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Reversible Switching of pRNA Activity on the DNA Packaging Motor of Bacteriophage phi29

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Abstract: This paper reports a reversible switching of the biological activity of an RNA molecule, packaging RNA (pRNA), which is a central component of the DNA packaging motor of bacteriophage phi29. The switching mechanism contains two components: (1) inhibition of pRNA by a short antisense DNA (asDNA) that can bind to the 3′ end of the pRNA and inactivate the packaging motor; and (2) reactivation of pRNA by isothermal removal of asDNA from pRNA through a strand displacement strategy. The switching process can be repeated for multiple cycles and has been demonstrated by gel electrophoresis and a virion assembly assay.

Introduction

The genomic DNA of bacteriophage phi29 translocates into a preformed protein shell (procapsid) during viral reproduction.[1] This energetically unfavorable process is driven by an ATP-powered DNA packaging motor. The motor has been studied extensively and reconstructed in vitro.[1,2] Recently, the DNA packaging motor and its components have also been explored as drug delivery vehicles for the therapy of cancers and viral infection,[3,4] and the motor components have been used as a target for developing new approaches in viral therapy.[5] The packaging motor contains an RNA component called packaging RNA (pRNA) that is indispensable for the motor activity.[6] The pRNA molecules fold into helix-rich secondary structures and can further interact with each other through loop-kissing interactions (Figure 1).[7,8] In the absence of viral procapsids, pRNA exists as monomers and dimers. With the native interacting loop sequences, pRNA exists dominantly as monomers, with a small fraction of dimers but no higher-molecular-weight oligomers (Figure 1b). In the presence of viral procapsids, pRNAs bind to procapsids (at the packaging porter) and associate with each other to form hexameric rings (Figure 1b). The pRNA hexameric complexes are templated and stabilized by the procapsids. In the final viral structures, the 5′/3′ helical domains of the pRNA molecules are at the outside of the viral particles and accessible to other molecules.

The ability to reversibly control the function of the DNA packaging motor is desirable for both fundamental biological studies on the DNA packaging mechanism and applications of the DNA packaging motor as nanodevices to actively transport DNA, RNA, and drugs into targeted cells.[9] In our previous studies, antisense oligonucleotides were used to target pRNA molecules, thereby inhibiting DNA packaging of the phi29 virus.[5] However, this inhibition was irreversible. The DNA−pRNA complex persisted. To effectively utilize the phi29 DNA packaging motor in nanodevices, it is desirable to restore the motor activity. Herein, we report a strategy for reversible on/off switching of the biological activity of the phi29 DNA packaging motor (Figure 1c).

A key to the reversible switching in this study is isothermal DNA strand displacement,[10,11] which is driven by the maximization of DNA base pairing. If there is a DNA duplex with a single-stranded overhang and a free single-stranded DNA (ssDNA) that is complementary to the long strand of the DNA duplex, the free ssDNA will displace the short strand in the original DNA duplex to form a longer DNA duplex, releasing the short ssDNA. The process is sequence-specific and takes place under isothermal conditions. Thus, other nonrelated DNA/RNA interactions are not affected. This strategy has been extensively used for DNA/RNA nanodevices[12−15] and DNA-based computations[16] in the past decade. However, the use of this strategy to reversibly regulate RNA molecules in the content

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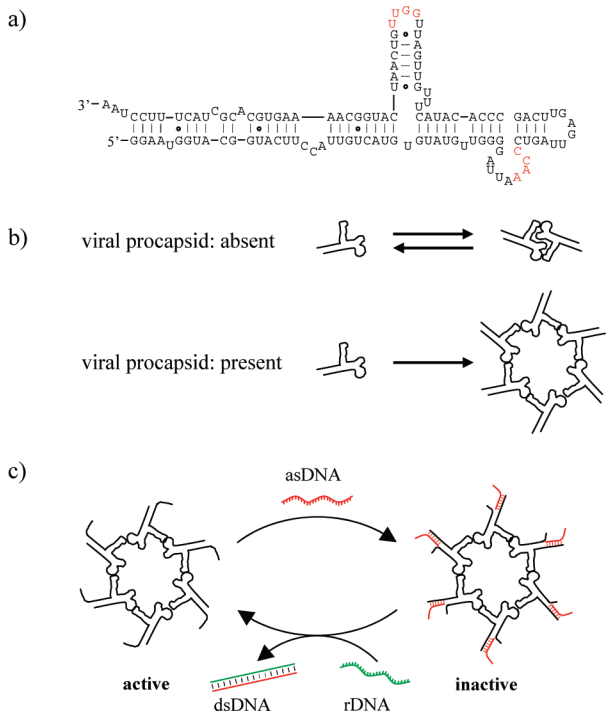

Figure 1. Reversible switching of the biological activity of the pRNA complex of the bacteriophage phi29 DNA packaging motor. (a) Secondary structure of the native pRNA molecule. The 3′ end of pRNA has been elongated in the current study to facilitate the asDNA binding. The two interacting loops are colored red. (b) pRNA self-association. In the absence of viral procapsids, pRNAs reversibly associate with each other to form dimers. The equilibrium prefers the pRNA monomer when the interacting loop sequences are as shown in (a). No higher oligomeric species can form. In the presence of procapsids, pRNA interacts with procapsids and forms hexameric rings around the packaging porters. (c) Regulation of the pRNA function by reversible and isothermal binding/removal of asDNA. It is not necessary for asDNA to bind to all of the pRNA to inhibit the packaging motor.

of complex biological systems has not yet been explored. Here we apply this strategy to controllably inhibit/activate the RNA-containing phi29 DNA packaging motor (Figure 1c). This strategy involves an antisense DNA (asDNA) and a removal DNA (rDNA). The pRNA is elongated at its 3′ end to contain a 3′-overhang, and the asDNA is complementary to the 3′ end of the pRNA. When the asDNA is bound to the pRNA, there is a single-stranded tail in the asDNA (Figure 1c). This tail facilitates the removal of the asDNA from the pRNA by the addition of an rDNA that is complementary to the asDNA. The formation of a longer DNA duplex promoted by a perfect match between the asDNA and the rDNA drives the dissociation of the asDNA from the pRNA and reactivates the packaging motor.

Materials and Methods

Oligonucleotides. All of the DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. and used without purification. The DNA sequences are as follows: asDNA, 5′-GTCAGATGTGGTAGGTTAGGAAAGTAGCGTGCACTTTTG-3′; rDNA, 5′-CAAAAGTGCACGCTACTTTCCTAACCTAC-CACATCTGAC-3′; and the shorter asDNA sequences, 5′-GTCA GATGTGGTAGGTTAGGAAAGTAG-3′ and 5′-G TCAGAT-GTGGTAGGTTAGGAAAG-3′.

Formation of asDNA−pRNA Complexes. For inhibition of the DNA packaging activity of the pRNA complex, unless otherwise stated, 1 μL of pRNA stock solution (10 μM), 1.5 μL of asDNA stock solution (10 μM), 1 μL of 10× TBM stock buffer, and 6.5 μL of DEPC-treated water were mixed to give a final pRNA and asDNA concentrations of 1 and 1.5 μM, respectively. The TBM buffer consisted of tris(hydroxymethyl)amino methane (Tris, 89 mM), boric acid (89 mM), and $MgAc_2$ (3 mM), pH 7.5. A pRNA/asDNA molar ratio of 1:1.5 was used to ensure that all of the pRNA molecules were bound to asDNA. The mixture was incubated at 22 °C for 2 h for hybridization. To remove the asDNA from asDNA−pRNA complexes, 1.5 μL of rDNA stock solution (10 μM) was added to the solution, which was then incubated at 22 °C for 2 h. For the second and third inhibition/activation cycles, asDNA and rDNA stock solutions were added to the solution resulting from the first cycle using the same procedure as for the first cycle. The molar ratio and incubation conditions were the same as for the first cycle. For agarose gel electrophoresis, 10 μL of sample solution was loaded into each well.

Native Gel Electrophoresis. A FisherBiotech mini-horizontal electrophoresis unit was used to run a 2% agarose gel at 4 °C with a constant voltage of 60 V (electric field strength: 6 V/cm). TBM buffer was used as the running buffer. After electrophoresis, the gel was stained by soaking in ethidium bromide (EB) aqueous solution (2.5 μg/mL) for 30 min, then destained by soaking in water for 30 min to reduce the background, and finally visualized under UV (λ = 365 nm) illumination (FisherBiotech Ultraviolet Transilluminator, FB-TIV-816A).

Preparation of pRNA. The synthesis and purification of pRNA were performed as reported previously.[17] The DNA duplex for the transcription of pRNA was prepared by PCR using a DNA template containing the pRNA coding sequence and two primers, 5′-TAA TAC GAC TCA CTA TAG GAA TGG TAC GGT ACT TCC-3′, which contained the T7 promoter (underlined), and 5′-CTT GCC AGG CAC CAT CGT AGG TTA GGA AAG TAG CGT GCA CTT TTG C-3′. The reaction solution contained 2.5 mM $MgCl_2$, each dNTP at 0.2 mM, each primer at 2 μM, and ∼1 μg of DNA template in GoTaq Flex buffer (Promega). After Taq DNA polymerase (Promega) was added, the solution was subjected to 22 thermal cycles (1 min at 95 °C, 2 min at 55 °C, and 30 s at 72 °C), then held at 72 °C for 5 min, and finally cooled to 4 °C. The pRNA was generated by in vitro transcription with T7 RNA polymerase. The reaction solution contained each rNTP at 5 mM, ∼50 ng/μL of DNA template from PCR, and T7 polymerase in transcription buffer (80 mM HEPES-KOH, 24 mM $MgCl_2$, 2 mM Spermidine, 40 mM DTT, pH 7.5). After the solution was incubated at 37 °C for 3 h, RNase free-DNase I (New England BioLabs) was added, and the solution was incubated at 37 °C for 10 min to digest the DNA template. pRNA was purified by 8% denaturing PAGE in TBE buffer (89 mM Tris-borate, 2.5 mM EDTA, pH 8.3) and 8 M urea. The sequence of the resulting pRNA was 5′-GGAAU-GGUACGGUACUUCCAUUGUCAUGUGUAUGUUGGGGAU-UAAACCCUGAUUGAGUUCAGCCCACAUACUUUGUUGA-UUGGUUGUCAAUCAUGGCAAAAGUGCACGCUACUUUC-CUAACCUACGAUGGUGCCUGGCAAG-3′.

Assay of Virion Assembly Activity of pRNA Complexes. The activity assay of pRNA via the in vitro phi29 assembly system was performed as reported previously.[2] After preincubation of pRNA with asDNA or rDNA molecules as indicated, pRNA or pRNA−DNA complexes were incubated with viral procapsids, protein gp16, and the phi29 genomic DNA−gp3 conjugate. The complexes were further assembled into virions to evaluate their infectivities against *Bacillus subtilis*. In brief, 1 μL of pRNA (0.1 mg/mL) or pRNA−DNA complexes was mixed with 10 μL of purified procapsids (0.3 mg/mL) and dialyzed on a 0.025 μm pore size membrane ("V" Series membrane, Millipore Corp.) for 15 min against TBE (89 mM Tris-borate, 2.5 mM EDTA, pH 8.3), after which the buffer was switched to TMS (50 mM Tris, 100 mM NaCl, 10 mM $MgCl_2$, pH 8.0) for 30 min of further dialysis. The pRNA−procapsid solution was mixed with 6 μL of gp16 (10 μg/mL), 1 μL of DNA−gp3 (0.2 mg/mL), and 3 μL of reaction buffer

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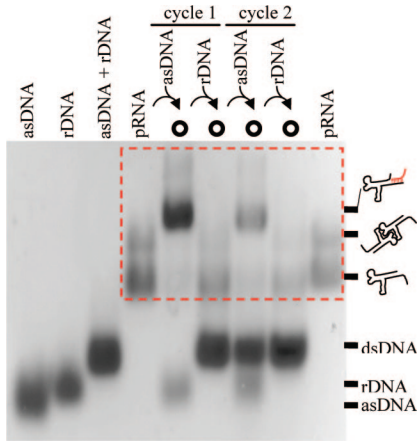

Figure 2. Electrophoretic assay of two cycles of reversible binding/dissociation of asDNA and pRNA by native agarose gel electrophoresis. Sample compositions are indicated above the gel, and chemical identities are indicated at the right. The pRNA and pRNA−asDNA complexes are located in the red box.

(10 mM ATP, 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, pH 8.0) to complete the DNA packaging reaction. After 30 min at ambient temperature, excess amounts of neck, tail, and morphogenic proteins were added to the DNA packaging reaction to complete the assembly of infectious virions. The assembled virions were plated with *B. subtilis* $su^{+44}(sup^{+})$ to calculate their plaque-forming units.

Results and Discussion

Reversible Binding of asDNA to pRNA. The reversible binding of pRNA (monomer or dimer) and asDNA was first demonstrated by native agarose gel electrophoresis (Figure 2). In the gel, pRNA itself shows a predominant rapidly migrating band (monomer) and a minor slow band (dimer). This phenomenon is consistent with previous reports.[2,7,8,17] The red box in the figure highlights the oscillation pattern of pRNA mobility in different states. In the presence of asDNA, pRNA (monomer and dimer) associates with asDNA, and the resulting pRNA−asDNA complex migrates more slowly than free pRNA. Upon addition of rDNA, the asDNA dissociates from the pRNA and forms a perfect asDNA−rDNA duplex, which frees the pRNA to resume its original electrophoretic mobility. Such a migration pattern cycles when another run of asDNA and rDNA is added, demonstrating that asDNA can reversibly bind to and dissociate from pRNA. To achieve reversible cycles of binding between asDNA and pRNA, addition of asDNA or rDNA dilutes the sample and causes serial decay of the pRNA band intensity in the gel for each successive cycle.

Biological Assay of Viral Assembly Activity of asDNA-Regulated pRNA. Binding of asDNA to pRNA reversibly switches the biological function of the pRNA, as demonstated by an in vitro phi29 virion assembly system. Briefly, pRNA was incubated first with DNA to form the pRNA−DNA complex. For the virion assembly assay, the resulting complex was further incubated with phi29 procapsids and then with the viral genomic DNA and the viral protein gp16.[2] The infectivity of the resulting virions was evaluated by plating on *B. subtilis*. The virions were counted as plaque-forming units (pfu) per milliliter. When bound to asDNA, the pRNA lost its function; the motor could not package its genomic DNA into the procapsid to assemble any infectious virion. However, after the addition of rDNA, the asDNA was removed from the pRNA, and the pRNA recovered its biological function, resulting in the production of infectious viruses. Addition of asDNA decreased the viral yield by a factor of ~1000 (Figure 3). Addition of rDNA restored the motor function to package the DNA and produce viruses. The inhibition/activation of the pRNA activity of the motor followed the addition sequence of asDNA and rDNA. In order to check whether random DNA can inhibit the DNA packaging, rDNA alone was used as a control, and no inhibition of the DNA packaging was found. When the asDNA was shortened at its 3′ end, its ability to bind to the pRNA decreased, and only minor inhibition was found for two slightly different shorter asDNAs. In two parallel experiments, the assembly activity decreased by a factors of 5.4 and 5.5 for one short asDNA and 15 and 5.4 for the other short asDNA. These control experiments confirmed that the asDNA worked as designed.

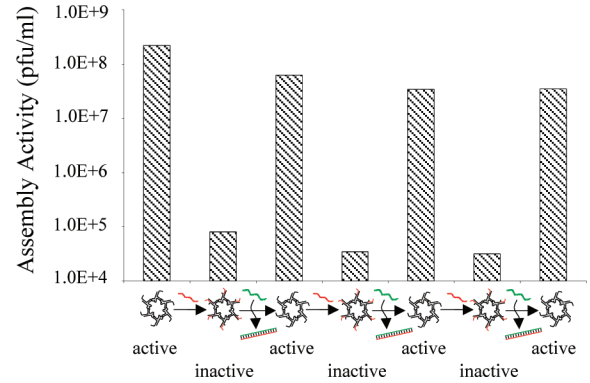

Figure 3. Virion assembly activity of the asDNA-regulated pRNA. Three cycles of inhibition/reactivation are shown.

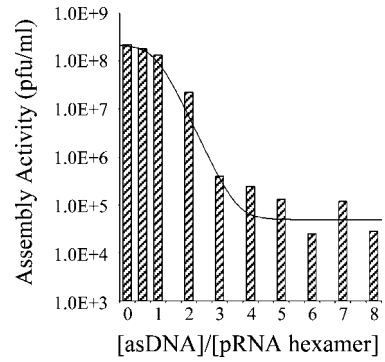

Figure 4. Effect of relative ratio of asDNA to pRNA hexamer on the activity of the pRNA hexameric complex. The columns are experimental data and the thin line is a guide to the eye.

Dependence of the Assembly Activity of pRNA on the Relative Ratio of pRNA and asDNA. The pRNA inhibition is efficient and dose-dependent. In the DNA packaging motor, pRNA molecules exist as hexameric ring-like complexes. It has been reported that incorporation of one inactive pRNA mutant into the pRNA complex completely blocks the function of the DNA packaging motor.[18] Thus, we reasoned that it should not be necessary for asDNA to bind to all six pRNA molecules to interrupt the biological function of the packaging motor. Instead, only one copy of asDNA binding to one copy of the pRNA in the hexameric pRNA ring should be sufficient to disrupt the biological activity of the entire pRNA complex. Therefore, an asDNA/pRNA molecular ratio of 1:1 should not be necessary. To test this hypothesis, we incubated asDNA and pRNA at

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various molecular ratios and then incubated with procapsids to check the corresponding efficiency of virion assembly (Figure 4). We found that the efficiency of the virion assembly was dramatically decreased by the addition of 2−3 asDNA molecules per pRNA hexameric complex on average. Further increasing the amount of asDNA did not significantly decrease the assembly efficiency.

Conclusions

We have developed an effective strategy for reversible switching of an RNA-containing biological nanomotor by introduction/removal of an antisense DNA. This work provides a method for controlling the biological activities of the phi29 DNA packaging nanomotor and paves the way for its technological application. We are currently investigating the use of the controllable nanomotor for active drug delivery. It is also conceivable that the same strategy could be applied for structural and functional control over other RNA-containing biological complexes.

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