Structural effects of linkage disequilibrium on the transcriptome

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ABSTRACT
A majority of SNPs (single nucleotide polymorphisms) map to noncoding and intergenic regions of the genome. Noncoding SNPs are often identified in genome wide association studies (GWAS) as strongly associated with human disease. Two such disease-associated SNPs in the 5’ UTR of the human FTL (Ferritin Light Chain) gene are predicted to alter the ensemble of structures adopted by the mRNA. High-accuracy single nucleotide resolution chemical mapping reveals that these SNPs result in substantial changes in the structural ensemble in agreement with the computational prediction. Furthermore six rescue mutations are correctly predicted to restore the mRNA to its wild-type ensemble. Our data confirm that the FTL 5’ UTR is a “RiboSNitch,” an RNA that changes structure if a particular disease-associated SNP is present. The structural change observed is analogous to that of a bacterial Riboswitch in that it likely regulates translation. These data further suggest that specific pairs of SNPs in high linkage disequilibrium (LD) will form RNA structure-stabilizing haplotypes (SSHs). We identified 484 SNP pairs that form SSHs in UTRs of the human genome, and in eight of the 10 SSH-containing transcripts, SNP pairs stabilize RNA protein binding sites. The ubiquitous nature of SSHs in the transcriptome suggests that certain haplotypes are conserved to avoid RiboSNitch formation.

Keywords: RiboSNitch; SNP; mRNA; partition function; transcript

INTRODUCTION
Noncoding but nonetheless transcribed regions of the human genome often play important regulatory functions in the cell (Griffiths-Jones et al. 2005; Rana 2007; Chu and Herschlag 2008; Tseng et al. 2009; Halvorsen et al. 2010). In particular, the 5’ and 3’ UTRs of genes are central components of the regulatory machinery (Lai et al. 1998; Pesole et al. 1999; Boffa et al. 2008; Halvorsen et al. 2010; Kilty et al. 2010). In bacteria, Riboswitches in 5’ UTRs will bind small molecules (often metabolite precursors) that alter their conformation to regulate protein expression levels (Tucker and Breaker 2005; Edwards and Ferre-D’Amare 2006; Weinberg et al. 2007; Stoddard et al. 2008; Wang et al. 2008a). Riboswitches exist because certain RNA sequences will adopt multiple, near isoenergetic conformations; small molecule binding stabilizes one conformation over the other (Wilkinson et al. 2005; Edwards and Ferre-D’Amare 2006; Weinberg et al. 2007; Stoddard et al. 2008; Wang et al. 2008a). Riboswitches exist because certain RNA sequences will adopt multiple, near isoenergetic conformations; small molecule binding stabilizes...
ensemble (Ferrari et al. 2006; Sanchez et al. 2006; Burdon et al. 2007; Halvorsen et al. 2010). Our data reveal the ensemble of conformations adopted by mRNA is exquisitely sensitive to specific mutations despite being described as “unstructured.” Furthermore, these structural changes can be rescued with multiple secondary compensating mutations. This raises the possibility that certain SNPs in high linkage disequilibrium (LD) stabilize critical regions in transcripts. A comprehensive analysis of common human genetic variation reveals that such “structure-stabilizing haplotypes” are ubiquitous in mRNA and in a majority of cases stabilize RNA binding protein (RBP) sites.

RESULTS

Disease-associated SNPs repartition the RNA conformational ensemble

Certain noncoding RNAs (for example the Ribosome and self splicing introns) have evolved to fold into unique three-dimensional structures with highly specific function (Woodson and Leontis 1998; Silverman et al. 2000; Weikl et al. 2004; Sykes and Levitt 2005; Laederach et al. 2007; Waldisch 2008). However, many regions in the transcriptome are under selective pressure that will not necessarily favor a single conformation (Peng et al. 1995; Rana 2007; Chu and Herschlag 2008; Regulski and Breaker 2008; Tseng et al. 2009; Halvorsen et al. 2010). Instead, they adopt an ensemble of conformations that cannot be described by a single structure (Russell and Herschlag 2001; Mathews 2004; Bernhart et al. 2006; Tyagi and Mathews 2007; Waldispuhl and Clote 2007). This is the case for a majority of human mRNA transcripts, including the 5' UTR of the human FTL gene.

To visualize the conformational diversity of the FTL 5' UTR, we performed Boltzmann sampling of suboptimal secondary structures and projected these onto a principal component decomposition of the ensemble (Ding et al. 2004, 2005). Unlike traditional minimum free energy methods for predicting RNA structure (e.g., mFold), Boltzmann sampling allows us to visualize the conformational ensemble of an mRNA (Halvorsen et al. 2010). Each dot in Figure 1A represents one suboptimal RNA structure, and the distance between them is correlated to the difference in structure. What is apparent from Figure 1 is that the human FTL 5' UTR is predicted to adopt three major conformations. The wild-type (WT) sequence (Fig. 1A) adopts primarily one conformation (red), while two disease-associated mutations (U22G and A56U, Fig. 1B,C, respectively) favor the alternative blue and green conformations. The FTL 5' UTR includes an Iron Responsive Element (IRE) which is indicated in light purple (Fig. 1A). Only for the WT sequence does a majority of structures adopt a conformation where the IRE is in the correct hairpin conformation. These calculations suggest a putative mechanism for the disease association. The Iron Responsive Element-Binding Protein (IREBP) binds to the IRE and represses translation (Sanchez et al. 2006). When the disease-associated SNP shifts the equilibrium away from the WT conformation (Fig. 1A), FTL translation is enhanced leading to the hyperferritinemia phenotype (Burdon et al. 2007).

A particularly important feature of computing RNA ensembles such as those illustrated in Figure 1 is that they also predict the probability of base-pairing for each nucleotide (Mathews 2004; Bernhart et al. 2006; Waldispuhl and Clote 2007). The frequency of formation for a base pair in the ensemble is proportional to the probability of that base pair (Ding et al. 2004, 2005). Figure 2A schematically represents the FTL 5' UTR, and Figure 2B is a heatmap of the predicted difference between the WT and U22G base-pair probabilities. We immediately observe another important aspect of this system. Even though the disease-associated mutation is not in the IRE, the largest predicted changes in base-pair probability are within the functional element.

Structure mapping confirms the presence of a RiboSNitch in the FTL 5’ UTR

Changes in base-pair probability are experimentally assayed using chemical mapping probes (Mitra et al. 2008; Quarrier et al. 2010). We transcribed the human FTL 5’ UTR and performed selective 2’-hydroxyl acylation analyzed by primer extension (SHAPE) chemical mapping experiments (Wilkinson et al. 2005, 2008, 2009). These experiments probe RNA structure with single nucleotide resolution and report the flexibility of the backbone, which is directly correlated with base-pair probability (Wilkinson et al. 2006). Figure 2C illustrates raw capillary (+) SHAPE traces for the WT, U22G, and a control G4A mutation (which is not predicted to affect the RNA structural ensemble, and is also not disease-associated). From these raw traces alone, we visually discern the effect of the U22G mutation on the structure of the FTL 5’ UTR. As predicted by Boltzmann sampling, the IRE nucleotides (C33-A49) have higher SHAPE reactivity for the U22G construct, indicative of a significant decrease in base-pairing. The full extent of the disease-associated SNP's effect on the RNA ensemble is observed when the peak areas are integrated, scaled, and averaged over multiple repeats (Fig. 2D; Mitra et al. 2008). Analogous experiments on the A56U construct reveal a similar effect, consistent with the ensemble calculation prediction (Supplemental Fig. 1). Furthermore the control SNP G4A has only minimal effect on the RNA structure, as predicted (Fig. 2E).

RiboSNitch rescue through double mutation

To further validate our ensemble model for the FTL 5’ UTR (Fig. 1) we performed a comprehensive SNPfold analysis to
identify secondary mutations that restore the ensemble to WT partitioning. These mutations are reported in Supplemental Table 1. We experimentally validated the top three mutations for both constructs. The SHAPE data for the U22G-G17C double mutants show that this mutation restores IRE structure to WT (Fig. 3A). This prediction is particularly interesting as it does not simply restore the U22-A58 base pair (Fig. 1, red conformation) through isosteric replacement of a canonical Watson-Crick pair (Lescoute et al. 2005). Instead it further destabilizes the first stem in the blue and green conformations favoring the WT structure.

We systematically collected SHAPE data on the WT, single and double mutants predicted to affect the IRE structure to evaluate the performance of the SNPfold algorithm. Figure 3B illustrates a subset of these data (the additional data are summarized in Supplemental Fig. 1C). The SHAPE data were used to calculate the True Positive, False Positive, True Negative, and False Negative rates of our SNPfold predictions for each base. We compared traditional minimum free energy predictions to our SNPfold algorithm using a Receiver Operator Curve (ROC) analysis (DeLong et al. 1985). The SNPfold algorithm performs better with an area under the curve of 0.86 (solid line, Fig. 3C) compared to that of the minimum free energy predictions (long dashes, Fig. 3C) with an area under the curve of 0.62. Furthermore, we evaluated SNPfold for identifying SNPs that cause a SHAPE reactivity change in more than eight nucleotides in the IRE of FTL. The performance of SNPfold for RiboSNitch detection is high (Fig. 3C, short dashes, area under the curve 0.97).
FIGURE 2. (A) Schematic of the human FTL 5' UTR and Exon 1; the IRE is indicated in green. (B) SNPfold prediction of the changes in the probability of a base being paired caused by the U22G hyperferritinemia-associated SNP (Cremonesi et al. 2001, 2003; Ferrari et al. 2006; Burdon et al. 2007) as a heatmap, with red indicating the disease-allele results in higher base-pair probability and blue indicating a lower probability. (C) Raw capillary traces of SHAPE analysis of the WT (black), U22G (cyan, disease-associated), and G4A (purple, not disease-associated) FTL 5' UTRs. (D) Quantification, averaging, and scaling of multiple repeats for WT (black) and U22G (cyan) SHAPE data. The thickness of the line indicates one standard deviation over six repeats of the experiment. The heatmap below the data is orthologous to the predictions made in B since changes in SHAPE reactivity correlate with changes in base-pair probability (Badorrek and Weeks 2005; Badorrek et al. 2006; Wilkinson et al. 2008). (E) Scaled and averaged repeats for G4A construct indicating the control mutation has no effect on base-pair probability as predicted. These experiments validate the SNPfold prediction and indicate that the FTL 5' UTR is indeed a RiboSNitch.
Structure-stabilizing haplotypes

Our results on the FTL 5’ UTR reveal that specific SNPs can have profound effects on the structural ensemble of non-coding regions of RNA and that the SNPfold algorithm accurately predicts these (Fig. 3C). It is important to realize that a majorit y of SNPs have little or no effect on the ensemble partitioning of RNA. An analysis of all possible FTL 5’ UTR mutations previously showed that the U22G and A56U SNPs are in the top 5% in terms of their predicted structural effect (Halvorsen et al. 2010). Our experimental results predict that an individual with the U22G-G17C (Fig. 3A) haplotype will not have the hyperferritinemia phenotype since this double mutation stabilizes the IRE in the FTL 5’ UTR. The U22G and A56U mutations are extremely rare, as is hyperferritinemia cataract syndrome. We performed a comprehensive search of HapMap haplotypes and did not find any individuals with the U22G or A56U SNPs (Stranger et al. 2007; Morton 2008).

We nonetheless expect to find pairs of SNPs in high LD that stabilize mRNA structure. We call these pairs of SNPs “structure-stabilizing haplotypes,” or SSHs. Our experimental results demonstrate that the SNPfold algorithm is highly predictive of SSHs (area under the curve 0.97) (Fig. 3C), having correctly identified six pairs in the FTL 5’ UTR. We comprehensively analyzed LD SNP pairs in the human genome that map to 5’ and 3’ UTRs to identify potential SSHs. A complete list of the 484 SSH pairs we identified in human UTRs is provided in the supplement (Supplemental Table 2). In Table 1, we report UTRs where more than four
SSH pairs were found. These are particularly noteworthy, since observing multiple SSH pairs in the same UTR is unlikely by chance (<1 in 100,000).

Figure 4A illustrates the subset of LD SNP pairs that are predicted SSHs in the 3′ UTR of RPA1 (replication protein A1) (Shao et al. 1999). In Figure 4B we plot the mean change in base-pair probability caused by all individual SNPs in the haplotype. This allows us to visualize the regions in the RNA that are stabilized by the SSH and we observe that these coincide with experimentally determined IGF2BP binding sites (Hafner et al. 2010a,b). We performed a similar analysis on the SSHs reported in Table 1, and find that, in eight of the 10 UTRs, the predicted regions of the UTR that are stabilized coincide with RBP or miRNA binding sites determined by photoactivatable-ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP) (Hafner et al. 2010a,b).

**DISCUSSION**

The SHAPE data we collected validate the presence of a disease-associated RiboSNitch in the FTL 5′ UTR as computationally predicted by SNPfold. Our results illustrate the extent to which specific disease-associated SNPs can impact mRNA transcript ensemble partitioning. The two hyperferritinemia-associated SNPs (U22G and A56U) cause similar large rearrangements in the ensemble partitioning of the RNA. The rearrangements favor alternative conformations where the IRE does not adopt a hairpin conformation, a prerequisite for IREBP binding (Kaygun and...

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**Table 1. Structure-stabilizing haplotypes in the human genome where more than four SNP pairs stabilize RNA structure**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Full name</th>
<th>UTR</th>
<th># SSHs</th>
<th>SNP Pairs</th>
<th>LOD score</th>
<th>Function</th>
<th>RBP sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPA1</td>
<td>NM_002945</td>
<td>3</td>
<td>12</td>
<td>C380G/G417A, G417A/T463C, G534A/C214A, C774T/A347G</td>
<td>17.89</td>
<td>Bind ssDNA during replication (Shao et al. 1999)</td>
<td>IGF2BP</td>
</tr>
<tr>
<td>HLA-DOA</td>
<td>NM_002119</td>
<td>3</td>
<td>12</td>
<td></td>
<td>17.47</td>
<td>Member of MHC class II, highly conserved (Naruse et al. 1999)</td>
<td>AGO, PUM2</td>
</tr>
<tr>
<td>KIAA1609</td>
<td>NM_020947</td>
<td>3</td>
<td>6</td>
<td>C2194G/C2131T, A2259G/T2034G</td>
<td>25.77</td>
<td>An ortholog for the EAK-7 protein in Caenorhabditis Elegans, which controls development by inhibiting DAF-16/FoxO activity (Alam et al. 2010)</td>
<td>IGF2BP,TNRC6</td>
</tr>
<tr>
<td>ADAMTS14</td>
<td>NM_139155</td>
<td>3</td>
<td>5</td>
<td>C700G/A1580G, G546A/G1185A</td>
<td>16.34</td>
<td>An amino-procollagen peptidase, mutations associated with Multiple Sclerosis (Bolz et al. 2010; Goertsches et al. 2005)</td>
<td>—</td>
</tr>
<tr>
<td>EP400</td>
<td>NM_015409</td>
<td>3</td>
<td>5</td>
<td>T73C/C193T, G57A/C193T</td>
<td>4.28</td>
<td>Destabilizes nucleosomes during double strand breaks as part of repair (Xu et al. 2010)</td>
<td>IGF2BP,PUM2</td>
</tr>
<tr>
<td>SPTBN1</td>
<td>NM_178313</td>
<td>3</td>
<td>4</td>
<td>T1310C/G1913A, G852A/T1926C</td>
<td>11.21</td>
<td>Interacts with calmodulin in a calcium-dependent manner (Hu et al. 1992)</td>
<td>IGF2BP</td>
</tr>
<tr>
<td>APOL4</td>
<td>NM_030643</td>
<td>5</td>
<td>4</td>
<td>C327T/C323A, C32A/A250G</td>
<td>12.38</td>
<td>Involved in lipid metabolism. Only present in primates (Monajemi et al. 2002)</td>
<td>—</td>
</tr>
<tr>
<td>QDPR</td>
<td>NM_000320</td>
<td>3</td>
<td>4</td>
<td>C510T/A468C, C408T/A403G</td>
<td>21.66</td>
<td>Regenerates tetrahydro-biopterin (needed for phenylalanine metabolism). Mutations associated with tetrahydro-biopterin deficiency (Thöny and Blau 2006)</td>
<td>IGF2BP</td>
</tr>
</tbody>
</table>

PAR-CLIP sites stabilized by the haplotype are indicated in the RBP sites column.
We find that the performance of the SNPfold algorithm is better than traditional minimum free energy approaches at identifying SNPs that alter mRNA structure (Fig. 3C) most likely because it takes into account the structural heterogeneity of the transcript (Mathews 2004; Waldispuhl and Clote 2007). Most importantly, our results indicate that transcripts, which are generally thought of as "unstructured," are nonetheless highly sensitive to specific mutations. The SNP-induced structural change in the FTL 5′ UTR is analogous in magnitude to that observed upon ligand binding in bacterial Riboswitches (Tucker and Breaker 2005). Furthermore, the effects on FTL protein expression are likely post-transcriptional, explaining the hyperferritinemia phenotype. Indeed, IREBP binding represses translation, such that, if the IRE is indirectly disrupted by SNP-induced ensemble repartitioning, excess protein will be translated. More importantly, secondary compensating mutations exist that restore WT partitioning, and the SNPfold algorithm correctly predicted six of them for both diseased alleles (Fig. 3B). Interestingly, these compensating mutations are not necessarily standard isosteric replacements of canonical base pairs (e.g., U22G-A58C), but also include more complex structural mechanisms (e.g., U22G-G17C) (Fig. 3A). These results suggest that certain SNP pairs are conserved in the human population because they stabilize specific ensembles of conformations in mRNAs. Of particular interest are genes where multiple SNP pairs stabilize structure (Table 1; Fig. 4). Combined with PAR-CLIP data that identify transcriptome-wide RBP binding sites, an intriguing interplay between population genetics, RBP binding, and mRNA structure is revealed. We have thus far found eight transcript UTRs where multiple SSHs stabilize RBP and/or miRNA binding sites (Table 1). This likely represents only a subset of the genes where SNPs stabilize RNA structure, since we have limited human genetic variation data and PAR-CLIP data for four RBPs and one miRNA. Furthermore, to maintain
computational tractability, we analyzed only SNP pairs in LD that map to the same UTR.

To identify pairs of SNPs in high linkage that stabilize RNA structure (i.e., SSHs) we defined a new metric, the Structure Recovery Ratio (SRR, Equation 1 below). Conceptually, the SRR measures the extent to which a pair of SNPs restores structure relative to the SNP that has the largest effect on that structure. Because it is a ratio, the numerical value of the SRR is not dependent on length of the RNA. We show this empirically in Supplemental Figure 2A, where there is no correlation between the SRR and length. We empirically chose an SRR cutoff value of two based on an analysis of the number of SSHs as a function of this threshold (Supplemental Fig. 2B). The mRNAs we identify in Table 1 are particularly interesting as four or more SSHs were found in either the 5’ or 3’ UTRs. We therefore expect that RNA structure likely plays an important regulatory role in these gene’s regulation.

Covariation is common in large structured RNAs such as the Ribozyme, tRNAs, and even bacterial Riboswitches (Michel and Westhof 1990; Gutell et al. 2002; Leontis et al. 2002; Wang et al. 2008b). In fact, covariation models are often used to identify structured RNAs in genomic data (Griffiths-Jones et al. 2005). Traditionally, programs such as CMfinder (Yao et al. 2006; Benjamin et al. 2007) or FOLDALIGN (Havgaard et al. 2005) are used to predict structure. These techniques require significant covariation to constrain a structure and make an accurate prediction. Linkage data do not provide sufficient information to determine a structure in this way, which is why we chose to combine our structural predictions with PAR-CLIP data (Fig. 4) to make biological predictions. Our data suggest that certain haplotypes in the human population are conserved because they stabilize specific ensemble partitioning of functional mRNA regions.

We conclude that RibosNitches are likely ubiquitous in the human genome, and that specific haplotypes are conserved because they preserve mRNA ensemble partitioning. We expect that SSHs in mRNA play an important regulatory role, and that our strategy of combining common genetic variation data with SNPfold analysis and PAR-CLIP data can pinpoint important new regulatory regions in the transcriptome. It is also likely that SNP-induced structure change affects mRNA stability. As more eQTL (expressed Quantitative Trait Loci) data become available, it will be possible to correlate structural change and mRNA stability, greatly expanding our understanding of post-transcriptional regulatory mechanisms (Majewski and Pastinen 2011).

MATERIALS AND METHODS

Computational methods

The SNPfold algorithm was used to predict how RNA structure is modified by SNPs (Halvorsen et al. 2010). The FTL SNPs were originally selected for their disease association. Secondary correcting mutations were predicted by identifying the SNP pair containing the primary mutant that resulted in the highest correlation coefficient to the WT base-pair probability plot as computed by the partition function (McCaskill 1990; Mathews 2004). The control SNPs were selected because SNPfold calculations showed they had the highest Pearson correlation coefficient (CC) in relation to the WT base-pairing. Rescue SNPs were identified by using SNPfold to calculate the CCs of the disease-associated SNP and a secondary SNP pair to the WT base-pairing and selecting those pairs with the highest CC.

From the hg18 genome assembly and hg18 “refgene” datafile, as downloaded from the UCSC dbSNP, 44,186 SNPs were identified. These SNPs satisfied the conditions that they were located in the untranslated region of genes and mapped to fully assembled chromosomes. Of these SNPs, 15,491 are located on the same spliced UTR and have linkage disequilibrium values (LOD scores) greater than or equal to one. The D’ values were extracted from LD flatfiles (available at the Hapmap webpage, http://hapmap.ncbi.nlm.nih.gov/downloads/ld_data/2009-04_rel27/) corresponding to the CEU population of Hapmap (release #27, Phase II and III).

The SRR is defined as

\[
SRR = \frac{1 - CC_{\text{min}}}{1 - CC_{\text{both}}}
\]

where \(CC_{\text{both}}\) is the correlation coefficient of the pairing probability of the RNA with both SNPs present, and \(CC_{\text{min}}\) is the correlation coefficient of the pairing probability to WT for the SNP in LD that has the largest effect on RNA structure (smallest CC). In both cases the correlation coefficients are computed with respect to the pairing probability of WT.

Principal component analysis

Principal components were calculated (as described previously; Halvorsen et al. 2010) from a total of 10,000 Boltzmann sampled structures generated equally from the five sequences: WT, C10U, C14G, U22G, and A56U. The projection of the binary representation of these 10,000 structures onto the first two principal components revealed three distinct clusters as identified by the k-means clustering algorithm. The centroid structure of each cluster was drawn using R2R. For a given SNP of interest, we project 10,000 structures, generated just from that sequence, onto the first two principal components with the projections colored according to which cluster they belong to.

SHAPE experiments

The WT FTL 5’ UTR sequence (GCAGTTCCGGCGTCCCGGG GTCTGTCCTTGTCTTCACAGTGTTTGGAGGGAACAGATCCGG GGGACTCCTTTCCAGCGTCCAGCGGCGGTCCCAGTTCCTC TCCGCGTTAATGTCCGGGACCATCCTTCTCGCCCATCCTCGCT TCCTCGGACGTCCGAGCGGTGTTTGGTGTGATTGCTCTTCTTC TGCCAAACC) was inserted between the SgfI and MluI sites of the C. The re-
Supplemental material is available for this article.

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Boffa MB, Maret D, Hamill JD, Bastajian N, Grinich P, Jenny NS, Teng Z, Macy EM, Tracy RP, Franco RF, et al. 2008. Effect of with a second round of amplification used to join the PCR fragments. The resulting PCR product was transcribed into RNA using Megascript followed by MegaClear according to Ambion’s protocol.

Selective 2°-Hydroxyl Acylation and Primer Extension (SHAPE) experiments were performed in the following way: A total of 10 pmol RNA was suspended in 12 µL of 0.5× TE buffer and then heated to 95°C for 2 min. Immediately after, the mixture was placed on ice for 2 min. With gentle pipetting, 6 µL of folding mix was added (3.3× folding mix; 333 mM HEPES, pH 8.0, 20 mM MgCl₂, 333 mM NaCl) to the mixture and then incubated at 37°C for 20 min. Following incubation, the mixture was split equally to obtain a negative control. The negative control was treated with 1 µL of dimethyl sulfoxide (DMSO) while the experimental mixture was treated with 1 µL of DMSO containing 65 mM N-methylisatoic anhydride (NMA). Both mixtures were incubated for 45 min at 37°C, or roughly five half-lives of NMA hydrolysis. RNA incubated at ~80°C for 30 min following the addition of 90 µL of water, 4 µL of 5 M NaCl, 4 µL of 5 mg/mL glycogen, 2 µL of 100 mM EDTA pH 8.0, and 350 µL of 100% ethanol. RNA was precipitated by centrifugation at 14,000 rpm at 4°C for 30 min. The RNA was rinsed with 70% ethanol after the removal of the supernatant. After being spun down and dried by speed-vac for 10 min, the RNA was suspended in 10 µL of 0.5× TE.

The resulting mixtures had 1 µL of 5 µM of Cy5-labeled primer solution added and mixed by pipetting. The primers were annealed to the RNA by incubation at 65°C for 5 min, then 35°C for 5 min, and finally placed on ice for 1 min. The solutions with the annealed primers had 7 µL of the reverse transcriptase buffer mix which contained 250 mM KCl, 167 mM Tris-HCl, pH 8.3, 1.67 mM magnesium chloride, 0.2 mM dNTPs, 17 mM DTT, 10 mM MgCl₂, and 40 U of Invitrogen’s RNaseOUT. The reverse transcriptase buffer mix was also added to the sequencing ladder reactions and, for the reactions lacking NMA, 1–2 µL of one dNTP (5mM) was also added. The samples were heated at 52°C for 11 min with 1 µL of Superscript III (Invitrogen) added after the first minute and mixed by pipetting. The resulting cDNA was cleaned up with the addition of 2 µL of 2 M NaOH and incubated at 95°C for 5 min. Following the incubation, the samples were treated with a solution containing 2 µL of 2 M HCl, 3 µL of 3 M Na-acetate, 1 µL of 100 mM MgCl₂, and 90 µL of 100% ethanol. The mixture was then centrifuged at 14,000 rpm at 4°C for 30 min and rinsed with 70% ethanol. The resulting cDNA pellets were dried, suspended in 40 µL of Sample Loading Solution (Beckman), and subjected to capillary electrophoresis on a Beckman CEQ 8000.

Taking advantage of the data independent Beckman ladders and using custom written Matlab software (available at http://ribsnotch.bio.unc.edu/The_Laeclerach_Lab/Software.html), the data from the CEQ were aligned. The ShapeFinder (Vasa et al. 2008) software package was used to analyze the aligned SHAPE data. The ShapeFinder data were non-normal with a P-value <0.001 and using outlier analysis as previously reported resulting in the majority of the residues being between 0 and 1.5. SHAPE experiments were carried out under standard RNA folding conditions (10 mM MgCl₂ and 100 mM KCl) as described in separate publications on the technique (Wilkinson et al. 2005). CAFA analysis of the SHAPE capillary traces was performed with CAFA and Shapefinder as previously described (Mitra et al. 2008; Vasa et al. 2008). The SHAPE data were normalized using standard outlier analysis (Vasa et al. 2008). ROC analysis is based on thresholding the SHAPE data (DeLong et al. 1985), a Welch’s t-test was used to determine significance over a minimum of three repeats per experiment. Raw SHAPE data were made available using the SNRNASM standard (Rocca-Serra et al. 2011) and the data are available at http://snrnasm.bio.unc.edu.

Heatmap graphs

With the exception of the G4A mutant having only three data sets, a minimum of four data sets per mutant were used to perform statistical tests. The Shapiro-Wilkes test comparing the data set to the normal distribution with the same mean and standard deviation was performed individually for each position to confirm the appropriateness of using a Welch’s t-test. The Welch’s t-test for mean difference was performed at each nucleotide with the null hypothesis being that that the mutant and WT data sets were equal. The degree of mean difference for positions with P-values >0.001 are color-coded red when the mutant has the higher value and blue when the WT has a higher value with the depth of the color indicating the degree of difference in 0.15 steps and a maximum value of 0.6. Even in our most extreme cases, <8% of the positions were non-normal with a P-value <0.05. All statistical tests were performed using version 2.8.1 of R (R development Core Team).

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