Diverse self-association properties within a family of phage packaging RNAs

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ABSTRACT

The packaging RNA (pRNA) found in phi29 bacteriophage is an essential component of a molecular motor that packages the phage's DNA genome. The pRNA forms higher-order multimers by intermolecular "kissing" interactions between identical molecules. The phi29 pRNA is a proven building block for nanotechnology and a model to explore the rare phenomenon of naturally occurring RNA self-association. Although the self-association properties of the phi29 pRNA have been extensively studied and this pRNA is used in nanotechnology, the characteristics of phylogenetically related pRNAs with divergent sequences are comparatively underexplored. These diverse pRNAs may lend new insight into both the rules governing RNA self-association and for RNA engineering. Therefore, we used a combination of biochemical and biophysical methods to resolve ambiguities in the proposed secondary structures of pRNAs from M2, GA1, SF5, and B103 phage, and to discover that different naturally occurring pRNAs form multimers of different stoichiometry and thermostability. Indeed, the M2 pRNA formed multimers that were particularly thermostable and may be more useful than phi29 pRNA for many applications. To determine if diverse pRNA behaviors are conferred by different kissing loop sequences, we designed and tested chimeric RNAs based on our revised secondary structural models. We found that although the kissing loops are essential for self-association, the critical determinant of multimer stability and stoichiometry is likely the diverse three-way junctions found in these RNAs. Using known features of RNA three-way junctions and solved structures of phi29 pRNA's junction, we propose a model for how different junctions affect self-association.

Keywords: packaging RNA (pRNA); RNA structure; RNA self-association; DNA packaging; nanomachines

INTRODUCTION

The only naturally occurring RNA known to form a biologically functional higher-order multimer by intermolecular interaction of identical molecules is found in the phi29 family of bacteriophage. This packaging RNA (pRNA) is an essential component of the packaging motor used to drive the phage genomic DNA into the empty capsid. This motor is unique in that in addition to multiple copies of a connector protein (gp10) and an ATPase (gp16), it also contains several copies of the phage-encoded noncoding pRNA (Guo et al. 1987a,b; Meijer et al. 2001). The motor uses ATP hydrolysis to package ~19.3 kb of dsDNA through its ring-like structure into the capsid in <5 min against a back pressure of ~57 pN (Fig. 1A; Guo et al. 1987a,b, 2006; Simpson et al. 2001; Smith et al. 2001; Guasch et al. 2002; Serwer 2003; Guo 2005; Fuller et al. 2007; Hugel et al. 2007; Morais et al. 2008; Rao and Feiss 2008; Aathavan et al. 2009; Lee et al. 2009; Fu et al. 2010; Ding et al. 2011). For phage phi29, the full-length transcribed pRNA is 174 nt long, but a 120 nt-long truncated version is suf-

ficient for in vitro packaging (Fig. 1B; Bailey et al. 1990; Reid et al. 1994a,b; Garver and Guo 1997; Bourassa and Major 2002). The pRNA self-associates to form a ring (Simpson et al. 2001; Shu et al. 2007; Morais et al. 2008; Ding et al. 2011), which encircles the dodecameric connector protein ring using a "hand-in-hand" or "kissing loop" interaction that involves base-pairing between complementary sequences in loops of adjacent pRNA molecules (Fig. 1C; Garver and Guo 1997; Chen et al. 1999, 2000). Although there has been some disagreement over the number of copies of pRNA in the ring (5 versus 6), it is clear that self-association is critical. Mutations that eliminate this kissing interaction and thus prevent self-association dramatically reduce the function of the motor and the infectivity of the phage (Guo et al. 1998; Garver and Guo 2000).

Studies of the phi29 pRNA have been motivated by the fact that it is a good model for understanding the specific,

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FIGURE 1. The phi29 packaging motor and phi29 packaging RNA (pRNA). (A, left) Partial model of the phi29 packaging motor based on a cryo-EM reconstruction (Morais et al. 2008), the crystal structure of phi29 packaging RNA (Ding et al. 2011), and the connector protein crystal structure (Simpson et al. 2001). The cryo-EM density and connector protein are shown in blue and pRNA in red. (Middle) Close-up view of just the motor. (Right) View of the connector protein and pRNA model only, viewed at $\sim 90^\circ$ angle rotation. (B) Secondary structure of the phi29 pRNA. The full-length pRNA is 174 nt, and the first 120 nt (boxed) is necessary and sufficient for DNA packaging in vitro. The gray shaded domain is not needed for multimerization or connector interaction with the connector protein. Paired/helical (P), loop (L), and junction (J) structural elements are labeled. The kissing interaction sequences are shown in red. (C) Cartoon of the “hand-in-hand” self-association of multiple pRNA copies that leads to the ring. A pentamer is shown to match the cryo-EM.

unique, and perhaps subtle properties that drive RNA self-association. In addition, because both naturally occurring and “designed” self-associating and self-assembling RNAs hold promise as ways to generate higher-order nanostructures (Westhof et al. 1996; Yan 2004; Ko et al. 2010; Dibrov et al. 2011; Lee et al. 2012; Ishikawa et al. 2013), the insight gained by studying pRNA can inform these efforts. Most published research on pRNA has focused on the phi29 strain, in particular the kissing hairpin interaction, the molecularity of the multimer (dimer, trimer, etc.), and how self-association contributes to phage infectivity (Chen and Guo 1997a,b; Garver and Guo 1997, 2000; Zhang et al. 1997; Guo et al. 1998; Chen et al. 1999; Trottier et al. 2000; Shu et al. 2007; Fang et al. 2008). In addition to the kissing interaction, it has been reported that the three-way junction within the phi29 pRNA has special properties including unique thermostability (Shu et al. 2011), or functionally important flexibility (Ding et al. 2011). Recent crystal structures of an engineered phi29 pRNA (Ding et al. 2011), and of an isolated phi29 pRNA three-way junction (lacking the kissing interactions) (Zhang et al. 2013) provided additional details of the intermolecular and intramolecular interactions that are important within this specific pRNA species. The knowledge resulting from these diverse studies of the phi29 pRNA has led to its adaptation as a polyvalent delivery vector and it has been useful for various types of RNA engineering (Guo et al. 2005, 2006; Li et al. 2009; Wendell et al. 2009; Shu et al. 2011;

Tarapore et al. 2011; Ye et al. 2012; Khisamutdinov et al. 2014).

The fact that basic studies of phi29 pRNA behavior has led to successful engineering suggests that even more insight into the properties that govern pRNA self-association will continue to inform engineering and therapeutic design efforts. One source of additional insight is the pRNAs found in other members of the phi29 bacteriophage family, which are surprisingly diverse in terms of their sequences (Bailey et al. 1990; Pecenkova and Paces 1999; Meijer et al. 2001). Most pRNA-centered studies have focused on the phi29 strain; in comparison the pRNAs from other strains have been relatively unexplored. Comparative studies of phylogenetically related but diverse RNAs are a powerful way to understand underlying principles of structure and function. Therefore, we took advantage of the diversity found in the pRNAs from other members of the phi29 family to examine how different pRNA sequences compared with regard to their multimerization properties. Available information predicts that within the M2, SF5, GA1, and B103 pRNAs the sequences and lengths of the putative kissing interaction sequences, and the sequences within other secondary structure features, are different (Bailey et al. 1990). We therefore initially aimed to understand if the multimers formed by these pRNAs differed in stoichiometry and thermostability, and if so, if these properties were due to different kissing interactions or other differences. During the course of our experimentation,

another study was published in which biophysical analyses of the pRNAs from several phi29 family showed that all form multimers but with some differences in their behavior that are not just a result of the kissing loop interaction (Gu and Schroeder 2011). This study did not, however, examine the behavior of these pRNAs at different temperatures and also did not directly probe the location and identity of the intermolecular kissing interactions. In addition, rather than using the full ~120 nt-long pRNAs, we focused our studies on a ~75 nt-long version of the pRNA that folds independently, can interact with the packaging motor, and self-associate (Bourassa and Major 2002). We chose this strategy because this isolated multimerization domain contains both the kissing interaction and three-way junction elements proposed to be important for multimer behavior, allowing us to focus on the contributions of these elements. Also, understanding the behavior of “minimal” multimerization domains could be useful in ongoing engineering efforts and potentially structural studies.

Here, we report our findings regarding the behavior of minimal multimerizing pRNAs from the M2, GA1, SF5, and B103 strains, comparing them directly to phi29. Using chemical probing, we developed experimentally supported secondary structure models that define which bases are involved in the kissing interaction in each pRNA. Using a biochemical and biophysical approach, we (1) determined that the stability and size (number of pRNA copies) of multimers varies between these pRNAs, (2) tested the hypothesis that changes to the pRNA sequence can dramatically alter its characteristics, and (3) found that while the kissing interaction is essential, the three-way junction-containing “scaffold” within which the kissing interaction occurs may be a more important determinant of multimer size and stability. In particular, we found that the pRNA from M2 forms the most stable multimers of highest stoichiometry in solution.

RESULTS

Characterization of phi29 pRNA multimerization by native gel electrophoresis

To establish an experimental framework for examining the multimerization properties of different pRNAs, we first examined the prototype phi29 pRNA. By confirming that in our assays this RNA behaved as reported, we would be able to use these assays to study other pRNAs. We made four RNAs comprising nucleotides 23–97 of the phi29 pRNA as this sequence is sufficient for self-association and binding to the gp10 connector protein (Garver and Guo 1997), and also this sequence contains the kissing hairpins and three-way junction elements. The four RNAs were as follows: wild-type (WT), two mutants in which one half of the kissing interaction was mutated to abrogate this interaction (MUT1 and MUT2) and a double mutant in which the potential to form the kissing interaction was restored (COM: compen-

satory mutant) (Fig. 2A). We also made a longer WT RNA (120 nt) that contained the minimal functional phi29 pRNA for direct comparison to the shorter version. We analyzed these RNAs by nondenaturing (native) polyacrylamide gel electrophoresis in both the presence of MgCl₂ and in the presence of EDTA and at three different temperatures: 4°C, 25°C, and 37°C. Different temperatures were used to assess the stability of the self-association interaction and its sensitivity to temperature. Native gel electrophoresis is exquisitely sensitive to differences in RNA conformations and in this case, differences in pRNA self-association.

At 4°C, all four truncated phi29 pRNAs migrated as a single band on an EDTA-containing gel. Their migration rates differed slightly, probably due to expected small local differences in secondary structure (EDTA-containing gels are not fully denaturing). However, the different pRNAs showed very different behavior on an Mg²⁺-containing gel (Fig. 2B). WT and COM migrated more slowly than either MUT 1 or 2, consistent with the expected ability of WT and COM to self-associate and the inability of MUT1 and MUT2 to self-associate. However, WT and COM were not identical; COM migrated as a tight band while the WT band was smeared. The most straightforward interpretation is that there is exchange occurring with the WT RNA that differs in degree or rate from that with COM; this could be intramolecular conformational exchange or intermolecular association–dissociation. The 120 nt-long version of WT also smeared, suggesting this is a genuine characteristic of the pRNA. At 25°C and 37°C, MUT1 and MUT2 migrated at a rate consistent with no self-association. WT and COM still migrated more slowly than either MUT1 or MUT2, but WT now migrated more quickly than COM. This is consistent with WT and COM self-association, but the two have different conformational properties and/or multimer stabilities.

To better link the observed migration patterns with self-association, we took advantage of the fact that although MUT1 and MUT2 were mutated to block self-association, they should be able to interact with each other to form multimers. To test this, MUT1 and MUT2 were mixed and run on native gels (Fig. 2C). Combining MUT1 and MUT2 resulted in a new slower-migrating band that migrated at the same rate as WT RNA; we interpret this as the intermolecular MUT1–MUT2 multimer. These results are consistent with the conclusion that the slowly migrating WT RNA band (and by extension, COM) is due to self-association of multiple identical molecules. Furthermore, although the native gel data cannot unambiguously establish the number of pRNA copies that self-associate, the fact that with equal amount of MUT1 and MUT2 only the multimer band was visible showing a 1:1 stoichiometry (Fig. 2C, lower gel) is suggestive of a dimer. The fact that WT ran similarly to the putative MUT1–MUT2 dimer suggests it (and COM) also formed dimers under these conditions. Although dimers are not the ultimate form of these RNAs within the packaging motor, they have been shown to be an authentic intermediate in the assembly of

FIGURE 2. Multimerization characterizations of phi29 constructs. (A, left) Secondary structures of the phi29 WT pRNA 75 nt-long constructs; the kissing sequences are shown in red. Cartoons of the secondary structure with mutated sequences visible in red are shown. (Right) Summary of mutations. (B) Native gel electrophoresis of different phi29 pRNA constructs at 4°C, 25°C, and 37°C. Gels containing 5 mM MgCl₂ are shown in the upper row, gels with 2 mM EDTA are shown in the lower row. (C) Native gel electrophoresis of the MUT1 and MUT2 titration experiment at 4°C. Two sets of experiments are shown, either adding increasing amounts of MUT2 into MUT1 (upper gel) or altering MUT1 and MUT2 ratios while keeping the total RNA amounts the same (lower gel). Both experiments show a new band consistent with intermolecular interactions, highlighted by a red arrow. (D) Dimethyl sulfate (DMS) probing gels of phi29 pRNAs. (RT) reverse transcription control; (-) controls without added DMS; (+) reactions with DMS; "4" or "37" indicates temperature (°C). A sequencing ladder is included and labeled. The locations of the CE and E loops are highlighted with bars and labels to the right, nucleotide locations are indicated to the left, and the location of the primer band is shown. To the right, the analyzed data are mapped onto the secondary structures. Nucleotides modified at both 4°C and 37°C are indicated with magenta while those only modified at 37°C are shown in green. The nucleotides involved in forming the kissing hairpin are colored red. The primer binding location is shaded gray; these nucleotides were not visible on the gel.

TABLE 1. Analytical ultracentrifugation measurements

Name	Parameter	12°C		25°C	
phi29WT	c (AU)	28%	72%	100%	
	$S_{20,w}$ (S)	3.69	5.51	2.98	
	$D_{20,w}$ ($\times 10^7$ cm ² /sec)	7.78	3.98	5.84	
COM	c (AU)	7%	93%	100%	
	$S_{20,w}$ (S)	3.00	4.95	3.35	
	$D_{20,w}$ ($\times 10^7$ cm ² /sec)	5.88	5.55	6.18	
MUT1	c (AU)	100%		100%	
	$S_{20,w}$ (S)	2.80		3.30	
	$D_{20,w}$ ($\times 10^7$ cm ² /sec)	6.22		6.33	
MUT2	c (AU)	100%		100%	
	$S_{20,w}$ (S)	3.16		4.61	
	$D_{20,w}$ ($\times 10^7$ cm ² /sec)	7.31		11.1	
M2	c (AU)	77%	23%	30%	70%
	$S_{20,w}$ (S)	5.35	4.64	5.01	8.09
	$D_{20,w}$ ($\times 10^7$ cm ² /sec)	37.2	1.16	16.49	9.32
SF5	c (AU)	18%	82%	100%	
	$S_{20,w}$ (S)	3.35	5.64	5.13	
	$D_{20,w}$ ($\times 10^7$ cm ² /sec)	9.34	4.1	18.7	
GA1	c (AU)	100%		100%	
	$S_{20,w}$ (S)	3.27		4.53	
	$D_{20,w}$ ($\times 10^7$ cm ² /sec)	9.61		1.08	
B103	c (AU)	46%	54%	27%	73%
	$S_{20,w}$ (S)	4.52	4.90	5.02	8.23
	$D_{20,w}$ ($\times 10^7$ cm ² /sec)	20.9	1.36	13.6	7.77
SF5_phi29	c (AU)			12.6%	87.4%
	$S_{20,w}$ (S)			4.39	6.69
	$D_{20,w}$ ($\times 10^7$ cm ² /sec)			14.3	7.77
SF5_M2	c (AU)			100%	
	$S_{20,w}$ (S)			5.21	
	$D_{20,w}$ ($\times 10^7$ cm ² /sec)			23.8	

higher-order multimers (Chen et al. 2000). This is consistent with the previous results, which report dimer formation by the full-length phi29 pRNA at 4°C and no evidence of higher-order multimers (e.g., hexamers) on a native gel (Gu and Schroeder 2011). In addition, this shows the ~75-nt long version of phi29 pRNA that we used behaves similar to the full-length pRNA reported in the literature.

Examination of phi29 pRNA self-association by analytical ultracentrifugation

To augment the native gels we used sedimentation velocity analytical ultracentrifugation (SV-AUC), which has been successfully used previously to study pRNA behavior (Robinson et al. 2006; Gu and Schroeder 2011). Experiments were performed in the presence of magnesium and at two temperatures, 12°C and 25°C. At 12°C, fitting of the data suggested MUT1 and MUT2 each exist as a single species, while data from WT and COM are best fit by a multispecies model (Table 1). For COM and WT, one of the species has parameters that are similar to those of MUT1 and MUT2. This is consistent with MUT1 and MUT2 being monomers while WT and COM self-associate, forming a mixture of monomer and multimer (hereafter interpreted as dimer). This matches

our interpretation of the native gels (Fig. 2B). Notably, the data are best fit with parameters in which COM has a higher percentage of dimer than does WT (93% to 72%, respectively), correlating with its migration on the native gel. When SV-AUC was conducted at 25°C, all phi29 pRNAs were best fit with a single-species model and the measured parameters were consistent with a monomer. We noted that at 25°C, WT and COM showed dimer-like migration patterns on a native gel; this suggests there are subtle differences in what each technique detects, and motivated us to develop an additional experiment for querying pRNA self-association.

Detecting self-association by chemical probing

Since the kissing hairpin is critical in self-association, we developed a method to directly monitor this interaction that could be applied to different pRNAs. Dimethyl sulfate (DMS) probing detects base-pairing as the compound methylates the N3 of cytosine and the N1 of adenine, which are protected if the bases are in Watson–Crick base pairs (Tijerina et al. 2007). In pRNA the kissing loops

contain adenines and cytosines, thus DMS probing should detect self-association by a decrease in modification in the kissing nucleotides. We used the aforementioned four phi29-based pRNAs to validate this approach. We observed that the bases in the kissing hairpins of WT and COM were modified more by DMS at 37°C than at 4°C, consistent with self-association through the kissing interaction at 4°C but destabilization of the interaction at 37°C (Fig. 2D, cf. lanes 3 and 5 for WT and lanes 8 and 10 for COM). In contrast, in MUT1 and MUT2 kissing loop nucleotides were modified at both 4°C and 37°C, consistent with no self-association (Fig. 2D, cf. lanes 17 and 19 for MUT1 and lanes 22 and 24 for MUT2). Adenines and cytosines which were not involved in the kissing interaction were modified at similar levels regardless of temperature, showing that the primary site of intermolecular interaction is the kissing interaction that can be readily monitored using DMS probing. Overall, the native gel, AUC and DMS probing results for the phi29 test ~75 nt constructs provides a set of self-consistent results indicating multimer (in this case, likely dimer) formation, consistent with studies of larger phi29 pRNAs (Gu and Schroeder 2011), showing we have an experimental framework suitable for exploring additional pRNAs in the phi29 family.

Determining the secondary structures of pRNAs from the phi29 family

To apply the experiments described above to pRNAs from other members of the phi29 family, we required an accurate model of the secondary structures of these RNAs and identification of the nucleotides involved in the kissing interaction. Two different secondary structure models exist in the literature for pRNAs from strains M2, SF5, GA1, and B103 (Bailey et al. 1990; Chen et al. 1999; Gu and Schroeder 2011). Although preliminary enzymatic probing was performed on M2 and SF5 (Bailey et al. 1990), these data do not provide enough resolution to unambiguously determine which of these models are correct and which nucleotides form the kissing interaction. To address this issue, we performed Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) (Wilkinson et al. 2006), probing truncated pRNAs from the M2, SF5, GA1, and B103 strains (Fig. 3). Based on these data, it was clear that the secondary structures of Chen et al. (1999) were largely correct. These models suggest different loop composition and kissing interactions than those previously used to design mutants (Gu and Schroeder 2011).

FIGURE 3. phi29 family constructs SHAPE analyses. SHAPE analysis gels of different phi29 family pRNAs are shown. (RT) reverse transcription control; (–) controls without added NMIA; reactions with increasing NMIA are indicated with white arrow. A sequencing ladder is included and labeled. The locations of the CE and E loops are highlighted with bars and labels to the *right*, nucleotide locations are indicated to the *left*, and the location of the primer band is shown.

Native gels of different pRNAs reveal diverse behavior

On native gels at 4°C, pRNAs from M2, SF5, GA1, and B103 had similar migration rates on the EDTA-containing gel, but very different rates and behaviors on the Mg^{2+} -containing gel (Fig. 4B). M2 and B103 pRNAs migrated primarily as a slow-moving band (relative to WT phi29 pRNA) with some smearing underneath the main band. In contrast, SF5 pRNA migrated at approximately the same rate as phi29 pRNA and was more smeared, while GA1 was a fast-migrating single band. At 25°C and 37°C, M2 and B103 were faster and less smeared while SF5 and GA1 were single bands with similar mobility. Our preliminary interpretation is that these RNAs have different inherent propensities to self-associate and the resultant multimers have different stabilities. Specifically, M2 and B103 readily multimerize and are likely forming higher-order multimers (greater than dimer) based on their migration. This is similar to the behavior reported for larger versions of M2 pRNA (Gu and Schroeder 2011). SF5 has an intermediate ability to self-associate, and GA1 shows little self-association behavior under these conditions even at low temperature. Full-length GA1 was previously reported to have some dimerization ability and full-length SF5 was reported to associate into higher-order complexes (Gu and Schroeder 2011); we did not observe these behaviors with our RNAs under these conditions. The difference in the amount of smearing of the different pRNAs suggests different degrees of conformational flexibility or rates of monomer–multimer exchange.

Analytical ultracentrifugation confirms a variety of self-association stabilities

We used SV-AUC at two temperatures in a manner identical to that done for the phi29 pRNAs (Table 1). Consistent with the native gel, data for GA1 at both temperatures were best fit by a single-species model and the measured hydrodynamic properties are those of a monomer. In contrast, SF5 was best fit by a two-species model at 12°C but by a single-species model at 25°C, suggesting multimers (likely dimer based on migration) are formed at lower temperature but are not stable at the higher temperature. Finally, M2 and B103 can both self-associate at both the low and high temperatures and were best fit with a model in which a significant amount of the RNA is in a multimer state (likely greater than dimer). These results correlate well with our native gel observations.

Mapping the kissing interactions with chemical probing

To verify that the sites of interaction were as predicted by our experimentally determined secondary structures, we conducted DMS probing on several different pRNA constructs under several conditions. We first probed GA1, which showed no multimerization by either SV-AUC or native gels (Fig. 4C). DMS strongly modified the run of four A bases in the L_E

FIGURE 4. Multimerization characterization of pRNAs from the phi29 family. (A) Secondary structures of pRNAs from M2, SF5, GA1, and B103 phages. The putative kissing sequences are shown in red. (B) Native gel electrophoresis of the pRNAs in panel A at different temperatures. Gels containing 5 mM MgCl₂ are shown *above* while those with 2 mM EDTA are *below*. (C) DMS probing of the pRNAs from panel A. (RT) reverse transcription control; (-) controls without added DMS; (+) reactions with DMS; “4” or “37” indicates temperature (°C), and Mg²⁺ or EDTA indicates conditions with magnesium or EDTA. A sequencing ladder is included and labeled. The locations of the CE and E loops are highlighted with bars and labels to the *right*, nucleotide locations are indicated to the *left*, and the location of the primer band is shown. *Below* the gel, the analyzed data are mapped onto the secondary structures. Nucleotides modified at both 4°C and 37°C are indicated with magenta while those only modified at 37°C are shown in green. The nucleotides involved in forming the kissing hairpin are shown in red. The primer binding location is shaded gray; these nucleotides were not visible on the gel.

domain at 4°C and 37°C, consistent with the formation of this loop in the secondary structure and with no involvement in self-association (Fig. 4C, lanes 29 and 31). Likewise, the A and C bases of the L_{CE} loop were modified similarly at both temperatures. It is worth noting that the degree of modification of the bases in L_{CE} was not as high as those in L_E . This is likely due, in part, to the very strong RT stops at four heavily modified A bases that precede this loop and attenuate the RT signal. The SHAPE probing indicates conformational dynamics. Due to the location of the reverse-transcription primer on the RNA, the other half of the kissing interaction (loop L_D) was not observed. Overall, the DMS probing is consistent with the conclusion that this GA1 pRNA construct does not stably self-associate to a detectable level under these conditions.

DMS probing of the M2 and B103 constructs showed behavior different than the GA1 pRNA. Specifically, like the GA1 pRNA, the L_E loop was modified (in this case a single A base) to the same degree at all temperatures, consistent with no involvement in self-association. However, in the presence of Mg^{2+} (necessary for multimer formation), the L_{CE} loop was modified less at 4°C than at 37°C (Fig. 4C, cf. lanes 7 and 11 for M2, lanes 16 and 20 for B103). Notably, the nucleotides that were modified more at 37°C are those predicted to participate in the kissing interaction. To ensure that this change in modification was not a temperature effect, we probed these two RNAs at 4°C and 37°C in the presence of EDTA, which allowed secondary structure to form but not tertiary structure or self-association. In the presence of EDTA, the A and C bases of the loop were modified to the same degree at both temperatures, consistent with a lack of self-association (Fig. 4C, cf. lanes 9 and 13 for M2, lanes 18 and 22 for B103). Overall, the chemical probing provided information clarifying which nucleotides are involved in the kissing interaction for each pRNA and confirmed the behavior and temperature sensitivity seen in the native gels.

Determining multimer size by sedimentation equilibrium analytical ultracentrifugation

The ability to form different-sized multimers could be an important feature of diverse pRNAs and could explain the large mobility differences on the native gels (Fig. 4B). Therefore, we rigorously explored whether these 75 nt-long multimerization domains from diverse pRNAs form different-sized multimers (e.g., dimer, trimer, etc.) using sedimentation equilibrium analytical ultracentrifugation (SE-AUC). As representative RNAs, we used the M2, phi29, and MUT2 pRNAs, based on their large migration differences on native gels. To determine the suitability of this technique, we performed measurements in the presence of Mg^{2+} , at three different pRNA concentrations, at 4°C and 25°C, and at three different speeds. The resultant data were analyzed using global fitting procedures in the Heteroanalysis program to fit the curves. Examples of raw data and fit curves for the MUT2, phi29,

and M2 pRNAs at a single concentration (4°C: [phi29 RNA] = 0.6 μ M, [MUT2 RNA] = 0.73 μ M, [M2 RNA] = 0.58 μ M; 25°C: [phi29 RNA] = 0.86 μ M, [MUT2 RNA] = 1.0 μ M, [M2 RNA] = 0.82 μ M) and speed (12K rpm) are shown in Figure 5A. At both temperatures and for all of these RNAs, the fits are excellent.

The shapes of the SE-AUC curves indicate differences in the self-association properties of the MUT2, phi29, and M2 pRNAs at equilibrium under these conditions. Specifically, a curve that rises more steeply qualitatively indicates higher molecular weight particles and thus more self-association. Both the phi29 and M2 curves show more self-association than the MUT2 (Fig. 5B), as expected based on the native gel, SV-AUC, and chemical probing results. The differences in M2 pRNA's curve compared with phi29 pRNA's curve qualitatively suggests that in addition to forming more stable multimers, the M2 pRNA multimers are of higher order. This latter observation is consistent with the native gels in which M2 pRNA migrated more slowly than phi29 pRNA. At 25°C, the curves are consistent with MUT2 and phi29 pRNAs existing as monomers and M2 self-associating, again consistent with the native gel, chemical probing, and SV-AUC data.

A more quantitative analysis of the SE-AUC data was done to verify the molecularity of various pRNAs and their stabilities (Table 2). At 4°C, the data for MUT1 pRNA was best fit by a model in which this RNA showed no self-association (100% monomer). Likewise, when the MUT2 and GA1 pRNAs were analyzed by SE-AUC at 4°C, they also did not show self-association, well-correlated to native gel and the SV-AUC results. The phi29 pRNA contained a mix of monomer and dimer with a higher percentage of dimer at the highest analyzed concentration (the curves reflect the populationally weighted average of monomer or multimer states). However, attempts to fit the phi29 pRNA data into models with multimers greater than dimer led to substantially poorer fits (Fig. 2). The M2 pRNA was well fit by monomer + dimer and monomer + trimer models (Table 2). These SE-AUC data alone cannot unambiguously distinguish between these models, but the native gels and its SE-AUC curve strongly suggest it can form a higher-order multimer than phi29, likely a trimer. At 25°C the curves of MUT2 and phi29 pRNAs were best fit by a monomer-only model, while M2 fit to a monomer + dimer model (GA1 and MUT1 data were not studied at 25°C). The fit percentage of M2 pRNA dimer increased with higher RNA concentration, consistent with concentration-dependent self-association.

The presence of mixed populations for the phi29 and M2 pRNAs raised the question of interconversion between these species. SE-AUC can distinguish between reversible association (exchanging species) and mixture of fixed species (kinetically inert) molecules. We compared the fits of phi29 and M2 pRNAs at 4°C at two conditions: (1) higher pRNA concentration at lower centrifugation speed and (2) lower pRNA concentration at higher centrifugation speed. For phi29, condition (1) fit as 20% monomer and 80% dimer and condition

FIGURE 5. Sedimentation equilibrium analytical ultracentrifugation of three pRNAs. (A, top) SE-AUC data for MUT2, phi29 and M2 pRNAs at 4°C. Each graph contains the raw absorbance data as a function of position in the cell (black dots) with the global fit shown as a black line. The graph below contains the residuals of the fit, showing the good correlation of the fit to the raw data. (Bottom) SE-AUC data, fit, and residuals at 25°C. (B) Overlaid, fit SE-AUC data from MUT2 (black), phi29 (blue), and M2 (red) pRNAs at a single concentration and speed. (Left) 4°C, (right) 25°C.

TABLE 2. Sedimentation equilibrium analytical ultracentrifugation measurements

Name	Concentration	4°C	25°C
MUT2	Low concentration	100% monomer	100% monomer
	Mid concentration		
	High concentration		
phi29WT	Low concentration	45% monomer, 55% dimer	100% monomer
	Mid concentration	15% monomer, 85% dimer	
	High concentration	20% monomer, 80% dimer	
M2	Low concentration	Fit 1: 11% monomer, 89% dimer Fit 2: 55% monomer, 45% trimer	68% monomer, 32% dimer
	Mid concentration	Fit 1: 43% monomer, 57% trimer	20% monomer, 80% dimer
	High concentration	Fit 1: 11% monomer, 89% dimer Fit 2: 55% monomer, 45% trimer	30% monomer, 70% dimer
MUT1	Low concentration	100% monomer	
	Mid concentration		
	High concentration		
GA1	Low concentration	100% monomer	
	Mid concentration		
	High concentration		

(2) fit as 22% monomer and 78% dimer. The similarity of the monomer:multimer ratios under both conditions suggests reversible association. Assuming M2 forms trimers at 4°C, condition (1) fit as 55% monomer and 45% trimer and condition (2) fit as 65% monomer and 35% trimer. The remarkable consistency between the SE-AUC data and the native gel analysis suggests that native gels are a reliable means to rapidly assess the self-association stoichiometry and stability of diverse pRNAs.

Initial pRNA engineering through kissing interaction exchange

Because the kissing loop interaction is known to be essential for pRNA self-association, we explored whether the different behaviors of different pRNAs could be due to differences in the specific kissing loop interactions in each. We tested this by determining if the characteristics of one pRNA could be transferred to another by exchanging kissing interaction elements. Using the knowledge gleaned from our SHAPE and DMS chemical probing to choose the correct kissing sequences, the kissing loops from the stable trimeric M2 pRNA were placed into the poorly self-associating GA1 pRNA. Unfortunately, this chimeric pRNA formed an aberrant secondary structure and this precluded further analyses (for this and all other chimeric RNAs, formation of the correct secondary structure was confirmed by SHAPE probing, data not shown). Therefore, a different set of chimeric RNAs was designed using the SF5, phi29, and M2 pRNA sequences. The phi29 or M2 kissing interaction sequences were moved into the SF5 pRNA “scaffold” to make SF5_phi29 and SF5_M2 (Fig. 6A). SHAPE probing verified that these chimeric pRNAs formed the correct secondary structures (data not shown). If the kissing interaction is the primary driver of multimerization, the expectation was that on native gels SF5_phi29 would act similar to phi29 pRNA, and SF5_M2 would be like M2 pRNA.

At 4°C, SF5_phi29 migrated at a rate consistent with dimer and similar to SF5 and phi29 WT pRNAs, while at 25°C it migrated more slowly, implying that it self-associates more stably than either SF5 or phi29 pRNAs. Measured by SV-AUC, at 25°C the SF5_phi29 chimera existed in a monomer–multimer mix (Table 1), while both SF5 and phi29 WT were only monomers. At 37°C SF5_phi29 migrated at a rate similar to phi29 WT and SF5, consistent with all being monomeric. In DMS probing experiments, SF5_phi29’s kissing loop L_{CE} was modified less at 4°C than at 37°C, consistent with multimer formation at the lower temperature but not the higher (Fig. 6C, cf. lanes 7 and 8 to lanes 10 and 11). Thus, combining parts of SF5 with the kissing interaction of phi29 results in a chimeric “daughter” that is more stable than one of the unmodified “parents.” The SF5_M2 chimera exhibited different behavior. Specifically, its migration on the native gel at 4°C indicated a dimer, compared with trimeric M2 pRNA (Fig. 6B). Furthermore, at 25°C SF5_M2 was monomeric

while M2 pRNA was a dimer. SV-AUC and DMS probing indicated the self-association was unstable (Fig. 6C, cf. lanes 18 and 19 to lanes 21 and 22). Hence, M2-like behavior did not transfer with the M2 kissing interaction sequence. Analysis of these two chimeric pRNAs showed that some structural elements other than the kissing interaction are fundamental to pRNA multimer stabilization.

Exploring the role of the three-way junction-containing ‘scaffold’

We hypothesized that the “scaffold” on which the kissing interaction sequences are displayed is important and defined this scaffold as the overall pRNA structure excluding the kissing hairpin, to include the J_U three-way junction (3wj) present in all pRNAs but whose sequence differs dramatically between strains. To test this, chimeras containing the SF5 pRNA’s kissing interaction on either the phi29 scaffold (phi29_SF5) or the M2 scaffold (M2_SF5) (Fig. 7A) were designed and tested by native gel at the stringent temperature of 25°C. If the kissing hairpin is the only determinant of self-association behavior, then phi29_SF5 pRNAs will be similar to phi29 pRNAs, and M2_SF5’s multimers will be less stable (and perhaps smaller) than M2 pRNA trimers. Conversely, if the scaffold is more important than the kissing interaction, M2_SF5’s will act similarly to M2 pRNA. At 25°C, phi29_SF5 migrates similarly to SF5 pRNA; at the higher temperature it is more SF5-like (monomeric at this temperature). In contrast, M2_SF5’s major band migrates similarly to M2 pRNA. Thus, the scaffold of M2 pRNA, the pRNA that is consistently the most stable and of highest stoichiometry, was able to overcome a “mediocre” kissing interaction. These results are consistent with the assertion that the scaffold, and therefore the 3wj, plays a crucial role in determining pRNA self-association characteristics, and furthermore suggests some features of the M2 pRNA scaffold are particularly favorable for forming multimers of higher stoichiometry and stability.

DISCUSSION

The pRNA is the only identified naturally occurring RNA that forms multimers via intermolecular interactions of identical molecules. Here, we studied the properties of minimal self-association of pRNAs from several members of the phi29 family, finding that they have diverse abilities to form multimers of different stoichiometry and thermostability; the latter characteristic has not previously been compared between different pRNAs. In all these pRNAs, the kissing interaction appears to be the point of intermolecular contact, but a key determinant of their diverse behaviors is likely the 3wj which differs dramatically between pRNAs of different strains. Placed into context with published results, our data should be useful for future pRNA engineering.

Because a recent study contained experiments similar to ours (Gu and Schroeder 2011), it is important to compare

FIGURE 6. Characterizing multimerization properties of the chimeric constructs. (A) Secondary structures of the first set of chimeric RNAs. Wild-type SF5 RNA is included for comparison. The predicted kissing sequences are in red, other nucleotides introduced by mutation are in cyan. (B) Native gel electrophoresis of the chimeric pRNAs of panel A at different temperatures. Gels containing 5 mM MgCl₂ are shown *above* while those with 2 mM EDTA are *below*. (C) DMS probing of the pRNAs from panel A. (RT) reverse transcription control; (–) controls without added DMS; (1,2) reactions with DMS; “4” or “37” indicates temperature (°C). A sequencing ladder is included and labeled. The locations of the CE and E loops are highlighted with bars and labels to the *right*, nucleotide locations are indicated to the *left*, and the location of the primer band is shown. To the *right*, the analyzed data are mapped onto the secondary structures. Nucleotides modified at both 4°C and 37°C are indicated with magenta while those only modified at 37°C are shown in green. The nucleotides involved in forming the kissing hairpin are shown in red. The primer binding location is shaded gray; these nucleotides were not visible on the gel.

the results, particularly where they differ. We found that the GA1 pRNA has little or no ability to self-associate although Gu and Schroeder (2011) detected some dimers at 4°C with a longer pRNA, high [RNA] and 10 mM Mg²⁺. A phylogenetic study divided the phi29 phage family into three subclasses with GA1 in its own separate subclass (Bailey et al. 1990; Pecenkova and Paces 1999; Meijer et al. 2001). Perhaps GA1 pRNA may use a different mechanism to form a stable multimer, or weak multimerization of GA1 pRNA might imply that the ring requires stabilization by interaction with other motor components. Likewise, we found SF5 pRNA forms a dimer while previous data suggest SF5 forms higher-order multimers at 4°C. Results from some of

chimeric RNAs in our study led to different conclusions as well. The differences could be due to differences in the conditions under which the experiments were conducted such as ionic strength, temperature, pRNA concentration, the secondary structure models used to design mutants, and the use of shorter (~75 nt) RNAs comprising just the multimerization domain. Based on our SHAPE probing for different pRNAs (Fig. 3), it was clear that the secondary structures of Chen et al. (1999) were largely correct and this is the model we used (Chen et al. 1999). Overall, the studies are complementary and their differences indicate the subtle behaviors of pRNAs and how readily the behaviors of these RNAs can be modulated.

FIGURE 7. Native gel electrophoresis of chimeric mutants testing the M2 scaffold. (A) Secondary structures of the chimeric RNAs. Both chimeric RNAs have SF5's kissing hairpin with either phi29 scaffold (phi29_SF5) or M2 scaffold (M2_SF5). Wild-type phi29, M2, and SF5 RNAs are included for comparison. The predicted kissing sequences are shown in red. (B) Native gel electrophoresis of the RNAs in panel A. Gels containing 5 mM MgCl₂ are shown *above* while those with 2 mM EDTA are shown *below*.

One novel aspect of our studies is a systematic examination of the ability of different pRNAs to multimerize at different temperatures. pRNA self-association has been extensively studied using native gel electrophoresis; however, most of the studies only examined multimerization at a single temperature (4°C) (Guo et al. 1998; Chen et al. 2000; Gu and Schroeder 2011). Our native gel results at three different temperatures indicate that pRNAs from different strains have different abilities to maintain the multimeric state and different stoichiometries at higher temperature (25°C and 37°C). For example, while M2 pRNA forms a trimer at lower temperature, it switches to primarily a dimer at 25°C. This suggests that future applications of pRNA in engineering or therapeutics should carefully examine temperature effects, which could be exploited in a rational way. In addition, although sedimentation velocity analytical ultracentrifugation (SV-AUC) has been applied to characterize pRNA multimerization (Gu and Schroeder 2011), our study is the first to use sedimentation equilibrium analytical ultracentrifugation (SE-AUC) to examine a diverse family of pRNAs. We demonstrate the suitability of this method for exploring self-associating RNA, particularly when coupled with other methods. Because the SE-AUC method provides stoichiometric information independent of molecular size and shape, it should be useful for more detailed thermodynamic examinations of diverse mutants under equilibrium conditions.

Our data support the conclusion that the differing abilities of various pRNAs to self-associate and the stability of the resultant multimers are determined by multiple factors. One feature that affects the ability is the number of base pairs the kissing interaction can form. For example, the GA1 pRNA we tested putatively has only two base pairs in the kissing interaction and we do not detect multimers at any temperature, while those with four or five potential pairs form multimers. However, the multimer stability does not directly track with the number of potential base pairs. SF5 might form five pairs in the kissing interaction but does not multimerize at 25°C, while M2 and B103, which have the potential to form only four base pairs, multimerize at this temperature. This might be due to several effects: (1) differences in the ability of the base pairs to stack and thus form helical elements of different stabilities, (2) structures within the kissing hairpins that form additional noncanonical interactions, (3) the presence of competing structures, or (4) perhaps the number of base pairs formed in each kissing interaction differs from that predicted. Furthermore, the fact that these different pRNAs form multimers of different stoichiometry is not readily explained by differing kissing interactions, and the fact that the characteristics of one pRNA species are not readily transferred to another through the kissing interaction strongly suggests that other features are important for different pRNA behaviors.

Our results show that in general the constructs built on the M2 pRNA scaffold and containing the M2 3wj more stably self-associate and form larger multimers than those built on the phi29 pRNA scaffold. Because the 3wj was the only other major structural element present in the RNAs we used and it differs substantially between pRNAs, we hypothesize that it plays a major role in determining the individual self-association characteristics of each pRNA.

RNA 3wjs often facilitate long-range contacts by arranging the emerging helices at specific angles (Lescoute and Westhof 2006), and in pRNAs the structure of the 3wj may facilitate self-association by placing the kissing loops in a geometry that favors formation of specific multimers (Zhang et al. 2012; Zhao et al. 2012). Although only the 3wj specifically from the phi29 pRNA has been studied, those studies may lend insight into how the junctions of other pRNAs could modulate self-association. The crystal structure of a tetrameric form of phi29 pRNA showed helices P_A and P_C to be coaxially stacked with helix P_C emerging at an angle of ~70° (Ding et al. 2011). The crystal structure of the isolated phi29 3wj core

showed similar helical arrangements, with some difference in the angle between P_A and P_C (Zhang et al. 2013). Interestingly, when the phi29 pRNA was studied using solution methods such as single-molecule FRET and electron paramagnetic resonance, the resultant models show a coaxial stacking of P_C and P_D , with P_A emerging at an angle, a conformation also suggested by computer modeling (Hoeprich and Guo 2002; Shu et al. 2010; Zhang et al. 2012). In addition, it has been shown that the UUU triplet at nucleotides 50–52 in the phi29 pRNA 3wj is important for helical conformation and phage DNA packaging, leading to the proposal that inherent flexibility in the junction is important for function (Zhao et al. 2012). This feature would explain the different conformations observed in the biophysical and structural studies. These data suggest a mechanism for phi29 pRNA self-association in which the 3wj dynamically samples a conformational landscape until intermolecular interactions stabilize one state in a form of “tertiary capture.” The plasticity in arrangements of the helices would allow different oligomeric states depending on conditions and explain the published observations; e.g., in solution a dimer is thermodynamically favored, crystal contacts could drive tetramer formation, and interactions within the packaging motor favor even higher-order multimers.

The aforementioned features of the phi29 pRNA can now be used to develop a model for how the other pRNA 3wj's could induce different behaviors. Using the fact that 3wjs have been cataloged into families based on topology, the classification rules place phi29 pRNA's 3wj into family “A,” a form in which conformational flexibility is a feature (Lescoute and Westhof 2006). In the crystal structure of the phi29 pRNA in tetrameric form (Ding et al. 2011), the junction does have some family A characteristics: P_A and P_C stack, and there are few tertiary contacts in the junction. However, P_D emerges from the junction at $\sim 70^\circ$ angle, a defining characteristic of family “B.” This analysis suggests that to multimerize, the state favored by the phi29 pRNA 3wj in the monomeric form (family A) must be “captured” in a different state (family B); this is consistent with inherent flexibility in the junction. If we apply this analysis to the 3wj from M2's pRNA, a different situation emerges. The 3wj of M2 pRNA is predicted to be a member of family B, the conformation observed in the tetrameric crystal structure and thus likely favorable for self-association. Thus, the ability of M2 pRNA to form multimers of higher stoichiometry and stability than that of phi29 is likely due to the 3wj favoring a specific conformation conducive to self-association. By extension, the diverse self-association behavior of pRNAs from different strains is likely dependent on subtle differences in the flexibility of each junction and the specific conformation landscape sampled by each.

More detailed analysis of 3wj's other than those found in pRNAs may lend additional tools and insight useful for the development of nanostructures through RNA engineering. The fact that different versions of the pRNA have different stabilities, indicated by diverse behaviors at different temperatures, is an interesting feature that impacts the use of pRNAs

in nanotechnology engineering. Exploiting the pRNA's multimerization properties as a tool for gene therapy or for delivery of various other RNA cargos may require pRNAs with different multimerization stabilities. The pRNA from phi29 is the most studied and tested, but it may not be the best if multimerization at temperatures approaching 37°C are needed. Although a full exploration of different kissing interaction–scaffold combinations is beyond the scope of this study, our results suggest that the most stable multimers could be built on the M2 scaffold. In fact, it may be possible to engineer or select for other artificial scaffolds that are even more conducive to multimerization.

Finally, the question could be asked as to why we did not observe multimers above trimer by any pRNA in any of our experiments. Although there is some disagreement in the literature about whether five or six copies exist in the fully assembled ring (Garver and Guo 1997, 2000; Guo et al. 1998; Chen et al. 2000; Shu et al. 2007; Morais et al. 2008), it is clear that the pRNA forms higher-order complexes in its final biologically functional state. It is very likely that hexamers, pentamers, or tetramers are thermodynamically unstable when free in solution. Indeed, our observations of dimers and trimers are consistent with the aforementioned previous study (Gu and Schroeder 2011); stable production of a substantial population of larger-order complexes in solution may require very specific conditions not readily replicated. While we cannot exclude the possibility that the lack of larger multimers is due to the use of our minimal (~ 75 -nt long) self-association constructs, it seems more likely that interactions with other components of the motor stabilize the larger ring structures. Indeed, it has been proposed for phi29 that smaller complexes are intermediates on the pathway to larger rings (Chen et al. 2000), and so a reasonable model is that the interaction of these intermediate-sized complexes with the other components of the motor leads to rearrangement into the larger ring structure.

MATERIALS AND METHODS

RNA preparation

RNAs for native gels, analytical ultracentrifugation and DMS probing were made using DNA generated by PCR using overlapping primers to make the DNA template (Kieft et al. 2007). RNAs were generated by *in vitro* transcription using the PCR products, and purified on 10% polyacrylamide (29:1 bisacrylamide:acrylamide) 7 M urea denaturing gels. Bands were identified using UV shadowing and then excised and eluted in RNase-free water overnight at 4°C . The eluted solution was then concentrated using Amicon Ultra filters (Millipore). pRNA concentrations were measured by UV absorbance at 260 nm (Nanodrop 2000c, Thermo Scientific).

Nondenaturing gel electrophoresis

Nondenaturing gel electrophoresis experiments were performed as described (Kieft et al. 2007). Three micrograms of RNA in a total

volume of 10 μL was loaded. The pRNA sample was renatured by heating to 85°C for 15 sec in Mg-Buffer (66 mM Tris, 34 mM HEPES, 5 mM MgCl_2 , pH 7.4) or EDTA-Buffer (66 mM Tris, 34 mM HEPES, 2 mM EDTA, pH 7.4) then snap cooled on ice. Samples were mixed with equal volume of 2 $\times$ native gel loading buffer (132 mM Tris, 68 mM HEPES, 10% glycerol, 0.5 mg/mL bromophenol blue, 0.5 mg/mL xylene cyanol, pH 7.4 with either 10 mM MgCl_2 for Mg^{2+} gels or 4 mM EDTA for EDTA gels) and were run on an 8% polyacrylamide native gel (29:1 bisacrylamide:acrylamide with Mg-buffer for Mg^{2+} gels or EDTA-buffer for EDTA gels). Both gel and buffer were preincubated at desired temperatures and the gels were prerun for 20 min at 5 W. Gels were run at 5 W for 10 h and then stained with 1% ethidium bromide for visualization.

Sedimentation velocity analytical ultracentrifugation

RNA samples for SV-AUC experiments were diluted to obtain an absorbance at 260 nm of ~ 0.7 , then dialyzed into AUC buffer (10 mM sodium cacodylic acid, 5 mM MgCl_2 , pH 7.5). Experiments were carried out at 12°C and 25°C in an Optima XL-I Analytical Ultracentrifuge (Beckman) using cells with two-sector centerpieces and quartz windows in an An-60Ti 4-Place Analytical Rotor (Beckman). Samples were initially spun at 3000 rpm and the absorbance recorded to verify sample loading concentration and no leakage, then spun at 40,000 rpm for 12 h to record scans. Absorbance profiles were recorded at 260 nm at regular intervals. The sedimentation process was considered complete when 10 consecutive scans did not show a change as a function of time. Results were analyzed with Svedberg (Philo 1997; Schuck and Demeler 1999).

Sedimentation equilibrium analytical ultracentrifugation

SE-AUC was performed in a Beckman-Coulter XL-A centrifuge using UV absorption optics, cells with six-sector centerpieces and quartz windows, and an An-60Ti 4-Place Analytical Rotor (Beckman). Samples are prepared at three different initial loading concentrations from 1 to 10 μM in AUC buffer (10 mM sodium cacodylic acid, 5 mM MgCl_2 , pH 7.5). Experiments were performed at two temperatures, 4°C and 25°C, and three different speeds (10, 12, and 20K rpm at 4°C; 12, 15, and 24K rpm at 25°C). Samples were centrifuged at each speed for 12 h and absorbance scans were obtained every 3 h at 270 nm. Equilibrium was achieved when no change in these concentration profiles as a function of time was observed. Data were analyzed by Heteroanalysis software version 1.1.58 (Analytical Ultracentrifugation facility, Biotechnology Bioservices Center, University of Connecticut). Data at each rotor speed were fitted globally to a single molecular species model:

$$S(r, \lambda) = \delta_\lambda + \varepsilon_\lambda C_0 \exp \left[M(1 - \bar{v}\rho) \frac{\omega^2}{2RT} (r^2 - r_0^2) \right],$$

where $S(r, \lambda)$ is the radially-dependent signal at wavelength λ , δ_λ is the baseline offset at wavelength λ , ε_λ is the molar extinction coefficient at wavelength λ , C_0 is the molar concentration of the monomer at the arbitrary reference distance r_0 , and M is the molecular weight of the monomer. Values for ρ , the solvent density of AUC buffer and $\bar{v}$, the partial specific volume, were obtained from previously published

work (Wood et al. 2005); $\rho = 0.998 \text{ g/cm}^3$, $\bar{v} = 0.455 \text{ mL g}^{-1}$ was used in this study. ω is the angular velocity of the rotor in radians/sec, R is the molar gas constant (83,144,000 erg/mol/K), and T is the absolute temperature. Data were also fitted to a reversible self-association monomer– N mer model to analyze samples that could form mixtures of monomer, dimer, and trimer species:

$$S(r, \lambda) = \delta_\lambda + \varepsilon_\lambda C_0 \exp \left[M(1 - \bar{v}\rho) \frac{\omega^2}{2RT} (r^2 - r_0^2) \right] + N\varepsilon_\lambda C_0^N \exp \left[\ln K + NM(1 - \bar{v}\rho) \frac{\omega^2}{2RT} (r^2 - r_0^2) \right],$$

where N is the stoichiometry of the association reaction and $\ln K$ is the natural logarithm of the association constant, K .

Radiolabeling primers for probing

RNA primers were 5'-radiolabeled in a reaction containing 40 μM of purified primer, 2 μL of 10 $\times$ polynucleotide kinase (PNK) buffer (70 mM Tris–HCl at pH 7.6, 10 mM MgCl_2 , 5 mM DTT, New England Biolabs), 2 μL of T4 PNK (10,000 U/mL, New England Biolabs), 1 μL of γ - ^{32}P -ATP (PerkinElmer, 6000 Ci/mmol) in a final volume of 20 μL . Reactions were incubated at 35°C for 1 h and then added to 10 μL 9 M urea loading buffer and loaded on a 10% polyacrylamide (29:1 bisacrylamide:acrylamide) 7 M urea denaturing gel (16.5 cm $\times$ 3.25 cm $\times$ 3.1 mm) and electrophoresed at 25 W for 15 min. Gels were visualized by autoradiography, then the primer bands were excised and eluted passively in elution buffer (0.5 M sodium acetate, 0.1% SDS, pH 5.3) overnight. This was followed by ethanol precipitation and the dried pellet was resuspended in RNase-free water prior to use.

SHAPE analysis

Six picomoles of RNA in 8 μL of RNase-free water was heated to 85°C for 30 sec, then cooled on the desktop. Two microliters of RNA folding buffer (333 mM HEPES pH 8.0, 20 mM MgCl_2 , 333 mM NaCl) was added. One microliter of DMSO was added to the DMSO controls and 1 μL of 50, 100, or 150 mM *N*-methylisatoic anhydride (NMIA) prepared in DMSO was added to the experimental reactions. Reactions were incubated at 37°C for 1 h and then desalted using Micro Bio-Spin 30 Tris Chromatography Columns (Bio-Rad). Reverse transcription (RT) was done with 1 μL of one of the following primers: M2 primer (5'-GGGTAAGTGGGTATCAAT-3'), SF5 primer (5'-GGGCAAGAGTGTTTACGCTC-3'), GA1 primer (5'-GGTAGTATTGCTACCTCGCAAC-3'), or B103 primer (5'-GGGTAAGTGGGCTATCAATCC-3'), which were 5'-end labeled with ^{32}P and diluted to 30,000 cpm/ μL . Reverse transcription was performed as described (Kieft et al. 2007; Filbin and Kieft 2011). Gels were aligned using the Semi-Automated Footprinting Analysis (SAFA) software (Das et al. 2005) and different lanes were normalized to total radiation using ImageQuant software followed by background subtraction of the control lanes in Microsoft Excel.

Dimethyl sulfate probing

Ten picomoles of RNA in 8 μL of RNase-free water with 2 μL of 5 $\times$ DMS probing buffer (400 mM sodium cacodylic acid pH 7.2, 500 mM KCl and 25 mM MgCl_2 for Mg^{2+} probing or 10 mM EDTA

for EDTA probing) was heated to 85°C for 30 sec, then cooled on the bench. Two microliters of 95% ethanol was added to the controls and 2 μ L of 2.64 M DMS (Sigma) in 95% ethanol was added to the reactions. Reactions were incubated at desired temperature for 10 min and then quenched with 5 μ L DMS stop buffer (1.0 M Tris-HCl pH 7.5, 0.1 M EDTA, and 1 M β -mercaptoethanol). Micro Bio-Spin 30 Tris Chromatography Columns (Bio-Rad) were used to clean up the reactions. Reverse transcription (RT) was done with 1 μ L of one of the following primers: phi29 wt (5'-GGGATGATT GACAACCAATCAAC-3'), COM and MUT1 primer (5'-GGGATG ATTGACCCGTAATC-3'), MUT2 primer (5'-GGGATGATTGAC AACCAATC-3'), M2 primer (5'-GGGTAAGTGGGTTATCAAT-3'), SF5 primer (5'-GGGCAAGAGTGTTCACGCTC-3'), GA1 primer (5'-GGTAGTATTGCTACCTCGCAAC-3'), B103 primer (5'-GGG TAAGTGGGCTATCAATCC-3'), SF5_phi29 primer (5'-GGGCAA GAGAACCACTCCCC-3'), or SF5_M2 primer (5'-GGGCAAGA GTTATCAATCTC-3'), which were 5'-end labeled with 32 P and diluted to 30,000 cpm/ μ L. Reverse transcription reactions and sequencing gels were run as described (Tijerina et al. 2007; Filbin and Kieft 2011). Gels were aligned using the Semi-Automated Footprinting Analysis (SAFA) software and different lanes were normalized to total radiation using ImageQuant software followed by background subtraction of the control lanes in Microsoft Excel.

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