Construction of phi29 DNA-Packaging RNA Monomers, Dimers, and Trimers with Variable Sizes and Shapes as Potential Parts for Nanodevices

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Recently, DNA and RNA have been under extensive scrutiny with regard to their feasibility as parts in nanotechnology. The DNA-packaging motor of bacterial virus phi29 contains six copies of pRNA molecules, which together form a hexameric ring as a crucial part of the motor. This ring is formed via hand-in-hand interaction by Watson-Crick base pairing of four nucleotides from the left and right loops.

Here we report that this pRNA tends to form a circular ring by hand-in-hand contact even when in dimer or trimer form, thus implying that the pRNA structure is flexible. Stable dimers and trimers have been formed from the monomer unit in a protein-free environment with nearly 100% efficiency. The dimers and trimers could be isolated by density gradient sedimentation or purified from native gel. Dimers and trimers were resistant to pH levels as low as 4 and as high as 10, to temperatures as low as -70 °C and as high as 80 °C, and to high salt concentrations such as 2 M NaCl and 2 M MgCl₂. Further study showed that pRNA dimers or trimers with variable lengths could be constructed. Seventy-five bases were found to be the central component in this formation. The elongation of RNA at the 3’ end up to 120 bases did not hinder their formation. Other conditions, including the salt requirement for the formation of monomers, dimers, and trimers, have been investigated. RNA monomers, dimers, and trimers with variable lengths are potential parts for nanodevices.

Keywords: pRNA, DNA Packaging, Virus Assembly, Bacteriophage phi29, Dimerization, Trimerization, Hand-in-Hand Interaction, RNA/RNA Interaction, Loop/Loop Interaction.

1. INTRODUCTION

One of the most intricate translation processes in biological systems is the genomic DNA encapsidation of linear dsDNA viruses, including herpesviruses, poxviruses, adenoviruses, and the ds-DNA bacteriophages. During replication, the lengthy genome of dsDNA viruses is translocated with remarkable velocity into a limited space within the procapsid. This energetically unfavorable DNA condensation process is accomplished by a DNA-packaging motor that uses ATP as an energy source. Bacteriophage phi29 encodes a 120-base RNA (pRNA) that plays a novel and essential role in its genomic DNA translocation (Fig. 1). The pRNA by itself binds ATP.

Procapsids with pRNA attached are competent to package DNA with the aid of the DNA-packaging protein gp16 and ATP. We are able to package phi29 genomic DNA into procapsids in vitro with up to 90% efficiency, using only purified proteins from cloned gene products. Furthermore, the DNA-filled capsids can be converted into infectious virions in vitro with the addition of the tail protein (gp9), the neck proteins (gp11 and gp12), and the morphogenic factor (gp13), which is also produced from cloned genes. We can assemble up to 10⁹ plaque-forming units (pfu) per milliliter of infectious phi29 virions in vitro with nine purified protein components as well as pRNA and genomic DNA presynthesized in vitro. Omission of pRNA will not result in plaque formation, thereby providing a system with a sensitivity of eight orders of magnitude for functional assay of the pRNA.

The phi29 packaging motor includes a 12-subunit connector. Six pRNAs form a hexameric ring as a crucial component of the DNA-packaging motor. Quantitative methods have been used to reveal that two single-stranded loops of RNA are involved in inter-RNA interaction to form a pRNA hexamer for phi29 DNA transportation. These two loops interact alternately to generate interlocking chains. To facilitate the description, the two loops have been named as the Right loop (close to the 5’ end) and the Left loop (close to the 3’ end), respectively. Intermolecular interaction of pRNA is referred to as “hand-in-hand” interaction. Here we report the construction of pRNA monomers, dimers, and trimers with variable lengths, using part of the pRNA as a central component (Figs. 2–4). Because of the diversity in sequence and the complementary properties in interstrand hybridization, DNA has been under
The truncated 23/97 RNAs were synthesized by single-stranded DNA template transcription. Equal amounts of ssDNA template and T7 top strand were mixed to form an annealed template (0.5 μM final) before being added to the transcription mixture, which was composed of 4 mM NTPs, 40 mg/ml PEG 8000, 25 mM MgCl₂, 0.026 mg/ml T7 RNA polymerase, 4 U/ml IPP (inorganic pyrophosphates), 0.77 mg/ml DTT, 0.25 mg/ml spermidine, 0.05 mg/ml BSA, and 40 mM Tris-Cl, pH 8.0. After 3 h of incubation at 37 °C, the transcription reaction was stopped by 8 M urea denaturing loading buffer.

2.2. Native TBM PAGE for Dimer and Trimer Detection

Ten percent native polyacrylamide gels were prepared in TBM buffer (89 mM Tris, 200 mM boric acid, 5 mM MgCl₂, pH 7.6). An equal molar ratio of each of the pRNAs was applied to study the formation of dimers and trimers. After running at 4 °C for 3 h, the RNA was visualized by ethidium bromide staining. Images were captured with an Eagle Eye II system (Stratagene).

2.3. Isolation of Dimers and Trimers from Native PAGE

Tritiated pRNA A-b' was mixed with B-a' for dimers and (B-e') plus (E-a') for trimers and was subjected to electrophoresis in 10% native PAGE made in TBM. The pRNA dimer and trimer bands were excised from the gels and eluted overnight with the same TBM buffer at 4 °C. These complexes were then either kept in TBM buffer at 4 °C for further use or frozen at −20 °C.
2.4. Separation of pRNA Complexes by Sucrose Gradient Sedimentation

Linear 5–20% sucrose gradients were prepared in TBM buffer. The pRNA mixtures containing multimers were loaded onto the top of the gradient. To separate dimers from trimers, samples were spun in an SW55 rotor at 45,000 rpm for 13 h at 4 °C. To separate dimers from monomers, samples were spun at 50,000 rpm for 14.5 h at 4 °C. After sedimentation, 15-drop fractions were collected and subjected to scintillation counting.

2.5. In vitro phi29 Virion Assembly Assay

Ten microliters of purified procapsids (0.013 μM) was dialyzed on a 0.025-μm VS filter against TBE for 15 min at ambient temperature. Various amounts of pRNAs, including monomers and dimers, were dissolved in 1.5 μl TMS buffer and then added to procapsids. Only a small volume was used to ensure a high concentration of pRNAs in the reaction. The mixtures were then dialyzed against TMS for another 30 min. The pRNA-enriched procapsids were mixed with gp16, DNA-gp3, and reaction buffer (10 mM ATP, 6 mM 2-mercaptoethanol, 3 mM spermidine in TMS) to complete the DNA packaging reaction. After 30 min, the neck, tail, and morphogenic proteins were added to the DNA packaging reactions to complete the assembly of infectious virions, which were then assayed by standard plaque formation.

3. RESULTS AND DISCUSSION

3.1. Construction of Variable-Length RNA Monomer, Dimer, and Trimer

To make our description more understandable, we will use uppercase letters to describe the right loop of the pRNA and lowercase letters to represent the left loop. The same letters in uppercase and lowercase indicate complementary
sequences, whereas different letters mean noncomplementary loops. For example, pRNA S’/S’/(A-b’) represents a full-size pRNA with noncomplementary right loop A (S’/-G45/GAC) and left loop b’ (3’/-U85/GCG) (Figs. 2–5).

The monomer of full-size (S’/S’/) and truncated (23/97) noncomplementary pRNAs such as 5’/3’(A-b’), (B-c’), (E-a’), or 23/97 (A-b’), (B-c’), (B-c’), or (E-a’) (Table 1) migrate faster in native gels (Fig. 5).

When the S’/S’/ or the 23/97 (A-b’) were mixed together with equal ratios of S’/S’/ or 23/97 (B-c’). RNAs shifted into slower migrating bands in native gels and proved to be dimers (Figs. 5 and 6). The band of dimer with heterosized subunits was between that

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**Table 1. Sequences of pRNAs.**

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>RNA 5’/3’ A-b’ (120 nt)</td>
<td>5’G’-Same---3’UGC---Same---2’UGG---Same---A120’3</td>
<td>RNA 5’/3’ B-c’ (120 nt)</td>
<td>5’G’-Same---3’UGC---Same---2’UGG---Same---A120’3</td>
</tr>
<tr>
<td>RNA 5’/3’ B-c’ (120 nt)</td>
<td>5’G’-Same---3’UGC---Same---2’UGG---Same---A120’3</td>
<td>RNA 5’/3’ E-a’ (120 nt)</td>
<td>5’G’-Same---3’UGC---Same---2’UGG---Same---A120’3</td>
</tr>
<tr>
<td>RNA 23/97 A-b’ (75 nt)</td>
<td>5’GUCAUGGUAA-AUGUGUGGGG-AUAUGUGGACU-GAUGUGAGUGCUGACCCCAUCUAUCGUAAAGUUGCGCUUAA23/3</td>
<td>RNA 23/97 B-a’ (75 nt)</td>
<td>5’GUCAUGGUAA-AUGUGUGGGG-AUAUGUGGACU-GAUGUGAGUGCUGACCCCAUCUAUCGUAAAGUUGCGCUUAA23/3</td>
</tr>
<tr>
<td>RNA 23/97 B-c’ (75 nt)</td>
<td>5’G’-Same---3’UGC---Same---2’UGG---Same---A120’3</td>
<td>RNA 23/97 B-c’ (75 nt)</td>
<td>5’G’-Same---3’UGC---Same---2’UGG---Same---A120’3</td>
</tr>
<tr>
<td>RNA 23/97 E-a’ (75 nt)</td>
<td>5’G’-Same---3’UGC---Same---2’UGG---Same---A120’3</td>
<td>RNA 23/97 E-a’ (75 nt)</td>
<td>5’G’-Same---3’UGC---Same---2’UGG---Same---A120’3</td>
</tr>
</tbody>
</table>
of the dimer 5'/3'(A-b')- 5'/3'(B-a') and 23/97(A-b')-23/97(B-a'). When three full-size or truncated RNAs with interlocking loops such as (A-b')(B-e')(E-a') (in this paper, RNA without a prefix is full-length 5'/3' pRNA, unless otherwise indicated) were mixed at equal molar concentration, a band in the native gel with a migration rate slower than that of a dimer was found and confirmed to be a trimer by sucrose gradient sedimentation (Fig. 6) and cryo-AFM (atomic force microscopy) (Fig. 3). Nucleotides 23–97 are the central components in the formation of both dimers and trimers. The ability to form dimers or trimers is not disturbed by 5' or 3' end truncation; one or two truncated pRNAs can be incorporated into dimers while one, two, or three truncated pRNAs can be incorporated into trimers.

When analyzed by sucrose gradient sedimentation, [3H]pRNA monomers, dimers, and trimers sedimented to fractions 12, 8, and 6, respectively (Fig. 6). A plot of hypothetical molecular weight vs. the log of migration distance (the fractional number) in gradient showed a linear relationship (data not shown). Thus the peaks of fractions 12, 8, and 6 could stand for monomer, dimer, and trimer, respectively. The purified monomers, dimers, and trimers are further confirmed by AFM imaging (Fig. 3).

3.2. pRNA Has a Strong Tendency to Form a Circular Ring by Hand-in-Hand Contact Regardless of Whether the pRNA Will Enter a Dimer, Trimer, or Hexamer Form

As reported previously,5 if a pRNA dimer or trimer contained a pair of noncomplementary loops, the dimer or trimer was unstable. A closed ring could not be expected, because of this faulty linkage. Results suggested that the formation of a closed ring by hand-in-hand interaction was required for the formation of a stable dimer or trimer complex in the solution (Figs. 1–4). It also suggested that pRNA has a strong tendency to form a circular ring by hand-in-hand interaction, regardless of whether the pRNA is in dimer, trimer, or hexameric form. It is obvious that the angles between the two loops in dimers and the two loops in trimers are different. Therefore, the pRNAs in dimers have adopted a different structure for intermolecular contact than the pRNAs in trimer, suggesting that the structure of pRNA is flexible and amendable.

Fig. 7. Inhibition of phi29 viral assembly by assorted inactive dimers and trimers. Different amounts of assorted competitor dimers or trimers were mixed with a constant amount of wild-type pRNA before being applied to in vivo assembly assays. Inhibition of phi29 assembly by assorted dimers or trimers suggests that the assorted dimers and trimers, though inactive, contain an unchanged conformation for procapsid binding.
3.3. Elongation of RNA at the 3' End of the 120 Bases Did Not Hinder Dimer and Trimer Formation

Variable lengths of nucleotide sequences were extended from the 3’ end of the pRNA. The extended pRNAs were tested for dimer and trimer formation. It was found that elongation of RNA at the 3’ end of the 120 bases (Table I) did not hinder the formation of dimers or trimers (data not shown). The C18C20A20 bulge was found to be dispensable for both RNA dimer and trimer formation.

3.4. Inhibition by Truncated 23/97 RNA Dimer and Trimer in in Vitro Viral Assembly

Truncated 23/97 RNA is inactive in DNA packaging. As discussed previously, we found that the 23/97 segment RNA is a dimerization and trimerization unit. The inhibition study showed that the truncated dimer (A-b')(23/97B-a') or the trimer (A-b')(B-c')(23/97E-a') can partially inhibit wild-type monomer pRNA activity (Fig. 7). This means that a truncated dimer or trimer still has its correct biological folding. The reduced activity of wildtype pRNA in the presence of a dead truncated dimer or trimer is due to the fact that the truncated dimer/trimer is still able to bind and occupy the RNA binding site in the procapsid in a competitive way. This competitive binding nature was further confirmed by the fact that the truncated dimer (A-b')(23/97B-a') and trimer (A-b')(B-c')(23/97E-a') can strongly inhibit the plaque formation of wild-type dimer (A-b)(B-a) and trimer (A-b)(B-e)(E-a) (Fig. 7).

3.5. Testing the Stability of Dimer and Trimer by Ion Requirement, Salt Concentration, pH, Temperature, Electrophoresis, and Sedimentation

To detect the minimum ion concentration for pRNA oligomerization, equal amounts of tritiated (A-b') and unlabeled (B-a') were mixed and loaded onto the top of a 5–20% sucrose gradient in TB buffer along with a variable amount of ions (Fig. 8). At a concentration of 5 mM Mg2+, about 45% of tritiated (A-b') centered at fraction 8, representing pRNA dimers. When the concentration was increased to 25 mM, about 90% of tritiated (A-b') centered at the dimer position. While at a 1 mM or lower concentration, the tritiated (A-b') remained as a monomer centered at fraction 2–4. The data indicated that at least 5 mM Mg2+ was required for detectable dimerization. The Mg2+ concentration requirement for dimer formation agrees with the data from polyacrylamide gel shift assays.

For circularly permuted cpRNAs,11 the Mg2+ concentration required for 50% trimer formation was about 4 mM; for pRNAs with wild-type 5'/3' ends, it was about 0.4 mM.37

A minimum 1 M concentration of monovalent ions is needed for pRNA oligomerization, although as little as 5 mM divalent ions is sufficient. Spermidine, a positively charged compound, can also stimulate oligomerization at a concentration of 5 mM, indicating that dimer or trimer formation is a result of a cation effect. CoCl2 or NiCl2 could not promote trimerization, whereas FeCl2, ZnCl2, and CdCl2 caused the precipitation of pRNA (Fig. 8). These data suggest that pRNAs could form oligomers in the presence of positively charged cations, including mono- or divalent cations, as well as spermidine. Formation of a multimeric ring is an intrinsic feature of pRNAs, and cations are a facilitator.

As shown in Figure 5, before dimer or trimer purification, stable dimers and trimers of pRNA were formed in a protein-free environment with nearly 100% efficiency.

Table II. Conditions affecting pRNA oligomerization and the stability of oligomers after complex formation.

<table>
<thead>
<tr>
<th>pH</th>
<th>Temperature (°C)</th>
<th>MgCl2 (M)</th>
<th>NaCl (M)</th>
<th>RNase A (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiments</td>
<td>4 6 8 10</td>
<td>−10 −20</td>
<td>0 4 37 55</td>
<td>80 0.25 1 2 0.25 1 2</td>
</tr>
<tr>
<td>Conditions affecting oligomerization</td>
<td>− − ±</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Conditions affecting the stability after oligomerization</td>
<td>− − − −</td>
<td>− − − −</td>
<td>− − − −</td>
<td>− − − −</td>
</tr>
</tbody>
</table>
The dimer and trimer were found to be stable and could be isolated by either density gradient sedimentation or purification from native gel (Figs. 5 and 6). Dimers and trimers were resistant to a pH as low as 4 and as high as 10, a temperature as low as -70 °C and as high as 80 °C, and a high salt concentration of 2 M NaCl and 2 M MgCl₂. (Table II, Fig. 9), 2397 RNA is unstable when exposed to pH 10 buffer.

4. DISCUSSION

DNA has been shown to be a useful nanoscale biological component for self-assembly. This lack of structural diversity due to the formation of predominantly double-stranded helices limits its usefulness in the building of flexible structures or the construction of nanodevices. Therefore, to make stable branched structures with greater structural complexity, sticking ends were used as bridges for linkage between DNA subunits.

Amazingly, another natural type of building block, RNA, can overcome the limitation of the DNA molecule. Unlike DNA, RNA generally exists in nature as a single-stranded conformation. The astonishing diversity in RNA function is attributed to the flexibility of the RNA structure. It has been shown that in most cases it is the structure, not the primary sequence, of RNA that determines its function. However, the primary sequence of RNA governs the 3D structure of RNA, which comprises helices, bulges, loops, stems, and hairpins. Phylogenetics analysis and complementary modification of RNA species have shown that the covariance of bases, if complying with certain rules, can lead to the formation of a defined 3D structure.

Here we have reported a set of RNA molecules that can be manipulated to form monomers, dimers, trimers, and hexamers. The information governing the assembly of the diverse structure is encoded in a self-folded region with 74 nucleotides. Within this 74-base self-folded region, four bases in the left loop and another four bases in the right loop determine the formation of monomer, dimer, trimer, or hexamer. This report reveals that the extension of the 3' end of the RNA does not interfere with its property of self-folding of the 74-base region. Thus, the 3' end could have a function similar to that of the sticky end of DNA in building the branched structures. Gain insight over DNA in the formation of helices and sticking end complementation, plus the intrinsic property of structural diversity, self-folding, and controllable length, this set of RNA offers a novel and unique way to assemble arrays or to serve as potential parts for nanodevices.

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References and Notes

42. K. Zito, A. Hutenhofer, and N. Pace, Nucleic Acids Res. 21, 5916 (1993).

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