in either the *s-cis* or the *s-trans* rotamer (Fig. 1). If there is a rotational preference for the methyl group, the effect of m^2G substitution may be specific to the sequence context depending upon which face of the base participates in hydrogen bonding.

Helix 45 of bacterial 16S rRNA provides an excellent example of how post-synthetic methylation can alter RNA function (4). Helix 45, the last stem in 16S rRNA, contributes to the formation of the 30S initiation complex, and forms part of the 30S/50S subunit interface (4,5). The helix is capped by a highly conserved, hyper-methylated GGAA tetraloop, that is modified to yield the sequence m^2G-G-m_2^6A-m_2^6A (where m_2^6A is N^6,N^6-dimethyladenine) (4). Methylation of the first G is conserved in bacteria (6). Methylation of both A residues is also conserved in bacteria, though the loss of adenosine dimethylase activity responsible for methylating these residues confers resistance to the antibiotic kasugamycin (7–9). In contrast, the loss of adenosine dimethylase activity is lethal in yeast (10). These results suggest that base methylation within helix 45 plays an important role in ribosome function.

The solution structure of the fully methylated tetraloop has been determined by NMR, and it is substantially different than the unmethylated GNRA (N = any base, R = A or G) tetraloops previously reported (11). One noticeable difference is the G·A sheared pair, which forms between the first G and the last A in standard GNRA tetraloops, is disrupted in the helix 45 tetraloop due to the dimethylation of the last A. The m^2G-G-m_2^6A-m_2^6A structure does not appear to be directly affected by the m^2G modification, though the structure of the m^2G-G-A-A variant (kasugamycin resistant form) has not yet been determined. In an effort to extend our characterization of the effects of m^2G substitution on RNA structure, we measured the thermodynamic consequences of the methylations in the helix 45 tetraloop from *Bacillus stearothermophilis*.

In addition to being of interest biologically, methylated nucleotides are also being employed in RNA structure/function analyses using a method termed Nucleotide Analog Interference Mapping (NAIM) (12–14). In this approach, the methylated nucleotide is tagged with an α-phosphorothioate linkage, incorporated into the RNA by *in vitro* transcription, and the active RNA molecules in the population separated from the inactive

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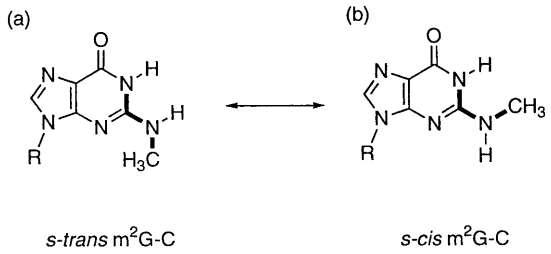

Figure 1. The two possible rotamers of the methylamine of m^2G. (**a**) *s-cis* m^2G. (**b**) *s-trans* m^2G.

variants by a selection assay. Sites intolerant of methylation are identified by iodine cleavage of the phosphorothioate linkages in the active population followed by analysis of the cleavage products (15). While identification of positions intolerant of methylation is fairly straight forward, interpretation of interference patterns can be more difficult. It is important to understand the thermodynamic effects methylation has on duplex stability so that secondary and tertiary interference effects can be distinguished from one another.

To help identify the role played by base methylation, we have determined the thermodynamic consequences of replacing G with m^2G in two different RNA base pairs within model duplexes. We have also examined its effect on the stability of the GNRA tetraloop in helix 45. These measurements include the effect of substituting m^2G in place of G in the context of three base pairing possibilities: an $m^2G \cdot C$ base pair where the methyl group must be *s-trans*, an $m^2G \cdot A$ sheared base pair where the methyl group must be *s-cis*, and an $m^2G \cdot U$ base pair where the methyl group can be in either configuration. In all of these contexts m^2G is essentially isoenergetic with G. We also re-investigated the effects of $m_2{}^6A$ substitution on the stability of the helix 45 tetraloop.

MATERIALS AND METHODS

RNA synthesis and purification

Previously synthesized N^6,N^6-dimethyladenosine and O^6-NPE-N^2-methylguanosine phosphoramidites (16) were incorporated into several RNAs using standard phosphoramidite chemistry. The RNA was cleaved from the solid support and the amino protecting groups were removed in a 3:1 mixture of ammonia (conc.) and ethanol (55°C overnight). To 1 µmol of synthetic RNA, 1.5 ml of neat triethylamine trihydrofluoride was added and agitated for 12–24 h. This mixture was diluted with 300 µl H_2O and precipitated with 10 ml *n*-butanol (–20°C for 4 h). After centrifugation the supernatant was decanted and the RNA pellet dried under vacuum. For oligoribonucleotides containing NPE-O^6 protected N^2-methylguanosine, the NPE group was removed by dissolving the product in 1.8 ml of 1.0 M TBAF in THF and agitating for 24 h. The RNA was precipitated by adding 600 µl of 1 M NaCl, 1.8 ml H_2O and 7.2 ml EtOH, and storing at –20°C for 4 h. After centrifugation the supernatant was decanted and the RNA pellet dried under vacuum. Fully deprotected RNAs were purified by denaturing (5 M urea) polyacrylamide (20%) gel electrophoresis. RNA was eluted from gel slices by repeated incubation (3×25 ml) in 1 M NH_4OAc at 55°C for 3 h. The RNAs were desalted using C_{18} Sep-pak cartridges (Millipore) (17).

Oligonucleotide melting studies and thermodynamics

Oligonucleotide concentrations were calculated from their absorbance at 260 nm. For oligonucleotides containing only A, C, G and U, molar extinction coefficients were calculated from the nearest neighbor approximation (18). For oligonucleotides containing methylated bases, values of the nearest neighbor approximations were based on the observation that plots of the extinction coefficients of X versus XY (X,Y = A, C, G or U) are linear. Thus, by using the molar extinction coefficient of $m_2{}^6A$ (ε_{260} = 11 000) and m^2G (ε_{260} = 13 000), the nearest neighbor contributions were estimated. Absorbance versus temperature curves were measured at 260 nm in 1.0 M NaCl, 1 mM EDTA and 10 mM sodium cacodylate (pH 7.0), for duplexes, and 50 mM NaCl, 5 mM cacodylate (pH 6.3), 1 mM EDTA, for hairpins, with a Cary 3E spectrophotometer using a heating rate of 1°C/min. Van't hoff enthalpies ($\Delta H°$) and melting temperatures (T_m) were determined using the derivative method of Gralla and Crothers (19). The entropy of formation ($\Delta S°$) was calculated using the following equations:

$$1/T_m = R/\Delta H° \ln Ct + \Delta S°/\Delta H°$$

for a self-complementary duplex, and

$$1/T_m = \Delta S°/\Delta H°$$

for monomolecular hairpins. The reported thermodynamic values are the average of at least three trials, usually at different total strand concentrations (*Ct*).

NMR spectroscopy

The RNA was dissolved in 170 µl 90% D_2O/10% H_2O [50 mM NaCl, 5 mM cacodylate (pH 6.3) and 1 mM EDTA] to a strand concentration of 1.8–2.2 mM. Spectra at 5°C were collected using a water flip-back pulse sequence (20) for water suppression on either a Varian Unity (11.7 tesla) or a Varian UnityPlus (14 tesla) spectrometer. Stem imino proton resonances were assigned from sequential imino/imino NOEs using 2D NOESY experiments (data not shown).

RESULTS AND DISCUSSION

RNA containing the modified nucleotides m^2G and $m_2{}^6A$ were prepared by solid phase synthesis (16). Terminal and internal m^2G-C and $m^2G \cdot U$ base pair stabilities were measured for the self-complementary duplexes 5′-XCCGGY-3′ and 5′-GYCUA-GXG-3′ (where X is either G or m^2G and Y is either C or U) (Table 1) (17,21,22). The thermal stabilities of methylated helix 45 variants were addressed in oligonucleotides with the sequence 5′-GGACCXGYYGGUCC-3′ (where X is G or m^2G and Y is A or $m_2{}^6A$). All the RNAs had a single melting transition with sloping upper and lower baselines. The stabilities of the methylated oligonucleotides are compared to those of the corresponding unmodified RNAs in Table 1.

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Table 1. Thermodynamic parameters for G/m^{2}G-C and G/m^{2}G · U base pairs and hairpin analogs of helix 45 from *B.stearothermophilis* 16S rRNA[a]

Pair (X, Y)	$-\Delta H^\circ$ (kcal/mol) [b]	$-\Delta S^\circ$ (cal/mol/K)[b]	T_m(°C)[c]	$-\Delta G^\circ_{37}$ (kcal/mol)	$\Delta\Delta G$[d] (m^{2}G versus G) per base pair
Watson–Crick pairs					
internal: G<u>Y</u>CUAG<u>X</u>C					
C-G[e]	81.63 (±1.88)	218.4 (±6.0)	71.9	13.9 (±0.1)	
C-m^{2}G	82.39 (±3.12)	219.9 (±9.2)	72.9	14.1 (±0.3)	–0.1
terminal: <u>X</u>CCGG<u>Y</u>					
C-G[f]	75.70 (±7.23)	205.8 (±21.0)	65.0	11.9 (±0.7)	
C-m^{2}G	74.50 (±2.61)	202.5 (±8.4)	64.4	11.9 (±0.1)	0.0
Wobble pairs					
internal: G<u>Y</u>CUAG<u>X</u>C					
U•G[g]	73.65 (±2.07)	212.0 (±6.5)	46.8	7.9 (±0.1)	
U•m^{2}G	74.37 (±1.66)	212.4 (±5.5)	49.3	8.5 (±0.0)	–0.3
terminal: <u>X</u>CCGG<u>Y</u>					
U•G[h]	64.15 (±1.34)	176.7 (±3.9)	56.0	9.4 (±0.2)	
U•m^{2}G	64.21 (±3.03)	176.7 (±9.9)	56.2	9.4 (±0.2)	0.0
Tetraloop stabilities:					
GGACC$^{\underline{X}}$G					
CCUGG$_{\underline{Y}}$<u>Y</u>					
				$-\Delta G^\circ_{70}$ [i]	
X = G; Y = A	38.27 (±4.07)	107.7 (±11.3)	82.2	1.3 (±0.2)	
X = m^{2}G; Y = A	41.32 (±4.66)	116.4 (±13.1)	81.8	1.4 (±0.2)	–0.1
X = m^{2}G; Y = m$_2$^{6}A	35.17 (±5.70)	100.0 (±16.4)	78.8	0.9 (±0.1)	+0.4

[a]Values in this table represent at least three melting experiments performed in 1 M NaCl, 10 mM sodium cacodylate (pH 7.0) and 1 mM EDTA for the duplexes or 50 mM NaCl, 10 mM sodium cacodylate (pH 6.3) and 1 mM EDTA for the hairpins.
[b]Four significant figures are reported for ΔH° and ΔS° to avoid rounding errors.
[c]For the duplexes the T_m is at an RNA concentration of 100 µm.
[d]Relative stabilities are calculated by subtracting $-\Delta G^\circ$ for the parental RNA from the ΔG° for the methylated RNA. For the RNA duplexes, the effect per substitution is calculated by dividing the $\Delta\Delta G$ by two. A negative value in the column indicates that the methylated RNA is more stable than the unmethylated RNA.
[e](33). [f](34). [g](17). [h](22).
[i]The ΔG° values for the hairpins are calculated at 70°C to avoid large extrapolation errors.

The melting data indicate that replacement of G by m^{2}G in G-C pairs has no effect on helix stability at either internal or terminal positions. This result is consistent with mononucleoside association studies performed in organic solvents, which showed that the equilibrium constants for G-C and m^{2}G-C pairs are approximately equal (23). However, these data are in sharp contrast to a previous study done using poly(m^{2}G) · poly(C) duplexes (24). If this were also true for mixed sequences, then we would have expected a substantial difference between the stability of an internal and a terminal m^{2}G-C pair, because the methyl group would be less sterically constrained at the end of a helix. The fact that we do not observe such a difference suggests that the RNA minor groove can accommodate a methyl group on the *s-trans* face of the G exocyclic amine. The large disparity between our results and those obtained with poly(m^{2}G) may simply be due to the difference in the levels of m^{2}G incorporation. In the present study, the duplexes contain isolated m^{2}G substitutions, which is typical in natural RNAs; whereas runs of m^{2}G were present in the polynucleotide experiments. Thus, taken together, these two studies suggest that consecutive m^{2}G substitutions have a destabilizing effect on the duplex, presumably due to steric clashes between neighboring methyl groups in the minor groove.

m^{2}G · U pairs are slightly more stable (0.3 kcal/mol) than G · U pairs at internal positions within a helix (Table 1). This stabilization may reflect increased hydrophobic interactions between the methylated nucleotide and the neighboring base pairs (17,25,26). Consistent with this interpretation, the analogous substitution at the end of the helix did not have a positive effect presumably because increased hydrophobicity is less stabilizing in the context of poorly stacked, terminal base pairs.

We also characterized methylated versions of the GGAA tetraloop from 16S rRNA helix 45 to determine the effects of methylating the G in a sheared G · A pair (specifically the $G_6 \cdot A_9$ pair; see numbering in Fig. 3a). To form a sheared m^{2}G · A pair, the methyl group must adopt the *s-cis* rotamer, instead of the *s-trans* rotamer that is required for m^{2}G-C base pairing (Fig. 2). The melting data show that the stabilities of the GGAA and the m^{2}GGAA tetraloops are the same. Although the exocyclic

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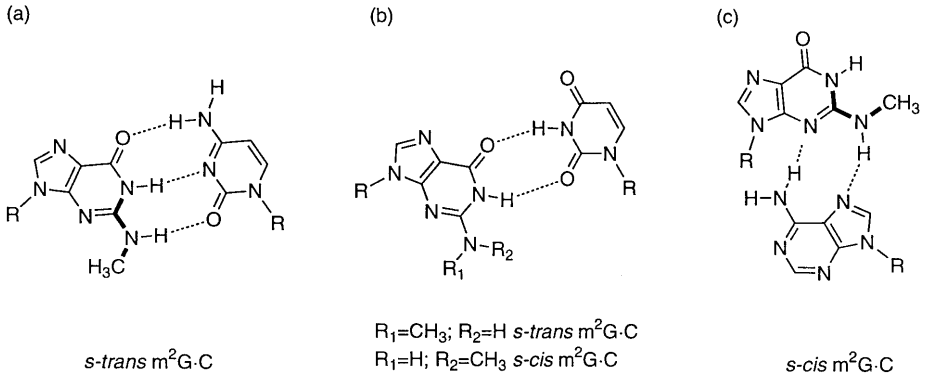

Figure 2. The three types of m^2G pairings investigated. (**a**) In an m^2G-C base pair the methyl group must adopt the *s-trans* rotamer, which places the methyl group into the minor groove of the double helix. (**b**) In an $m^2G \cdot U$ wobble base pair, the methyl group can adopt either the *s-cis* or the *s-trans* configuration, because the exocyclic methylamine is not involved in hydrogen bonding. (**c**) In a sheared $m^2G \cdot A$ base pair the methyl group must adopt the *s-cis* configuration.

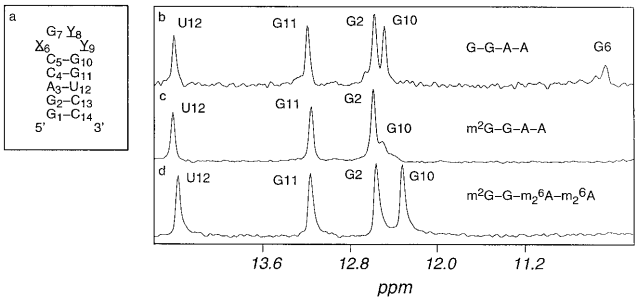

Figure 3. The imino regions from one dimensional ^1H spectra collected at 5°C for three helix 45 tetraloop analogs variants. (**a**) The hairpin sequence and nucleotide numbering is shown and the tetraloop sequence for each sample ($X_6G_7Y_8Y_9$) is shown above each spectrum. (**b**) The imino region of the unmodified parental sequence (X = G; Y = A). The assignment of G_6 was based on previous reports of other GNRA hairpins NMR assignments (31). (**c**) The imino region of the helix 45 tetraloop (X = m^2G; Y = A). The imino resonance of m^2G_6 is absent and the intensity of the G_{10} imino resonance is reduced compared to the corresponding imino resonance in (b). (**d**) The imino region of the wild-type helix 45 tetraloop (X = m^2G; Y = $m_2{}^6A$). As with the spectrum in (c), the m^2G_6 imino resonance is absent; however, the intensity of the G_{10} imino resonance is restored to full strength.

amineof the first G in a GNRA tetraloop contributes ~0.6 kcal/mol to loop stability, our data indicate that there is no net loss in energy when that amine is methylated (27). This implies that the methyl group in m^2G can adopt the *s-cis* conformation in the context of a sheared base pair (Fig. 2).

These results contradict an earlier study that suggested m^2G destabilizes the helix 45 tetraloop by 1.3 kcal/mol (28). The earlier report was ambiguous, however, because the RNA molecules compared in that study differed in the length of their helical stems, and the authors had to include a correction factor to account for this difference. The hairpins used in the present study differ only in the methylation state of G_6, and therefore no correction factor is necessary. In agreement with previous reports, $m_2{}^6A$ substitution of A_8 and A_9 had a modest destabilizing effect on the tetraloop (0.2 kcal/mol per substitution) (29). The primary cause of this loss of stability is likely to be the disruption of the sheared $G_6 \cdot A_9$ pair, which based upon the NMR structure of this tetraloop, does not form when A_9 is methylated (11).

The net iso-energetic effects observed for the G-C/m^2G-C and $G \cdot A/m^2G \cdot A$ pairs are likely to be a balance between weak stabilization due to improved hydrophobic interactions and modest destabilization due to the loss of rotational entropy of the methyl group or weakened hydrogen bonding effects. The values of $\Delta S°$ and $\Delta H°$ do not change significantly in going from a G-C base pair to a m^2G-C base pair and from a $G \cdot A$ base pair to a $m^2G \cdot A$ base pair.

To characterize the structural consequences of tetraloop methylation, we collected one-dimensional, imino proton NMR spectra for all of the variants of the helix 45 tetraloops studied thermodynamically (Fig. 3). In this experiment, imino proton resonances can be observed only if their exchange with solvent is slowed by base pairing or stacking (30). Although the imino proton of G_6 is not involved in a base–base hydrogen bond within the $G_6 \cdot A_9$ sheared pair (Fig. 2), this proton was observed as a weak upfield resonance within the unmethylated hairpin, which is typical of GNRA tetraloops (31). The corresponding resonance was not observed for either the m^2GGAA or the m^2G-G-$m_2{}^6$A-$m_2{}^6$A hairpins. Furthermore, the intensity of the imino proton resonance within the closing G_{10}-C_5 pair is reduced in the spectrum of the m^2GGAA hairpin. This indicates that there is greater solvent exchange for the imino protons of G_6 within the loop and of G_{10} in the closing base pair of the stem, even though the overall stability of the hairpin is unaffected (Table 1). Based on other GNRA tetraloop structures, the exocyclic amine of G_6 in the GGAA tetraloop is close to a non-bridging oxygen of the A_9 phosphate (27,31,32). Introduction of a methyl group in this region may displace the phosphate away from G_6, and lead to increased solvent exchange.

It is difficult to predict the effect of m^2G on RNA secondary or tertiary structural stability because it can exist in either of two rotamers, *s-cis* or *s-trans* (Fig. 2) (23). In this study we substituted m^2G for G in the context of three different base pairs (Fig. 2): (i) an m^2G-C base pair where the methyl group must be *s-trans*, (ii) an $m^2G \cdot A$ sheared pair where the methyl group is likely to be *s-cis*, and (iii) an $m^2G \cdot U$ wobble pair where the methyl group could adopt either conformation. The thermodynamic data suggest that m^2G readily pairs with C, U and A, and that its methyl group has no rotational preference. By contrast, N^6-methyladenosine and N^4-methylcytidine are not so versatile. In both cases the *s-cis* rotamer is preferred (23). Given that m^2G does not effect the

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stability of RNA secondary structure, alternative explanations must be sought to explain their occurrence in a wide variety of RNAs. Its methyl group may prevent the formation of tertiary interactions that could disrupt normal RNA folding, or may create hydrophobic patches on RNA surfaces that contribute to protein–RNA interactions. Because m^2G can form iso-energetic base pairs with several nucleotides, it may be a useful analog to probe tertiary interactions within RNA–RNA and protein–RNA structures (12).

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