Effects of Refolding on Large-Scale RNA Structure
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Supporting Information

ABSTRACT: Understanding RNA-mediated functions requires a detailed characterization of the underlying RNA structure. In many cases, structure probing experiments are performed on RNA that has been “refolded” in some way, which may cause the conformation to differ from that of the native RNA. We used SHAPE–MaP (selective 2′-hydroxyl acylation analyzed by primer extension, read out by mutational profiling), to probe the structure of the Dengue virus (DENV) RNA genome after gentle extraction of the native RNA from intact virions (ex virion) and after heat denaturation and refolding. Comparison of multiple SHAPE-informed structural features revealed that refolded RNA is more highly structured and samples fewer conformations than the ex virion RNA. Regions with similar structural features are generally those with low SHAPE reactivity and low Shannon entropy (lowSS regions), which correspond to elements with high levels of well-determined structure. This high-structure and low-entropy analysis framework, previously shown to make possible discovery of functional RNA structures, is thus now shown to allow de novo identification of structural elements in a refolded RNA that are likely to recapitulate RNA structures in the ex virion RNA state. Regions with less well-defined structures, which occurred more frequently in the more native-like ex virion RNA and may contain RNA switches, are challenging to recapitulate using refolded RNA.

The ability of RNA to fold back on itself to form three-dimensional structures is crucial for its ability to regulate transcription, translation, gene silencing, protein trafficking, and RNA splicing.1,2 To understand the functions of RNA, the underlying RNA secondary and tertiary structures must be well understood. Ideally, RNA structure should be probed at comprehensive nucleotide resolution in healthy cells,3–7 however, experimental requirements often necessitate studying RNA that has been purified under denaturing conditions or transcribed in vitro. These samples are often heat denatured and refolded to promote the formation of secondary and tertiary structure.

Evidence suggests that RNA structure formation begins during transcription.5,8 Co-transcriptional folding processes can be affected by many factors, including the rate of transcription and the cellular environment. These factors differ greatly between in-cell and simplified in vitro environments. In-cell RNA folding can also be influenced by RNA chaperones that facilitate folding into the native structure.9 Finally, full-length cellular RNAs may sample various distinct, functionally important conformations as they encounter specific ligands and proteins.2,11 Every in-cell RNA molecule potentially has a unique folding history, and vestiges of these former structures may be evident in the native RNA structure. Thus, RNA structures in cells and in virus particles could differ substantially from that of refolded RNAs. A number of studies have examined the effects of refolding on small RNAs or small regions of large RNAs,4,12–14 but the effects of heat denaturation and refolding on large RNAs, with complex and heterogeneous structures, are poorly understood.

We investigated the effects of heat denaturation and refolding on the structure of a Dengue virus serotype 2 (DENV2) RNA genome. We measured the local nucleotide flexibility for each position in the 10723-nucleotide genome using selective 2′-hydroxyl acylation analyzed by primer extension, read out by mutational profiling (SHAPE-MaP).15–17 These data were used to model the secondary structure of protein-free DENV2 RNA under two conditions: gently extracted from virions (termed ex virion) and heat-denatured and refolded RNA (termed refolded). We identified 25 structural elements in the refolded RNA with well-determined secondary structures as defined by the experimentally determined level of RNA structure and conformational entropy. The structures of these specific elements agree well with the ex virion RNA secondary structure. We also identified extensive differences between the refolded and ex virion RNAs. We found that refolded DENV2 RNA generally has a conformation that is more well-defined than that of the more native-like ex virion RNA. Ultimately, many regions of the refolded RNA sample a single structure, whereas these same regions in the more native-like ex virion RNA sample multiple conformations.

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SHAPE-MaP RNA Structure Probing. Details about DENV2 RNA sample preparation and data processing have been described previously. Briefly, RNA was gently extracted from purified DENV2 viral particles (strain S16803, GenBank GU289914; Microbix Biosystems) under non-denaturing conditions. For the ex virion RNA experiment, extracted RNA was exchanged into 1.1X SHAPE-MaP buffer [50 mM HEPES (pH 8.0), 200 mM potassium acetate (pH 8.0), and 3 mM MgCl₂, at a 1X concentration]. For the refolded RNA experiments, extracted RNA was exchanged into 0.5X TE [5 mM Tris (pH 7.5) and 0.5 mM EDTA] (Qiagen), heated at 85 °C for 5 min, and cooled on ice for 5 min. SHAPE-MaP buffer was added to a final concentration of 1.1X buffer. Both ex virion and refolded RNA samples were incubated under identical final conditions at 37 °C for 30 min. RNA was treated with 0.1 volume of 1-methyl-7-nitroisatoic anhydride (1M7), 1-methyl-6-nitroisatoic anhydride (1M6), or N-methylisatoic anhydride (NMIA). No-reagent controls were prepared in parallel by addition of neat dimethyl sulfoxide. A single set of denaturing controls were prepared using 1M7, 1M6, and NMIA. DNA libraries were prepared for massively parallel sequencing as denaturing controls were prepared using 1M7, 1M6, and NMIA. No-reagent controls were prepared under identical conditions. SHAPE-MaP buffer was also included.

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Sequencing data were analyzed using ShapeMapper software, which has been extensively validated relative to reference structures. Raw sequencing FASTQ files were aligned to a reference DENV2 sequence, and SHAPE reactivities were generated using the following parameters: minPhred = 10, minLength = 25, mapping quality = 30, and minPhredToCount = 20. SHAPE reactivities for nucleotides with a read depth of <2500 or with a high no-reagent mutation rate (>0.03) were excluded from analysis. We obtained 1M7 SHAPE reactivities for 99% of the nucleotides in the DENV2 genome in the ex virion and refolded states. Full biological replicate experiments for both ex virion and refolded RNAs yielded Pearson correlation coefficients of ≥0.9.

Kolmogorov–Smirnov Test. A Kolmogorov–Smirnov (KS) permutation test was used to evaluate whether the distributions of ex virion and refolded 1M7 SHAPE reactivities were significantly different. The null hypothesis of the KS test is that two data sets are drawn from the same distribution. First, 10% of the reactivities (~1072) of each data set were randomly sampled. A KS test was performed on these two sample sets, and the p value was calculated. This process was repeated for a total of 1000 iterations. Control KS permutation tests were performed by comparing ex virion sample sets (or refolded sample sets) to each other. To account for multiple testing, a Bonferroni-corrected significance threshold value was used, α/n, where α equals the significance threshold (0.01) and n equals the number of hypotheses tested (1000) to give a corrected significance threshold of 0.00001. In the ex virion and refolded KS tests, all p values were <0.00001 (Figure S1). Thus, the ex virion and refolded 1M7 SHAPE reactivity distributions are significantly different. As expected, the p values of the control KS tests were all greater than the Bonferroni-corrected α value. The same KS permutation test procedure was also used to compare two refolded RNA 1M7 SHAPE reactivity replicates, collected months apart in full biologically distinct experiments (Figure S1). These two replicates had a Pearson correlation coefficient (R) of 0.88. Of the 1000 KS tests, 999 failed to reject the null hypothesis, indicating that the two replicates of refolded RNA SHAPE reactivities were drawn from the same distribution. Thus, the differences observed between ex virion and refolded SHAPE reactivities were not due to variability in the SHAPE-MaP approach but were a result of the refolding treatment.

Secondary Structure Modeling of Refolded RNA. The SHAPE-derived secondary structure model for the ex virion RNA was reported previously, and the same methods were used here to characterize the secondary structure of refolded RNA. Briefly, pseudoknots were predicted using 1M7 SHAPE reactivities as constraints in the program ShapeKnots, implemented in RNAstructure version 5.6. The maximum base-pairing distance was set to 500 nucleotides; thus, in our analysis, we could examine nucleotide interactions that are within this pairing distance. All of the pseudoknots previously predicted in the ex virion RNA were also predicted to occur in the refolded RNA. Two additional pseudoknots were predicted in the refolded RNA: a small pseudoknot with the 5’-most nucleotide at position 3863 and a pseudoknot in the 3’-dumbbell structure in the 3’-untranslated region (3’-UTR). Pseudoknotted nucleotides were forced to be single-stranded in subsequent structure calculations.

Both 1M7 and differential SHAPE reactivities were input as constraints using SuperFold, which uses RNAstructure Partition (version 5.6) to generate base-pairing probabilities for all possible canonical base pairs. Base-pair probabilities were used to calculate a Shannon entropy value for every nucleotide. We used SuperFold, which calls RNAstructure Fold (version 5.6), to generate a minimum free energy structure. Pseudoknotted base pairs were added to the structure. The sensitivity and positive predictive value (ppv) of the refolded model compared to the previously reported ex virion RNA model were calculated, treating the ex virion structure as the reference state. These values are termed refolded-sensitivity (RF-sens) and refolded-ppv (RF-ppv) throughout the text. For whole-genome calculations, RF-sens is the percentage of base pairs in the ex virion structural model that are present in the refolded RNA model. RF-ppv is the percentage of base pairs in the refolded structural model that are also present in the ex virion model.

Identification of Well-Structured Elements. Structural elements with both 1M7 SHAPE reactivities and Shannon entropies below their respective medians were identified. The minimum length of selected regions was required to be at least 40 nucleotides. Some regions were combined or expanded to include all nucleotides of bisecting helices. An exception was made for element 6. This element was not expanded to include the intersecting pseudoknotted helix, because the pseudoknot contains numerous low-probability, long-range base pairs that would have required expanding the element by >750 nucleotides. This single pseudoknot helix was ignored in the RF-sens and RF-ppv calculations of individual structural elements (discussed below).

The RF-ppv values for individual structural elements were calculated as described above for the entire genome, again treating the ex virion structure model as the reference state. The RF-sens was calculated differently, because the region of interest in the ex virion model often contained nucleotides for only one-half of a helix, with base-pairing partners located outside of the region of interest. Ignoring these base pairs would have artificially increased the calculated RF-sens values. Instead, for the individual element RF-sens calculations, we defined RF-sens as the percentage of base-paired nucleotides in the ex virion...
model that have the same base-pairing pattern in the refolded RNA model.

Comparison of SHAPE-Directed Models to an Accepted DENV2 Structure. An accepted DENV2 secondary structure was created by unifying structural elements proposed in homology-based studies and as supported by subsequent structural studies of DENV2 RNA fragments, mini-genomes, and full-length genomes. Detailed descriptions of how the accepted structure was defined and comparisons of the SHAPE-predicted models to the accepted DENV2 structure are provided in the Supporting Information. Sensitivity (sens) and ppv values for the ex virion model were calculated relative to those elements for which well-determined accepted models exist, which together span 7% of the Dengue genome; these are elements 1 (nucleotides 1–105), 2 (nucleotides 148–308), and 24 (nucleotides 10247–10723). The sens value was defined as the percentage of accepted base pairs accurately predicted by the ex virion RNA model, and ppv was defined as the percentage of base pairs in the ex virion structural model that are also present in the accepted model.

■ RESULTS

Effects of Refolding Treatment on SHAPE Reactivities and Shannon Entropies. To understand the effects of heat denaturing and refolding on large-scale RNA structure, we obtained nucleotide-resolution SHAPE-MaP structural data for an entire DENV2 genome (strain S1680324) under two folding conditions. Under the first condition (ex virion), DENV2 RNA from intact virions was extracted under gentle, nondenaturing conditions (Figure 1). Under the second condition (refolded), isolated DENV2 RNA was denatured by heating to 85°C in the absence of monovalent and divalent ions and cooling on ice. Both RNAs were allowed to fold at 37°C in 50 mM HEPES (pH 8.0), 200 mM potassium acetate (pH 8.0), and 3 mM MgCl₂ before modification with 1M724 or the differential SHAPE reagents 1M6 and NMIA.25,26 DNA libraries were prepared for massively parallel sequencing, and SHAPE reactivity profiles were generated.

Generally, nucleotides with high SHAPE reactivities (>0.85) are conformationally flexible and likely to be single-stranded. Conversely, nucleotides with low SHAPE reactivities (<0.4) are structurally constrained and likely base paired.9,27 A nucleotide with an intermediate reactivity may be only partially structured (for example, base-stacked but not base-paired) or may sample multiple conformations or participate in a structural switch. In the latter cases, two or more stable secondary structures may exist in equilibrium, and the measured SHAPE reactivity is the average flexibility of a nucleotide across all conformations. The median 1M7 SHAPE reactivity profiles of refolded and ex virion RNA are very similar across the genome (Figure 2A). This similarity indicates that DENV2 RNA likely adopts broadly similar architectures in the ex virion and refolded states.

Differential and 1M7 SHAPE reactivities were used in a partition function calculation to determine base-pairing probabilities for each base pair in the predicted ensemble of structures for both RNA states. The probabilities were summed to calculate a Shannon entropy for each nucleotide.22 We emphasize that even though SHAPE data are an input into the Shannon entropy calculation, Shannon entropy provides additional information beyond that provided by raw SHAPE reactivities. SHAPE reactivity is a measure of the degree of RNA structure. Shannon entropy is a measure of our certainty or confidence in a predicted secondary structure. A region of RNA with low SHAPE reactivity indicates that the region is highly structured but does not report on our ability to model its structure using available structure probing tools. If multiple, different secondary structures are consistent with the SHAPE reactivity pattern, then this region will have a high Shannon entropy even though it might be highly structured. As expected, because these two metrics report on distinct features of RNA structure, they are not strongly correlated with each other. Pearson’s R values between Shannon entropy and SHAPE reactivity for the ex virion and refolded RNAs are −0.25 and −0.19, respectively (Figure S2). The Shannon entropy profiles for refolded and ex virion RNAs are similar across the genome (Figure 2B), indicating that, overall, the two RNAs are structurally similar.

The Structure of Refolded RNA Is More Well-Determined Than That of Ex Virion RNA. Despite the broad similarities between the median SHAPE reactivities and Shannon entropies across the ex virion and refolded RNAs, there are nonetheless clear differences between the two RNA states. We first examined the frequency distribution of SHAPE reactivities across the two states, which is correlated with the overall level of structure of an RNA. A large number of low and high reactivities suggest an RNA samples a single, well-defined structure. Conversely, an RNA with many intermediate reactivities is less structured or samples multiple conformations.26 The distributions of 1M7 SHAPE reactivities for refolded and ex virion DENV2 RNA are very different (Figure 2C). A greater number of nucleotides with low and high SHAPE reactivities are observed in refolded RNA, whereas a greater proportion of nucleotides with intermediate reactivities are detected in the ex virion RNA. These differences were significant at the p < 0.01 level [Kolmogorov–Smirnov permutation test (Figure S1A)]. A similar analysis of two replicate refolded RNA
data sets showed that the two replicates were statistically the same (Figure S1B). Thus, the differences between ex virion and refolded RNA SHAPE reactivities suggest that ex virion RNA is less structured or samples multiple conformations relative to the refolded RNA. In addition, the Shannon entropies of nucleotides in the refolded RNA were almost always lower than those of the ex virion RNA; the median Shannon entropies of the refolded and ex virion RNA are 0.062 and 0.085, respectively (Figure 2B). The smaller Shannon entropy of the refolded RNA suggests that the secondary structure of refolded RNA is more well-determined and likely composed of fewer heterogeneous structural variants.

The differential SHAPE experiment uses reagents 1M6 and NMIA to specifically detect nucleotides involved in non-canonical and local tertiary interactions. For example, in prior work, differential SHAPE reactivities were measured for the thiamine pyrophosphate (TPP) riboswitch in the presence and absence of a TPP ligand. The ligand-bound RNA forms stable tertiary interactions and is characterized by a small number of relatively strong differential SHAPE reactivities. In contrast, the ligand-free TPP riboswitch does not form significant tertiary structure and its differential reactivity profile is distinct in two ways: there are more nucleotides with differential reactivities, and the magnitudes of their reactivities are smaller. This pattern is consistent with the view that the ligand-free state of the TPP riboswitch contains less stable or only transient tertiary structure. A similar trend was observed between refolded and ex virion DENV2 RNA. Refolded RNA had fewer (n = 583) differential reactivities than ex virion RNA (n = 2770), but the reactivities of the refolded RNA were larger in magnitude on average (Figure 2D and Figure S1C). Thus, the differential SHAPE reactivity profiles qualitatively suggest that refolded RNA has more nucleotides that participate in stable tertiary and noncanonical structural interactions. In summary, although the two DENV RNA states are broadly similar, heat denaturation and refolding result in a more structured state overall, containing more well-defined secondary and tertiary structure interactions than does the ex virion state.

**Effects of Refolding Treatment on Secondary Structure.** The complete three-reagent SHAPE approach was used to model the secondary structures of the entire ex virion and refolded DENV RNAs (Figure 3). Extensive benchmarking supports the view that the three-reagent approach yields RNA structure models in which a large fraction of correct base pairs are recovered accurately in regions with a stable, well-determined structure. We compared the entire refolded SHAPE-derived minimum free energy (MFE) structure to the ex virion MFE structure in terms of its sensitivity (RF-sens) and positive predictive value (RF-ppv), which are 66% and 59%, respectively (Figure 3C). We define RF-sens and RF-ppv by treating the ex virion structure (the more direct biological state) as the reference. The RF-sens then reveals the percentage of the ex virion base pairs that the refolded structure accurately predicts. The RF-ppv gives the percentage of the refolded structure base pairs that are common between the two structures. The RF-ppv gives the percentage of the refolded structure base pairs that are common between the two structures. The RF-ppv is smaller than the RF-sens because the refolded RNA model contains more base pairs (2766 bp) than the ex virion model (2447 bp). The differences in base-pairing patterns could represent real changes in secondary structure upon refolding treatment. Alternatively, base-pairing
differences may not be significant if they occur in regions with high Shannon entropies, which are not structurally well-defined by our MFE models.

Stable, well-defined structural elements in large RNAs are those with both low SHAPE reactivity (corresponding to a high level of structure) and low Shannon entropy (reflecting a single highly probable structure). In prior analyses of SHAPE-MaP data for HIV-1, hepatitis C virus, and Dengue virus, regions of low SHAPE reactivity and low entropy are indicated by light gray boxes and are numbered. Red asterisks indicate structural elements that were identified as low SHAPE and low entropy in the (more highly structured) refolded RNA but not in the ex virion RNA. (B) Ex virion RNA base-pair probability plot. Arcs and elements of low SHAPE and low entropy are colored as described above. Blue asterisks indicate structural elements that were identified in the ex virion RNA but not in the refolded RNA. (C) Minimum free energy (MFE) secondary structure models. Base pairs common to both ex virion and refolded RNA are colored black. Base pairs unique to the ex virion and refolded models are colored blue and red, respectively. The dagger indicates a dumbbell pseudoknot structure that forms in the refolded RNA but not in ex virion RNA.

Figure 3. SHAPE-directed structure models for refolded and ex virion RNA states. (A) Refolded RNA base-pair probability plot. Base pairs are shown as arcs and are colored by their probability (see the key), with green arcs indicating the most probable helices. Black arcs indicate pseudoknots. Structural elements with both low SHAPE reactivity and low Shannon entropy are indicated by dark gray boxes. Regions that were expanded to include entire helices that intersect each low-SHAPE and low-entropy region are indicated by light gray boxes and are numbered. Red asterisks indicate structural elements that were identified as low SHAPE and low entropy in the (more highly structured) refolded RNA but not in the ex virion RNA. (B) Ex virion RNA base-pair probability plot. Arcs and elements of low SHAPE and low entropy are colored as described above. Blue asterisks indicate structural elements that were identified in the ex virion RNA but not in the refolded RNA. (C) Minimum free energy (MFE) secondary structure models. Base pairs common to both ex virion and refolded RNA are colored black. Base pairs unique to the ex virion and refolded models are colored blue and red, respectively. The dagger indicates a dumbbell pseudoknot structure that forms in the refolded RNA but not in ex virion RNA.

more than half were shown to be under clear evolutionary pressure. The same lowSS analysis for the refolded DENV2 RNA yielded 25 lowSS elements (Figure 3A, numbered gray boxes). The 25 structural elements of the refolded RNA overlap well with the 24 previously identified lowSS elements in ex virion RNA (Figure 3B, gray boxes). Four of the 24 identified elements are unique to the refolded RNA analysis (elements 5, 15, 17, and 18 (Figure 3A, red asterisks)).

We compared the structures of the ex virion and refolded RNAs across all 25 refolded RNA structural elements by calculating their refolded RNA model sensitivity (RF-sens) and RF-ppv. We also calculated the RF-sens and RF-ppv over the remaining or intervening 22 regions (with lengths of >50 nucleotides) that did not meet the low-SHAPE and low-entropy
criteria. The median RF-sens and median RF-ppv of the 25 low-
SHAPE, low-entropy elements are 96% and 89%, respectively
(Figure 4A); these values are much higher than the RF-sens and
lowSS elements also show much stronger conservation of base-
paired secondary structure after refolding treatment than does
the RNA as a whole.

The refolded and ex virion DENV structures are very similar
in all elements containing known functional structures that were
identified prior to our whole-RNA-genome SHAPE analysis
[elements 1 (5′-UTR), 2 (5′ portion of the capsid-coding
region), and 25 (3′-UTR)]19–35 with RF-ppv values of 0.97,
0.97, and 0.85, respectively (Figures 3 and 4A). The main
difference between the two models in these three regions is that
a “dumbbell” pseudoknot in the 3′-UTR is predicted to form in
the refolded RNA but is reactive by SHAPE and appears to be
unstable in ex virion RNA (Figure 3, dagger).

There are four structural elements in which the RF-sens is
relatively low (<70%): elements 3 and 16–18 (Figure 4A). In
these regions, the ex virion RNA structure contains many base
pairs with low probability (Figure S3). The ex virion base-pair
probability plot contains many of the base pairs from the
refolded MFE structure, but they are predicted with lower
probabilities. Thus, in these four elements, the data support a
model in which ex virion RNA samples multiple conformation;
one of these is the MFE structure adopted by the refolded RNA,
but this structure does not dominate the ex virion conforma-
tional ensemble.

Comparison of SHAPE-Predicted Models to Accepted
DENV2 Structures. There are three regions in the Dengue
RNA genome for which there are phylogenetically supported
accepted structures.17,31 Many of these structures have been
modeled in previous SHAPE studies of the UTRs of full-length
DENV or DENV minigenomes.17,32–38 These accepted
structures are in the 5′-UTR, the 5′ portion of the capsid-
coding element, and the 3′-UTR. Strikingly, we find that all of
the accepted structures fall in lowSS regions. Both the ex virion
and refolded models accurately recover every accepted
structural feature in the 5′-UTR and 3′ portion of the capsid-
coding region (Figure S4), recover most of the major structural
features in the 3′-UTR (Figure S5), and correctly model two
pseudoknots located in the 5′-UTR and 3′-UTR. Both SHAPE-
directed models miss several short pseudoknots in the 3′-UTR
and predict a medium-range helix (involving positions 10247–
10270 and 10640–10661) that is not included among accepted
DENV2 models but that nonetheless has strong experimental
support for the specific DENV2 strain (S16803) studied here.17
Sens and ppv values for each SHAPE model were calculated for
each element with an accepted secondary structure (Figure 4C);
the sensitivities of the two models for these three elements range
from 86 to 100%. Values for ppv are smaller than for sens,
because the SHAPE-predicted models contain more base pairs
than the accepted DENV2 structure. In summary, SHAPE-
directed models, based on the ex virion or refolded RNAs,
recover most accepted base pairs, as expected, as all accepted
structures reside in lowSS regions.

■ DISCUSSION

By either necessity or convenience, researchers often study
protein-free RNAs that have been refolded after denaturation.
There is little information about how commonly used refolding
approaches affect the structure of long RNAs, especially relative
to more native-like cellular or virus-based states. Analyses of
1M7 SHAPE reactivities, differential SHAPE reactivities, and
Shannon entropies all suggest that refolded DENV2 RNA has
many features that are similar to those of a viral RNA genome
that is likely in a native-like conformation (ex virion RNA) but

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Comparison between refolded and ex virion RNAs

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Figure 4. Comparison of DENV2 RNA structure models as a function of refolding and relative to regions with accepted structures. (A) RF-
sens and RF-ppv values for the 25 elements of low SHAPE reactivity and
low Shannon entropy (lowSS) identified in refolded DENV2 RNA,
relative to the ex virion structure model. (B) RF-sens and RF-ppv values
for all remaining regions of the DENV2 genome with lengths of at least
50 nucleotides. (C) RNA structure modeling accuracies relative to
accepted DENV2 structures. The sens and ppv values of ex virion
and refolded RNA models were calculated for elements 1 (5′-UTR), 2 (5′
portion of the capsid-coding region), and 24 or 25. In the ex virion and
refolded RNAs, the 3′-UTR corresponds to elements 24 and 25,
respectively (because there is one additional element in the refolded
RNA). For the 3′-UTR* calculation, nucleotides proposed to be
involved in a medium-range base-pairing interaction, which are not in
the accepted model but for which there is direct experimental
evidence,17 were omitted from the sens and ppv calculations.
For reference, the medium-range interaction is included in the 3′-UTR
calculation (no asterisk). See the Supporting Information for a
discussion.

RF-ppv of the entire RNA genome (discussed above).
Moreover, the median RF-sens and median RF-ppv for the 22
intervening (non-element, non-lowSS) regions are 54% and
47%, respectively (Figure 4B). The RF-sens and RF-ppv values
of the 25 structural elements versus those of the 22 intervening
regions are significantly different (p < 0.001; Kolmogorov–
Smirnov test). Thus, whereas previous studies have shown that
the lowSS metric is useful in identifying functional RNA
structures in a large RNA, this study newly demonstrates that
that there are also notable differences. In general, heat denaturation and refolding treatment results in a more structured and well-determined conformation. This difference suggests that the more native-like ex virion RNA samples a wider variety of states and may not uniquely form the most thermodynamically stable structure (Figure 5). Instead, some regions are only partially folded or sample multiple structures.

**Figure 5.** Model for the global effects of refolding on large-scale RNA structure. (A) Ex virion RNA contains multiple low-probability base pairs and a large number of low-magnitude differential SHAPE reactivities, suggesting that significant regions in the more native-like ex virion RNA are not structurally well-defined and instead sample multiple conformations. (B) Refolded RNA contains more highly probable base pairs and a smaller number of large differential SHAPE reactivities than the ex virion RNA, suggesting that heat denaturation and refolding result in a more structured and well-defined RNA conformation.

SHAPE reactivities were used to create secondary structure models for the entire DENV2 genome for the two RNA states. We were able to examine internucleotide interactions up to 500 residues apart. Most of the structural differences between the two models are located in regions that have high Shannon entropies and are thus intrinsically not well-defined by a single structural model. We identified 25 elements in the refolded RNA that are highly structured and predicted to favor a single, highly probable conformation. When only these elements were considered, the median RF-sens (percentage of the ex virion base pairs that the refolded structure accurately predicts) between the two RNA states was ∼96% (Figure 4A). This high RF-sens suggests that analysis of refolded RNA is predictive of native RNA structure in regions in which both SHAPE reactivities and Shannon entropies are low.

Studying refolded RNA is likely justifiable for small RNAs, such as those optimized and studied by X-ray crystallography and nuclear magnetic resonance, assuming that the full RNA motif is included. Even with small RNAs, it is clear that the native cellular environment can modulate their structure.4,13,14 A much greater degree of care must be taken when studying larger, more complex systems. This work shows that the native DENV2 genomic RNA is structurally more heterogeneous and contains fewer well-determined structural elements than its artificially refolded analogue. There are clear limitations to studying RNA motifs that are conformationally dynamic or involved in structural switches in refolded RNA, given that refolding of the long DENV2 RNA resulted in a more homogeneous and structured RNA architecture (Figure 5).

The low-SHAPE and low-Shannon entropy hypothesis has been evaluated previously, which revealed that regions with these characteristics overlap significantly with known functionally important regions in the HIV-1, HCV, and Dengue RNA genomes.15,17,28 The work described here illustrates a new and additional useful feature of the lowSS criterion: lowSS regions also and simultaneously identify structural elements in a refolded RNA that are likely to correctly recapitulate RNA structures in a native-like RNA state. Furthermore, these structural elements in the refolded RNA overlap well with the previously identified low-SHAPE and low-Shannon entropy elements in ex virion RNA, of which many were shown to be under evolutionary pressure and likely necessary for viral replication. A subset of these elements likely form local folds with higher-order and tertiary structures.15,17,28 The three regions in the DENV RNA genome with previously defined accepted structures also fall within lowSS regions for both ex virion and refolded models (Figure 4C). Thus, the lowSS structure and entropy metric appears to be highly useful for the discovery of important RNA structures, even in artificially refolded genomic RNA.

The lowSS framework emphasizes that not all predicted base pairs in a structural model should be given equal consideration and that structural well-determinedness can be established de novo. We strongly recommend reporting Shannon entropy values or related metrics alongside modeled RNA secondary structures to inform on the confidence (or lack thereof) in the predicted structural features across RNA genomes or transcriptomes. Moreover, the lowSS metric should find broad application in identifying regions that represent strong candidates for further functional evaluation25,31,32 and for which in vitro transcripts can be used as good facsimiles of native RNAs.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.8b01219.

Methods describing generation of an “accepted” DENV2 structure and detailed comparison to ex virion and refolded SHAPE-directed models, two figures showing statistical analysis of ex virion and refolded 1M7 and differential SHAPE reactivities and comparing the base-pair probability arcs for low-RF-sens elements, and two figures comparing the ex virion and refolded models to the accepted DENV2 structure (PDF).

Complete RNA SHAPE data sets for both ex virion and refolded states, predicted secondary structure models, and tables of lowSS regions for ex virion and refolded DENV2 RNA (ZIP)
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Notes
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Custom software used in this work is freely available from the Shape Mapper 2. RNA 24, 143−148.

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Custom software used in this work is freely available from the corresponding author’s Web site. Massively parallel sequencing data are available from the Sequence Read Archive, accession number SRP066015.

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