Secondary Structure Prediction for RNA Sequences Including N⁶-methyladenosine

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Abstract:

There is increasing interest in the roles played by covalently modified nucleotides in mRNAs and non-coding RNAs. New high-throughput sequencing technologies localize these modifications to exact nucleotide positions. There has been, however, an inability to account for these modifications in secondary structure prediction because of a lack of software tools for handling modifications and a lack of thermodynamic parameters for modifications. Here, we report that we solved these issues for N⁶-methyladenosine (m⁶A), for the first time allowing secondary structure prediction for a nucleotide alphabet of A, C, G, U, and m⁶A. We revised the RNAstructure software package to work with any user-defined alphabet of nucleotides. We also developed a set of nearest neighbor parameters for helices and loops containing m⁶A, using a set of 45 optical melting experiments. Interestingly, N⁶-methylation decreases the folding stability of structures with adenosines in the middle of a helix, has little effect on the folding stability of adenosines at the ends of helices, and stabilizes the folding stability for structures with unpaired adenosines stacked on the end of a helix. The parameters were tested against an additional two melting experiments, including a consensus sequence for methylation and an m⁶A dangling end. The utility of the new software was tested using predictions of the structure of a molecular switch in the MALAT1 lncRNA, for which a conformation change is triggered by methylation. Additionally, human transcriptome-wide calculations for the effect of N⁶-methylation on the probability of an adenosine being buried in a helix compare favorably with PARS structure mapping data. Now users of RNAstructure are able to develop hypothesis for structure-function relationships for RNAs with m⁶A, including conformational switching triggered by methylation.

Introduction:

It has long been appreciated that covalent modification of RNA is used by nature to expand the chemical repertoire of the four common nucleotides. tRNAs, in particular, are known to have prevalent modifications, and the roles of some of these have been elucidated¹. For mRNAs and long non-coding RNAs (lncRNAs), it had been harder to identify sites of modification until recently when new methods were developed using next generation sequencing technologies to identify modifications^{2,3}. Modifications including deamination to inosine^{4,5}, pseudo-uridylation⁶⁻⁸, 5-methylation of cytosine⁹, and N⁶-adenosine methylation¹⁰⁻¹⁵ now can now be localized transcriptome-wide.

N⁶-methyladenosine (m⁶A) is considered the most prevalent modification in mRNA, and m⁶A is also widespread in lncRNAs^{16,17}. It is known to have writers that apply the modifications to specific positions (methyltransferases including METTL3 and METTL14), readers that identify sequences with N⁶-methylation (RNA-binding proteins including YTHDF2 and the YTH family), and erasers (demethylases including FTO and ALKBH5) that can remove the modification, restoring the base to adenine¹⁸⁻²¹. Furthermore, there are hundreds of sites for which the m⁶A modification consensus site is conserved between the mouse and human genomes¹³. The impacts of N⁶-methylation are being elucidated^{22,23}. For example, N⁶-methylation is known to cause structural switches that, for example, can expose protein binding sites that are otherwise not available for binding²⁴. Additionally, m⁶A can regulate splicing²⁵.

RNA secondary structure prediction is in widespread use to help determine structure-function relationships^{26,27}, but has not been generally available for understanding the roles of covalent modifications²⁸. For unmodified sequences, secondary structure prediction has been used to identify microRNA binding sites²⁹, design siRNAs^{30,31}, identify protein binding sites³², and discover functional RNA structures³³⁻³⁵. These types of calculations have not been able to account for modifications without extensive user intervention because a set of nearest neighbor parameters are needed for estimating the folding stability of structures that include modifications^{28,36}. A number of studies have demonstrated an impact on folding stability by modifications³⁷⁻⁴², but no complete set of parameters have been available for RNA folding, as there are for RNA folding with the four prevalent bases⁴³. At the same time, no software has been available for handling a larger alphabet of sequences containing modifications. This led to chicken-and-egg problem; without software, there was no impetus to assemble parameters and without parameters there was no reason to write the software.

In this work, we developed a full set of nearest neighbor parameters for a folding alphabet of m⁶A, A, C, G, and U nucleotides. These parameters account for helix and loop formation, and they are based on optical melting experiments for 32 helices with m⁶A-U base pairs and 13 oligonucleotides with m⁶A in loop motifs. We also modified the RNAstructure software package to accept user-defined folding alphabets and to read and utilize thermodynamic parameters for these extended alphabets⁴⁴. Together, these advances allow the prediction of RNA secondary structures for sequences with m⁶A. We demonstrate, for calculations with human mRNA sequences known to contain m⁶A, that N⁶-methylation alters the folding landscape so that m⁶A is less likely to be buried in a helix, i.e. stacked between two base pairs. We also provide a model for the RNA secondary structures of the methylation-triggered conformational change in the lncRNA metastasis-associated lung adenocarcinoma transcript (MALAT1).

Results:

Overview of Methods: Secondary structure prediction for RNA sequences including m⁶A requires both a set of nearest neighbor folding parameters and software capable of using the set of parameters. An overview of the methods is illustrated in Figure 1. RNA secondary structure prediction requires both parameters for evaluating folding stability and a search algorithm to identify the optimal structure given the parameters^{26,27,45}. In our RNAstructure software, we use nearest neighbor parameters to estimate folding free energy change³⁶ and set of dynamic programming algorithms that predict optimal structures^{46,47}.

We built a database of optical melting experiments of oligonucleotides including m⁶A and then used linear regression to fit nearest neighbor parameters. We also extended the functionality of RNAstructure⁴⁴ to recognize modified nucleotides in sequences and to use parameters for sequence alphabets beyond the four common nucleotides. The m⁶A modification parameters are the first to take advantage of this new feature.

Helix Nearest Neighbor Parameters for m⁶A: The full set of Turner nearest neighbor rules for estimating RNA folding stability are based on optical melting experiments of 802 oligonucleotides and use 294 parameters^{36,48,49}. We have shown, however, that the precision of a subset of parameters is more important than others for the precise prediction of secondary structure⁵⁰. Following that work, we focused our experiments on estimating parameters for helices, dangling ends, and terminal mismatches.

Our first goal was to fit the 15 stacking nearest neighbor parameters for m⁶A-U pairs adjacent to Watson-Crick pairs, G-U pairs, or m⁶A-U pairs. For this study, 29 fully helical duplexes containing m⁶A-U pairs were synthesized and optically melted. This provides a total database of 32 fully helical duplexes with m⁶A-U base pairs. Table S1 provides the duplexes and the stabilities determined by optical melting. These specific oligonucleotide sequences were chosen, in part, because analogous model RNA helices with A in the m⁶A position had been previously studied by optical melting (with the exception of GGUAACC₂). This allows us to directly compare the folding stability with and without N⁶-methylation. We calculated the change in folding stability ($\Delta\Delta G^\circ_{37}$) per methylation as compared to the unmethylated duplex. Figure S1 shows that the $\Delta\Delta G^\circ_{37}$ is highly dependent on the adjacent sequence, ranging from +2.1 to -0.1 per methylation where positive free energies are destabilizing for methylation. Therefore, to estimate folding stabilities for duplexes with m⁶A-U pairs, a full nearest neighbor model is needed to account for the sequence dependency.

Linear regression was used to fit the nearest neighbor parameters for folding free energy change. Figure 2A shows the increments in comparison to the same stack with A-U pairs and Table S2 provides the values. The free energy changes range from -1.79±0.25 kcal/mol to +1.45±0.57 kcal/mol. As expected based on prior optical melting experiments for duplexes with m⁶A-U pairs^{37,39}, nearest neighbor stacks for methylated A-U pairs are less stable than stacks for unmethylated A-U pairs. On average, the stacks with m⁶A-U pairs are 0.4 kcal/mol less stable per methylation. There are exceptions, however; an m⁶A-U pair followed by a U-A pair is as stable as an A-U pair followed by a U-A pair (-1.10 kcal/mol). The most unstable stack has two m⁶A-U pairs. Like A-U pairs, when the m⁶-U pair is adjacent a G-C it is more stable than when adjacent to A-U. Also like A-U pairs, m⁶A-U pairs adjacent to G-U are less stable than those adjacent to A-U pairs.

An unexpected feature of terminal m⁶A-U pairs is that they require no terminal penalty, although terminal A-U pairs receive a $+0.45 \pm 0.04$ kcal/mol penalty per A-U pair at the end of a helix⁵¹. Two findings support this. First, when a terminal parameter is included as a parameter in the linear regression fit, the value is $+0.13 \pm 0.17$ kcal/mol, which is not significantly different from 0 kcal/mol. Second, our dataset includes two helices with the same nearest neighbor stacks, but with different helix ends (Figure 2B). Previously, it was noted that this pair of helices, when unmethylated, had markedly different stability (0.70 ± 0.28 kcal/mol), with the helix with A-U ends less stable⁵¹. For the methylated helices, the difference is small (0.18 ± 0.27 kcal/mol). This demonstrates that a terminal m⁶A-U base pair has overall similar stability to a terminal A-U base pair because a terminal A-U pair has a more favorable stack but requires the terminal A-U penalty.

Loop Nearest Neighbor Parameters for m⁶A: For secondary structure prediction, parameters need to also be extrapolated for loop formation. The stability of a 3' dangling m⁶A had been previously measured³⁷. Additional optical melting experiments were performed for two m⁶A 3' dangling ends, an m⁶A 5' dangling end, and seven terminal mismatches involving at least one m⁶A. One hairpin loop was measured with an m⁶A in the loop and not adjacent to the helix end. One 2x2 internal loop was measured with symmetric tandem G-m⁶A pairs. The loop sequences were chosen such that analogous sequences with A instead of m⁶A had been previously studied, so that the effect of methylation on stability can be quantified. Table S3 provides the measured stabilities for these model structures and Table S4 shows the stability of the loop motif in comparison to the motif with A.

As shown by Figure 3, an m⁶A as a dangling end or as a component in a terminal mismatch stabilizes secondary structure formation to a greater extent than an analogous A. On average, the m⁶A dangling end is -0.43 ± 0.15 kcal/mol more stable than the analogous A dangling end for the 3' and 5' dangling ends studied here. Terminal mismatches for m⁶A-m⁶A, G-m⁶A, m⁶A-G, and m⁶A-C on Watson-Crick or G-U terminal pairs are on average -0.28 ± 0.26 kcal/mol more stabilizing than the analogous A-A, G-A, A-G, or A-C terminal mismatches. This stabilizing effect is sequence dependent; the $\Delta\Delta G^\circ_{37}$ ranges from -0.74 kcal/mol (G-m⁶A mismatch on a U-G pair) to $+0.02$ kcal/mol (G-m⁶A mismatch on an A-U pair). An m⁶A-m⁶A mismatch on an m⁶A-U pair is more stable than the m⁶A-m⁶A mismatch on an m⁶A-U pair by -0.42 ± 0.40 kcal/mol.

The hairpin loop structure with m⁶A is marginally less stable than the analogous hairpin loop with A ($\Delta\Delta G^\circ_{37} = 0.23 \pm 0.24$ kcal/mol; Table S4). The 2x2 internal loop with tandem G-m⁶A pairs is also marginally less stable than the analogous loop with tandem G-A pairs ($\Delta\Delta G^\circ_{37} = 0.33 \pm 0.53$ kcal/mol; Table S4). Both stability changes are within the uncertainty estimates, suggesting that they are not substantial differences.

Additional Experiments to Test the Parameters: To test our parameters, we performed additional melts of duplexes. The first is a duplex with all base pairs, incorporating a consensus N⁶-methylation site, GGACU, where we determined the helix stability with and without methylation. The second is an additional 3' dangling m⁶A to test our assumption that dangling m⁶A are stabilized by -0.3 kcal/mol compared to dangling A. Table S5 provides the stabilities determined by optical melting and Table S6 shows how well the stabilities are estimated with our nearest neighbor parameters.

We conclude from these tests that the nearest neighbor parameters are accurate enough to be used for RNA secondary structure prediction^{48,50}. The estimates for the duplex stabilities are within the uncertainties propagated for the experiment and the nearest neighbor parameters ($\Delta\Delta G^\circ_{37}$ column of

Table S6). The unmethylated consensus duplex is estimated by nearest neighbor parameters to be more stable (by -0.48 ± 0.73 kcal/mol) than it is by experiment. The methylated consensus duplex is estimated by nearest neighbors to be less stable than it is (by 0.85 ± 0.97 kcal/mol). These deviations are 2.9% and 5.5% of the experimentally determined values. The estimated stability of the duplex with the dangling m⁶A closely matches the experimental value ($\Delta\Delta G^\circ_{37}$ of 0.01 ± 0.84 kcal/mol).

RNAstructure Software Modifications: To predict RNA secondary structures for sequences with A, C, G, U, and m⁶A, we modified the command line programs in the RNAstructure software package to accept extended alphabets of nucleotides⁴⁴. By default, the software interprets sequences as standard RNA, but a command line switch can specify an alternative alphabet. For example, the nearest neighbor parameters for a DNA alphabet composed of A, C, G, and T has long been available. Now, because of this work, the nearest neighbor parameters for an RNA m⁶A alphabet is available.

The key to an extended alphabet is the specification of the nucleotides and pairs (Figure S2). A common architecture across the RNAstructure programs means that the command line programs are capable of using the extended alphabets, which can include any number of characters. This includes the prediction of minimum free energy structures, base pair probabilities, maximum expected accuracy structures, and folding stability for structures. Each nucleotide must be encoded by a single-character, and we chose “6” or “M” as the character to encode m⁶A in sequences and in the m⁶A nearest neighbor parameter tables. The Methods section details our estimates for the m⁶A nearest neighbor parameters.

Nearest neighbor parameter tables are read from disk as programs start. Each parameter table requires additional rows and columns to provide the nearest neighbor parameters values for those nucleotides, although the dimensionality of the tables stays the same. For example, a base pair stack table is four-dimensional because the sequence of four positions is required to estimate the stacking stability of two pairs. When m⁶A is included with RNA, the size of each dimension is increased to five from four. The largest table is the 2×2 internal loop lookup table³⁶, which is eight dimensional because it includes the sequence of the two closing base pairs.

Modeling Conformational Changes as a Result of Methylation: It has been established that N⁶-methylation can alter RNA structure, and because of this m⁶A is considered a conformational switch. To test our new m⁶A nearest neighbor parameters and software, we made a quantitative prediction for the switching of the structure in the lncRNA MALAT1 that opens a binding site for heterogeneous nuclear ribonucleoprotein C (HNRNPC). This has been characterized by Tao Pan and co-workers in an *in vitro* system with a single stem-loop structure⁵². Filter binding experiments demonstrated that the methylated RNA is more accessible to protein binding than the unmethylated RNA. Additionally, enzymatic cleavage by RNase S1, which has specificity for loop regions of RNA, demonstrated increased cleavage 5' and 3' to the methylated A, supporting a conformational change.

We used RNAstructure to predict the secondary structure of the 32 nucleotide RNA using stochastic sampling of the structures from the ensemble⁵³. This can be used to characterize the structures of RNAs that can fold to more than one structure at equilibrium. We found two predominant structures in the ensemble, as demonstrated in Figure 4A. One of the two structures is that of the stem-loop that was previously predicted and has three of the five nucleotides at the HNRNPC site base paired⁵². Interestingly, the second major structure in the ensemble has two stem-loops and the HNRNPC site is more exposed for protein binding (a single U at the 5' end of the binding site is base paired). RNAstructure estimates a shift in the population from the closed (protein-occluded) structure

to the open (protein-accessible) structure in agreement with the experimentally measured shift in protein binding. In the absence of methylation, the ratio of closed:open is estimated to be 62:38, but in the presence of N⁶-methylation the ratio is estimated to be 41:59. This demonstrates a quantitative prediction of shift in ensemble folding behavior with methylation that explains how the methylation accomplishes the structural switching.

Furthermore, we probed the 32 nucleotide RNA (extended with a 3' structural cassette) by chemical mapping with CMCT (1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate), DMS (dimethyl sulfate), and kethoxal (Figure 4A). The chemical mapping data and the prior enzymatic mapping⁵² data are consistent with the sequence populating more than one structure; neither of the two proposed structures alone fully explains the data. Nuclease S1 prefers to cut in loop regions and Nuclease V1 prefers to cut in helical regions⁵⁴, but a number of cleavages occur in tandem at the same phosphodiester bond. This indicates a mixture of structures. The loss of V1 cleavages between U10 and U11 supports an increase in the population of the open structure upon methylation. The S1 cleavage that emerges 5' and 3' to m⁶A22 upon methylation is consistent with the increase in population of the open structure. The chemical mapping data also suggest a mixture of two structures; the three agents used react with moieties on the Watson-Crick faces and paired bases are generally more protected than unpaired bases⁵⁴. A number of base reactivities support a population of the closed structure for both sequences, including C16, A17, U19, and U20. The CMCT reactivities at U26 and U30 support the second hairpin loop in the open structure, both with and without methylation. The loss of reactivity upon methylation at C15 and the gain of kethoxal reactivity upon methylation at G29 are consistent with the shift towards the open conformation upon methylation. In summary, the structure predictions with m⁶A parameters provide a more complete picture of conformational switching, which is more complicated than a simple on-off switch and better reflected as a shift in the Boltzmann-weighted ensemble between conformations.

Transcriptome-Wide Predictions with m⁶A: To further test our new m⁶A nearest neighbor parameters and software, we predicted structures for 18,026 mRNAs that were identified as having N⁶A methylation by whole transcriptome sequencing⁵⁵ and for which PARS structure mapping data are available⁵⁶. We used the nearest neighbor parameters and RNAstructure package to estimate the probability that the methylation site is buried in a helix, i.e. in a base pair stacked between two other base pairs, for both the unmethylated and methylated sequence (Figure 4B). We used 800 nucleotide fragments of local sequence to estimate the pairing probability because we previously found that pairing probability estimates for 800 nucleotide fragments reasonably match those for global secondary structure prediction³⁰. This is a reasonable balance between accuracy and total calculation time.

We find that the unmethylated A at the methylation site is less likely to be buried in a helix than adjacent nucleotides (Figure 4B). This is intuitive because adjacent nucleotides at the consensus site are often G or C, and A is more predominant in loops in RNAs with known structure⁵⁷. There is a substantial shift in the probability of m⁶A being buried in a helix relative to A (21% for A and 13% for m⁶A). This suggests there could be widespread structural switching being affected by N⁶-methylation. We can also compare our results to PARS data for the same sequences (Figure 4C)^{39,56}. A PARS score quantifies the enzymatic cleavage estimate of local pairing and the experiment is performed transcriptome wide. A lower PARS score indicates greater nuclease S1 cleavage relative to nuclease V1 and thus a greater extent of unpairing because nuclease S1 has specificity for loops and nuclease V1 has specificity for helices^{54,58-60}. The PARS scores at the methylation site also demonstrate a propensity to be unpaired at

the methylation site, but the minimum average PARS score is at the nucleotide 5' to the m⁶A site. A possible explanation for the discrepancy is that PARS attributes S1 cleavages to the base 5' to the cleavage site, assuming that the base 5' to the cleavage is unpaired. Cleavage can also occur when the base 3' to the cleavage site is unpaired and therefore the PARS scores 5' to the methylation site might be overestimating the propensity of being unpaired, in that some of the propensity of being unpaired should be attributed to the methylation site. For example, the prior S1 mapping of 5S rRNA structure is consistent with cleavages both 5' and 3' to unpaired nucleotides (Figure S3)⁶¹. Prior analysis of PARS scores for methylation sites also concluded that the data indicate the m⁶A is positioned in structures at the transition between base paired regions and loop regions, consistent with our structure prediction estimates³⁹.

Discussion:

Here we provide the first complete nearest neighbor model for a folding alphabet including modified nucleotides. Because m⁶A is considered the most abundant modification in mRNA and is known to affect folding stability, we chose m⁶A as the first modification to study. The full nearest neighbor model for secondary structure prediction requires both helical stack parameters and also loop parameters. We know from a sensitivity analysis of secondary structure prediction that, for loops, accurate parameters are most important for dangling ends and terminal mismatches^{48,50}, accordingly we focused our experimental effort on these motifs. We also observed marginal differences in stability for hairpin and internal loops containing m⁶A as compared to the same sequences without the N⁶-methylation. Subsequent studies could be focused on understanding and modeling folding stability differences for loops with m⁶A.

The other component of this study was advancing RNAstructure to work with sequences with nucleotides beyond A, C, G, and U. We provide command line tools that are ready to make quantitative predictions of structure and folding stability for sequences with m⁶A. Given the software, we plan expand our work in the future to include alphabets with inosine and pseudouridine. Both have helical nearest neighbor parameters available for stacks on Watson-Crick pairs^{40,41,62}, and both could be extended to full nearest neighbor parameters sets with additional optical melting experiments.

The two loops studied here with N⁶-methylations both had marginally less folding stability than the analogous unmethylated loops. Solution structures are available for each of the A-containing loops, and these structures provide clues as to why the stabilities would be only marginally changed by methylation. The hairpin loop, GGCGUAAUAGCC, has the first A in the loop (A6; the site of our m⁶-methylation) stacked at the apex of the loop on the adjacent A (A7)⁶³. Because A6 is not hydrogen bonding in the structure, a methylation at N⁶ can be accommodated in the preferred syn orientation by the structure without change⁶⁴. For the internal loop with tandem G-A pairs, the pairs are trans-sugar-Hoogsteen pairs, i.e. the N⁶ position of the A is hydrogen bonded with the G at the N³ position⁶⁵. For each methylated A, one hydrogen of N⁶ is available to form this hydrogen bond, placing the methyl in the preferred syn orientation⁶⁴. However, the second hydrogen of A N⁶ is close to O4' of the G (ranging from 2.34 to 3.36 Å in the 15 deposited NMR models). This suggests the structures would need at least small changes to accommodate the syn methyl to avoid a steric clash^{39,64}.

Recent studies demonstrated the ability of computational methods to estimate folding free energy changes⁶⁶⁻⁷². In this work, we performed optical melting experiments to determine the folding stabilities of small model systems with m⁶A and fit nearest neighbor parameters to these data. Future work, however, could rely on computation or a mixture of computation and experimentation. Hopfinger et al., for example, estimated helical stacking nearest neighbor parameters for the eight stacks with m⁶A-U pairs adjacent to Watson-Crick pairs⁶⁶. Overall the agreement of their estimates against our experimental values is excellent, with a root mean squared deviation of 0.30 kcal/mol. The largest single deviation is for a U-m⁶A pair followed by a G-C pair, where their estimate overstabilized the stack by 0.6 kcal/mol (Figure S4). Loop folding stabilities continue to be more of a challenge to estimate using computational methods because the conformational flexibility requires extensive sampling⁷¹.

With this work, we demonstrate the position of m⁶A in a structure determines whether folding stability is increased, decreased, or unchanged relative to the same structure with A. It was previously known that N⁶-methylation of an A-U pair in the middle of a helix would decrease the helix folding stability^{37,39}. Our stacking parameters now quantify this sequence-dependent change (Figure 2A). It was also previously known that m⁶A stacking on the end of a helix would stabilize the helix more than an analogous A. In this work, we also discovered that an m⁶A-U base pair at the terminal position of a helix provides roughly the same folding stability as an analogous A-U base pair. This is because terminal A-U pairs destabilize helices with a penalty of +0.45 kcal/mol⁵¹ that is not needed for terminal m⁶A-U base pairs (Figure 2B). Recently, it was also discovered that terminal G-U base pairs in helices do not need an end penalty⁷³. These results, taken together, show why N⁶-methylation is a potent switch of secondary structure.

Our transcriptome-wide calculations also suggest that structure switches from N⁶-methylation might be widespread (Figure 4B). It will be interesting to perform similar calculations with other widespread covalent modifications, such as inosine. There is potential to identify structural mechanisms by which covalent modifications exert changes in protein binding, transcript stability, or gene expression.

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Competing Interests statement:

The authors declare no competing interests.

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Methods:

Synthesis of Oligonucleotides with m⁶A: Oligoribonucleotides were synthesized on a BioAutomation MerMade12 DNA/RNA synthesizer using β -cyanoethyl phosphoramidite chemistry and commercially available RNA phosphoramidites (ChemGenes, GenePharma) and protected N⁶-methyladenosine phosphoramidite, which was synthesized according to a standard protocol. (Synthesis of N⁶-methyl adenosine via Dimroth rearrangement followed by protection of the 5'-hydroxyl with dimethoxytrityl and 2'-hydroxyl with tert-butyldimethylsilyl. Next, 5'- and 2'-protected N⁶-methyladenosine was treated with 2-cyanoethyl N,N,N',N'-tetraisopropylphosphorodiamidite^{74,75}.) Oligoribonucleotides were deprotected with aqueous ammonia/ethanol (3/1 v/v) for 16 h at 55°C. Silyl protecting groups were cleaved by treatment triethylamine trihydrofluoride. Deprotected oligonucleotides were purified by silica gel thin layer chromatography (TLC) in 1-propanol/aqueous ammonia/water (55/35/10 v/v/v) as described previously^{51,75}.

Optical Melting Data: The thermodynamic measurements were performed for nine various concentrations of RNA duplex in the range of 0.1 mM – 1 μ M in buffer containing 1 M sodium chloride, 20 mM sodium cacodylate, and 0.5 mM Na₂EDTA, pH 7. Oligonucleotide single strand concentrations were calculated from the absorbance above 80°C and single strand extinction coefficients were approximated by a nearest-neighbor model⁷⁶. Absorbance vs. temperature melting curves were measured at 260 nm with a heating rate of 1°C/min from 0 to 90°C on JASCO V-650 spectrophotometer with a thermoprogrammer. The melting curves were analyzed and the thermodynamic parameters calculated from a two-state model with the program MeltWin 3.5⁷⁷. For most model RNAs, the ΔH° derived from T_M^{-1} vs. $\ln(C_T/4)$ plots is within 15% of that derived from averaging the fits to individual melting curves, as expected if the two-state model is reasonable.

Linear Regression: Linear least-squares fitting to determine RNA stacking stabilities was performed with a custom Python program using the statsmodels ordinary least-squares class (OLS)⁷⁸. For each duplex, to determine the stabilities to be fit, the fixed terms were subtracted, including the stability of base pair stacks with Watson-Crick and G-U pairs only, the duplex initiation term, the terminal A-U penalty term (when needed), and the symmetry term (when needed). The fit was excellent, with coefficient of determination, R^2 , of 0.984. Uncertainty estimates (Figure 2A and Table S2) are the standard errors of the regression. Table S7 shows the stability to be fit and the estimate of the fit. Table S8 shows the number of occurrences of each stacking parameter in the set of fit helices.

Loop Motif Stability Calculations: Loop motif stabilities (Table S4) are calculated by subtracting the helical component of stability.

For the dangling ends and terminal mismatches, twice the stability increment of the motif is determined by subtracting a reference helix stability from the stability of the duplex with the motif⁷⁹:

$$2 \times \Delta G^{\circ}_{37 \text{ motif}} = \Delta G^{\circ}_{37 \text{ duplex with two motifs}} - \Delta G^{\circ}_{37 \text{ reference duplex without the motifs}}$$

The factor of two is present because the self-complementary duplexes have two instances of the motif.

For the hairpin loop, the stability of the loop motif is determined by subtracting the stability of the helical stacks (estimated with nearest neighbor parameters) from the total stability⁸⁰:

$$\Delta G^{\circ}_{37 \text{ hairpin loop}} = \Delta G^{\circ}_{37 \text{ stem-loop}} - \Delta G^{\circ}_{37 \text{ helical stacks}}$$

The total helical stack stability is reported as the Reference ΔG°_{37} in Table S4.

For the internal loop, the stability is the total stability of the duplex minus the helical stacks (estimated with nearest neighbor parameters) and minus the stability cost of symmetry (because the duplex is self-complementary)⁸¹:

$$\Delta G^{\circ}_{37 \text{ internal loop}} = \Delta G^{\circ}_{37 \text{ duplex with internal loop}} - \Delta G^{\circ}_{37 \text{ helical stacks}} - \Delta G^{\circ}_{37 \text{ symmetry}}$$

The Reference ΔG°_{37} reported in Table S4 is the sum of the helical stacks and symmetry free energy increments.

Error Propagation: To estimate uncertainties in free energies (σ), we propagate uncertainty estimates for experiments and nearest neighbor parameters using the standard method for uncorrelated parameters:

$$\sigma^2 = \sum_i \left(\sigma_i^2 \frac{\partial \Delta G^{\circ}}{\partial \Delta G^{\circ}_i} \right)^2$$

where ΔG°_i is the i^{th} term and σ_i is the uncertainty in the i^{th} term^{50,82}. For the sum of terms used here, this simplifies to:

$$\sigma^2 = \sum_i (n_i \sigma_i)^2$$

where n_i is the number of occurrences of the i^{th} parameter.

For uncertainty estimates for optical melting experiments, we use 4% of the magnitude of the ΔG°_{37} . This was chosen as a conservative estimate of the precision of optical melting by Xia et al.⁵¹. It is twice the mean difference in free energies determined using the two fit methods for optical melting data (Average of Curve Fits and Analysis of T_M Dependence) for a database of optical melting experiments.

Nearest Neighbor Parameter Determination: Nearest neighbor parameters were developed to estimate the folding stability (ΔG°_{37}) of sequences with A, C, G, U, and m⁶A. Nearest neighbor parameters are inherited from the 2004 Turner Rules³⁶, where a summary of their derivation can be found in Zuber et al.⁴⁸ and examples for their use are available on the Nearest Neighbor Database (NNDB)⁴³ website. Helical stacking tables are from Xia et al.⁵¹ for Watson-Crick stacks and from Chen et

al.⁷³ for stacks that contain G-U pairs, supplemented with the new stacks determined for m⁶A-U pairs in this work. Following Chen et al., terminal G-U base pairs in a helix are not penalized.

Dangling end m⁶As are stabilized as compared to the analogous A dangling end by the mean additional stability found here (-0.4 kcal/mol). Dangling ends on m⁶A-U pairs are assumed to be the same stability as dangling ends on A-U base pairs. When the stability is measured by an experiment, the measured value is used (Table S4).

Terminal mismatches involving m⁶A are estimated to be more stable than the analogous A terminal mismatch by the mean value found for the terminal mismatches in this study (-0.3 kcal/mol). M⁶A-m⁶A terminal mismatches receive only -0.3 kcal/mol additional stability. A terminal mismatch on an m⁶A-U pair is also stabilized by -0.3 kcal/mol compared to the analogous mismatch on an A-U pair. These effects are additive; an m⁶A-containing terminal mismatch on an m⁶A-U pair receives an additional -0.6 kcal/mol stability than the analogous terminal mismatch with all A parameters. When the stability is measured by an experiment, the measured value is used (Table S4).

Hairpin, internal, and bulge loop initiation costs are length-dependent³⁶. The same length-dependent costs are used here, which assumes that m⁶A does not alter the initiation costs.

1×1, 2×1, and 2×2 internal loop stabilities are stored in lookup tables. The stabilities for loops with unpaired m⁶A are taken from the analogous loops with A. And m⁶A-U-closed loops are taken from analogous A-U-closed loops with one change. A-U-closed loops have a 0.7 kcal/mol stability penalty per closure³⁶; for m⁶A-U-closed loops, this cost has been removed compared to the analogous A-U-closed loop. Larger internal loops use a terminal stacking table to assign a stability increment for the sequence of the closing pair and first mismatch. Separate tables are used for loops of size 1×n, 2×3, and (>2)×(>2)³⁶. These terminal stack tables use the analogous A parameter for stacks with m⁶A. The one exception is that the +0.7 kcal/mol internal loop A-U pair closure penalty is removed for m⁶A-U closures.

Hairpin loop tables for triloop, tetraloops, and hexaloops are unchanged. These tables include stabilities for specific hairpin sequences known by experiment to not be well predicted using nearest neighbor rules³⁶. Other hairpin loops are estimated with the sum of a terminal mismatch and a length-dependent initiation. The terminal mismatches for m⁶A use the analogous A parameter.

Multibranch loop initiation parameters are from an experimental fit using a simple linear model^{83,84}. The ersatz functional form was found to perform well in a study testing alternative functional forms⁸⁵. Coaxial stacking is included in multibranch and exterior loops³⁶. Coaxial stacking between two adjacent helices is assumed to be as stable as a helical stack. For coaxial stacks with an intervening mismatch, there are two stacks. The coaxial stacking increment for the stack where the backbone is not continuous was previously found to be independent of sequence⁸⁶, and the sequence-independent value is used here for stacks involving one or more m⁶As. The other stack is identical to the terminal mismatch stack table.

Extended Alphabet Implementation in RNAstructure. RNAstructure is a software package written in C++, with a C++ class library that is also wrapped using SWIG to be available to JAVA or Python programs⁴⁴. It is open source and provided for free under the GNU GPL license version 2 at <https://rna.urmc.rochester.edu>. A number of the command line programs have been updated to be capable of using extended alphabets, including Fold⁸⁷ (secondary structure prediction by free energy

minimization), efn2⁸⁸ (estimation of folding free energy changes for secondary structures), partition^{47,89} (partition function calculations for estimating pair, motif, or structure probabilities). A number of programs that rely on the partition function calculations are therefore also able to consider extended alphabets, including AllSub⁹⁰ (prediction of all low free energy structures within an increment of the lowest), design⁹¹ (design of a sequence to fold to a specific secondary structure), EnsembleEnergy (calculation of the ensemble folding free energy change), MaxExpect^{84,92} (prediction of maximum expected accuracy structures), ProbKnot⁹³ (prediction of structures that can include pseudoknots), ProbScan⁹⁴ (estimation of motif probabilities), and stochastic⁵³ (stochastic sampling from the Boltzmann ensemble).

The command line tools read the thermodynamic parameters at startup. The switch --alphabet is used to specify the set of parameters to be used. The default is "rna", the current (2004) Turner rules for estimating RNA folding free energy changes^{36,51}. Included with the latest RNAstructure release (version 6.3) is also "m6A", the parameters discussed here, and "dna", a set of nearest neighbor rules for DNA secondary structure prediction. The files are in a plain text format that was updated (in version 6.0) for extended alphabets. The specification file (Figure S2) is read first, and defines the alphabet and base pairs. Dynamic memory allocation is used to provide the memory needed to store the tables. The parameters themselves are then read from the files.

The 2004 Turner rules gave a terminal base pair penalty for any base pair (A-U or G-U) at the end of a helix that contained a U^{36,51,88}. In this work, we found that terminal m⁶A-U pairs did not require this terminal base pair penalty. Additionally, the revised G-U parameters⁷³, used with the m⁶A parameters we derived, do not require a terminal base pair penalty. Therefore, we changed the implementation of the energy function to account for this change.

MALAT1 Calculations: The secondary structure of the 32-nucleotide fragment of MALAT1 was predicted with and without N⁶-methylation of A22 using the stochastic program in RNAstructure⁴⁴. The ensemble size was set to 100 structures. The predicted ensembles show fluctuations in pairs around the two predominant structures, as expected. As examples, terminal base pairs for helices are variably present and the lower helix in the Open structure of Figure 4A can be absent in the Open structure. To classify each sampled structure as Open or Closed, the hamming distance on base pairs was calculated to each the Open and Closed conformation, and the structure was assigned to the conformation with lower distance.

Chemical mapping of RNA and data analysis: DMS (to modify adenosine and cytidine), CMCT (to modify uridine and guanosine) and kethoxal (to modify guanosine) were used to chemically map secondary structure of 32 nucleotide RNA (with a 3'-structural cassette). The RNA (5'AACUAAUGUUUUUGCAUUGGACUUUGAGUUACCUUCCGGGCUUCGGUCCGGAAC) was synthesized using the phosphoramidite method on a MerMade synthesizer, deprotected and purified on a 12% denaturing gel. The RNA contained a structure cassette at the 3' end (underlined), which was designed using RNAstructure to fold independently and allow readout of whole structure of studied RNA⁹⁵. The RNA contained C16-2'-OMe instead of a standard C nucleotide at position 16, introduced to prevent nonenzymatic spontaneous cleavage between C16 and A17^{96,97}. For each reaction, 10 pmol of RNA was folded in buffer containing 300 mM NaCl, 10 mM Tris-HCl, 5 mM MgCl₂ pH 8.0. Briefly the appropriate amount of RNA was diluted in H₂O and heated 3 min in 80 °C followed by slow cooling. Then, at 50 °C a concentrated buffer was added to get final buffer solution and sample was continuously slowly cooled.

After 10 min incubation at 4 °C chemical mapping was conducted using two concentration of each reagent. To a 9 µl sample, 1 µl of 300 mM or 160 mM DMS in ethanol was added to give a final concentration of 30 or 15 mM DMS. For modification with CMCT, 9 µl of CMCT solution was added to the 9 µl of RNA sample. CMCT was diluted in a folding buffer to give a final concentration of 250, and 100 mM in the reaction mixture. Kethoxal was diluted in ethanol/water (1:3 v/v) to give a final concentration of 160 and 80 mM. After modification with kethoxal, 3 µl of 35 mM potassium borate solution was added to stabilize products of modification. Chemical modification reactions were incubated for 1.5 h at 4 °C. Reactions were stopped by precipitation with ethanol. The chemical modification reactions were repeated for a total of two replicates of each agent. The RNA in control reactions was treated the same, except no chemical reagents was added.

Modification sites were identified by primer extension. The DNA primer for reverse transcription (RT) was synthesized with 6-fluorescein (FAM) on the 5' end (5'FAMGTTCCGGACCGAAGCCCG). The DNA primer was complementary to 3' end of RNA (the cassette part). For each reverse transcription reaction, 10 pmol of primer was used. Primer extension was performed at 55 °C with SuperScript III reverse transcriptase using Invitrogen's protocol. Reactions were stopped by addition of loading buffer containing urea and 10 mM EDTA, then chilling on ice. Prior to separation and read-out of cDNA products the samples were heated for 5 min at 95 °C and then separated on a 12% polyacrylamide denaturing gel (Figure S5).

The gel image from the Phosphorimager was analyzed using SAFA program to quantify nucleotide reactivities⁹⁸. cDNA products were identified by comparing to sequencing lanes and to control lanes and the raw results from SAFA were normalized. To quantify chemical modification at each nucleotide, we first corrected for the background by subtracting the volume integral of the band in the control lane from the volume integral of experimental lane. For each of two experiments for each modification agent and each sequence, we characterized the modification extent by quartiles. When a nucleotide was in the highest quartile of RT stops in both experiments, we report the mapping as strong (Figure 4A). When a nucleotide was in the second highest quartile in both experiments or the highest quartile in one and the second highest quartile in the other, we report the cleavage as moderate.

Transcriptome-Wide Calculations: We downloaded the set of m⁶A positions reported in the human transcriptome by Schwartz et al.⁵⁵, which was available as their Supplementary Table S2. Using a Python program, for each entry for the human genome of “high confidence category” and with a RefSeq entry, we fetched the sequence from RefSeq⁹⁹ using the Bio.Entrez module from Biopython¹⁰⁰. To identify the exact position of the m⁶A in the transcript, we used the provided hg19 coordinates to identify the A in one of the expected sequence motifs (GGACA, GGACT, GGACC, GAACT, AGACT, AGACA, or TGACT) using the twobitreader Python package¹⁰¹ and the hg19 sequence downloaded from the UCSC genome browser¹⁰². Once the motif was identified in the genome, the sequence was found in the RefSeq sequence, and an 800 nucleotide FASTA sequence was generate with the m⁶A position at the 401st position. For sequences in which the m⁶A was too close to the 5' end or 3' end to be in the 401st position, up to 800 nucleotides were extracted with the m⁶A position at either the 5' end or 3' end. Sequences were generated with both A and 6 at the m⁶A position. In total, 18,155 high confidence m⁶A sites were found.

Next, the partition function was calculated for each 800 nucleotide sequence using the partition program from RNAstructure⁴⁴. To determine the probability that the m⁶A position was buried in a helix, a custom C++ program was written using inheritance of the RNA class⁴⁴. The probability of the ith

nucleotide being buried in a helix is the sum for all j of the probability the i - j base pair is sandwiched between the base pairs $(i-1)$ - $(j+1)$ and $(i+1)$ - $(j-1)$. Each of these can be determined using the partition function, Q , as a normalization factor and partial partition function for interior and exterior fragments:

$$P_i = \sum_{j=1}^N V'(i-1, j+1) \times K_{stack}(i-1, j+1, i, j) \times K_{stack}(i, j, i+1, j-1) \times V(i+1, j-1)$$

where P_i is the probability that nucleotide i is buried in a helix, N is the length of the sequence, $V'(i, j)$ is the partition function for the exterior fragment of nucleotides 1 to i to j and to N given that i is paired to j , $K_{stack}(i, j, i+1, j-1)$ is the equilibrium constant for the base pair stack of base pairs i - j and $(i+1)$ - $(j-1)$, and $V(i, j)$ is the partition function for the interior fragment from nucleotides i to j given that i is paired to j . Figure S6 diagrams V' and V . These arrays of partition functions for sequence fragments are also explained in a description of the partition function calculation⁸⁹.

PARS calculations: To calculate PARS scores for human transcripts, we downloaded the dataset deposited by Wan et al.⁵⁶ to the NCBI GEO (Gene Expression Omnibus)¹⁰³. We used the mapped reads available for S1-treated (accessions GSM1226157, GSM1226159, and GSM1226161) and V1-treated (accessions GSM1226158, GSM1226160, and GSM1226162) samples. We calculated the PARS-score using⁵⁶:

$$PARS_i = \log_2 \left[\frac{V1_i \times \left(\frac{S1_{total}}{V1_{total}} \right) + 5}{S1_i + 5} \right]$$

where $PARS_i$ is the PARS score for the i^{th} nucleotide, $V1_i$ is the number of reads in the V1-treated samples attributed to the i^{th} nucleotide, $S1_i$ is the number of reads in the S1-treated samples attributed to the i^{th} nucleotide, $S1_{total}$ is the total number of S1-treated sample reads, and $V1_{total}$ is the total number of V1-treated sample reads. The ratio of $S1_{total}$ and $V1_{total}$ is a normalization factor. The addition of 5 in the numerator and denominator is a pseudocount to reduce the magnitude of scores for positions with few reads⁵⁶. In total, entries were found for 18,026 transcripts of the 18,155 high-confidence m⁶A-containing transcripts found.

Author Contributions:

E.K. designed experiments, synthesized strands, performed optical melting experiments, performed chemical mapping experiments, and revised the manuscript, X.Z. contributed code to RNAstructure, R.M.W. contributed code to RNAstructure, R.K. synthesized phosphoramidites and strands and revised the manuscript, D.H.M. designed experiments, contributed code to RNAstructure, fit the nearest neighbor parameters, and drafted the manuscript.

Figures:

Figure 1. Overview. In this study, we advanced the RNAstructure software package⁴⁴ (at center) to be capable of predicting secondary structures for sequences with the m⁶A nucleotide. RNA secondary structure prediction by RNAstructure relies on nearest neighbor parameters for estimating folding stability and dynamic programming algorithms for estimating structures and base pair probabilities. Here we fitted nearest neighbor parameters for m⁶A to optical melting data and revised the dynamic programming algorithms to be capable of considering any sequence alphabet.

Figure 2. Panel A. The nearest neighbor parameters for helix stacks. The position of the m^6A is indicated by 6. The stacking parameters are compared for methylated (blue; i.e. m^6A -U base pairs) and unmethylated (red; i.e. A-U base pairs) sequences for analogous nearest neighbors. The unmethylated stacks (i.e. A-U base pairs) are those of Xia et al.⁵¹ for adjacent Watson-Crick pairs and those of Chen et al.⁷³ for adjacent G-U pairs. Stacks with m^6A -U pairs are generally less stabilizing than analogous stacks with A-U pairs. **Panel B. Terminal m^6A -U pairs are not destabilizing.** The top two sequences (Watson-Crick paired with a complementary strand) have the same nearest neighbor stacks, but the second helix has two terminal A-U pairs⁵¹. This costs 0.7 kcal/mol of stability. The bottom two sequences also have the same nearest neighbor stacks, but the second has two terminal m^6A -U pairs. Here the stability cost is 0.18 kcal/mol and not outside of the uncertainty estimate. On average, terminal A-U pairs cost 0.45 kcal/mol of stability⁵¹, but terminal m^6A -U pairs are not destabilizing.

Figure 3. M⁶A Stacking on a Helix End Stabilizes Secondary Structure as Compared to A Stacking. The $\Delta\Delta G^{\circ}_{37}$ (kcal/mol) for dangling ends and terminal mismatches as a result of N⁶-methylation (Table S4) is shown, where negative values mean greater folding stability for m⁶A than A. The motifs shown here have a terminal base pair (left side of motif), and either a dangling end or terminal mismatch right (right side of motif). On average, the methylated motifs are more stabilizing than the unmethylated motifs, although the extent of the stabilization is sequence dependent.

Figure 4. Tests of the new m⁶A nearest neighbor parameters and RNAstructure software. Panel A. The Conformational Switch of MALAT1 RNA. Secondary structure was predicted for the model methylation-activated switch for HNRNPC binding. In the absence of methylation, the left (closed) structure is predicted to predominate the ensemble, where the HNRNPC protein binding site (marked in red nucleotides) is occluded to binding. When A22 is methylated to m⁶A, the right (open) structure is predicted to predominate, where the HNRNPC binding site is more accessible. In the absence of methylation, the ratio of closed:open is estimated to be 62:38. This estimate switches to 41:59 for the methylated sequence. Chemical and enzymatic probing results are superimposed on the structures. Mapping data for the unmethylated sequence are superimposed on the closed conformation (left) and mapping data for the methylated sequence are superimposed on the open structure (right). The chemical agents act on Watson-Crick faces and prefer loop nucleotides, although they also act on helix ends and G-U pairs⁵⁴. Nuclease S1 prefers loop regions and Nuclease V1 prefers helical regions⁵⁴. The chemical and enzymatic mapping data support a mixture of the two structures both with and without N⁶-methylation. **Panel B. The Average Probability that A or m⁶A are Buried in a Helix at the Position of a High-Confidence m⁶A sites in the Human Transcriptome.** The mean probability that an A or m⁶A is base paired and stacked between two adjacent pairs for 18,026 sites of N⁶-methylation, as estimated by RNAstructure. Position 0 is the site of methylation. N⁶-methylation is estimated to further open the structure at the methylation site. **Panel C. The Average PARS Scores for Accessibility for the 18,026 sites of N⁶-methylation in the Human Transcriptome.** Lower PARS scores indicate higher counts of nuclease S1 cleavage relative to nuclease V1 cleavage and therefore a higher likelihood of being unpaired. The RNAstructure predictions and the PARS data both show considerable single-stranded character at the site of N⁶-methylation.