Inhibition of Phage φ29 Assembly by Antisense Oligonucleotides Targeting Viral pRNA Essential for DNA Packaging

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Received January 30, 1995; accepted June 16, 1995

A sensitive and efficient system for the functional assay of antisense oligonucleotides (oligos) was developed based on an in vitro viral assembly system. A 120-base RNA (pRNA), which dispensably participates in bacteriophage φ29 DNA packaging, was used as the target for antisense action. Antisense oligos bound to pRNA, as revealed by a slower electrophoretic mobility of pRNA/oligo complexes in comparison with native pRNA. Infectious viruses were assembled in vitro with synthetic pRNA and DNA, as well as with viral proteins produced from cloned genes. Up to 10^7 plaque-forming units per milliliter were obtained in the absence of antisense oligos, while as few as zero plaques were detected in the presence of certain antisense oligos. A 1-base mismatch greatly influenced the inhibitory effect of the antisense oligos, but this 1-based mismatch was not important when the mismatch was placed at the end of the oligo. Five oligos did not bind pRNA or inhibit the assembly of the virion, suggesting that the RNA sequences complementary to these oligos are nonessential or buried internally in the RNA. Viral assembly was strongly inhibited by antisense oligos P15 and P10, targeting either the 5'-or the 3'-end of the pRNA, respectively. Viral assembly was also strongly inhibited by oligo P6, targeting an internal region, residues 75-91, of pRNA. Oligo P6 inhibited DNA packaging activity by blocking the binding of pRNA to the procapsid, while P10 and P15 inhibited DNA packaging activity but did not block the binding of pRNA to the procapsid, suggesting that in addition to the reported internal domain for procapsid binding, pRNA contains another domain at the paired 5'-3' ends with a yet to be defined role in DNA translocation.

INTRODUCTION

Bacteriophage φ29 of Bacillus subtilis packages its genomic DNA into a preformed procapsid (Bjornst et al., 1981; Guo et al., 1986) with the aid of a DNA packaging ATPase, gp16 (Guo et al., 1987c). Purified procapsids, assembled from cloned gene products, are active for DNA packaging in vitro (Guo et al., 1991; Lee and Guo, 1994; Guo and Trotter, 1994). A 120-base RNA molecule (pRNA, "p" for packaging) encoded by the φ29 genome has a novel and indispensable role in viral DNA packaging (Guo et al., 1987a, b). The pRNA attaches to the portal vertex (connector) of the procapsid in the presence of Mg^2+ and leaves the portal vertex in the presence of EDTA. The pRNA-free procapsid is inactive in DNA packaging but becomes active following incubation with the purified pRNA (Guo et al., 1987a, b; Wichlaczewski et al., 1989). In addition to phylogenetic analysis, which has proposed the secondary structure of pRNA (Bailey et al., 1990), our recent study with mutant pRNAs has implicated that the 5'- and the 3'-ends of the pRNA are paired, and the paired ends, excluding the 3-base overhang at 3'-end (residues 118-120), were crucial for the packaging of φ29 DNA (Zhang et al., 1994, 1995). The pRNA has also been shown, by RNA footprinting, to contain a procapsid binding domain localized at residues 22 through 84 (Reid et al., 1994a, b).

Involvement of a nonprotein factor, such as pRNA, in viral DNA packaging provides a new insight into RNA/protein or RNA/DNA interactions and extends previously shown RNA functions. Studies of the interactions involving this pRNA molecule are likely to provide a model for a catalytic RNA molecule acting on DNA. Involvement of RNA in DNA packaging has been demonstrated in the phage λ/φ29 chimeric system (Donate and Carrascosa, 1991) and Mu-like phage D108 (Burns et al., 1990) and speculated in poxvirus (Parsons and Pickup, 1980) and adenovirus (Hoffman and Hearing, 1994). A novel and very efficient antiviral strategy has been developed with the use of mutant pRNA to confer intracellular immunization (Trotter et al., 1995). If involvement of such pRNAs in viral genome encapsidation is a general phenomenon, then RNA can be a potential target for antiviral drug design. Consequently, the antisense oligonucleotide (oligo) would be a subject for investigation.

A series of oligos were used in an inhibition assay to define the functional domains of the pRNA in DNA packaging. These oligos were complementary to either the paired 5'- or 3'-ends or the internal regions of three
pRNAs that are different in primary sequence and variable in predicted secondary structure yet similar in biological activity. Using such oligos in conjunction with a pRNA binding assay allowed further characterization of the functional domains of pRNA. These studies elucidated the mechanism by which viral assembly was inhibited, whether by inhibiting RNA procapsid binding or by inhibiting the RNA's involvement in DNA translocation.

**MATERIALS AND METHODS**

**Oligos**

Oligos P4, P9, P10, P11, and P13 were complementary to the 3'-end of the pRNA. P15 was complementary to the 5'-end of the pRNA, and all others were complementary to internal sequences of the pRNA. In addition to the T7 promoter sequence, oligos P7 and P8 contained 8 and 7 bases, respectively, sense to the 5'-end of the pRNA, thus serving as negative controls (Table 1).

**In vitro transcription of pRNAs with T7 RNA polymerase**

Mutant pRNAs were constructed as described previously (Zhang et al., 1994). Briefly, linear plasmid DNAs were used as templates to generate DNA fragments with primer pairs P7/P4, P7/P10, and P7/P11 (Table 1) from PCR, respectively. The purified PCR DNA fragments were used as templates to synthesize pRNAs 7/4, 7/10, and 7/11, respectively, by in vitro transcription with T7 RNA polymerase. The pRNA from the PCR DNA template produced with primer pairs P7 and P4 was called pRNA 7/4, and other pRNAs derived from different primer pairs were named in a similar fashion.

³H-labeled pRNA 7/11 was synthesized and purified with procedures similar to those for preparation of unlabeled pRNA 7/11, except for the addition of [³H]UTP to the in vitro transcription mixture.

**Purification of the recombinant procapsid, DNA-gp3, and gp16**

The purification of gp16 (Guo et al., 1986) and DNA-gp3 (Lee and Guo, 1994) has been described previously. Procapsids were overproduced and purified from Escherichia coli with a procedure derived from previously reported methods (Guo and Moss, 1990; Guo et al., 1991).

**Functional assay for antisense oligos with the φ29 assembly system**

The construction of the highly sensitive φ29 assembly system has been reported (Lee and Guo, 1984). To assay the effect of an oligo on phage assembly, 1 μl pRNA (4 μM or 0.15 μg/μl) was mixed with 1 μl of oligos (100 μM) and dialyzed on a 0.025-μm type VS filter membrane (Millipore Corp.) against TBE (89 mM Tris–HCl, pH 8.3, 89 mM boric acid, 2.5 mM EDTA) for 15 min at ambient temperature. To attach the pRNA/oligo complex to procapsids, 5 μl of purified procapsids was mixed with the pRNA/oligo complex for additional 15 min under the same conditions and then against TMS (50 mM Tris–HCl, pH 7.8, 10 mM MgCl₂, and 100 mM NaCl) for 30 min at ambient temperature. To package DNA-gp3, the pRNA-enriched procapsids were mixed with 3 μl reaction buffer (10 mM ATP, 6 mM spermidine, 3 mM β-mercaptoethanol, 50 mM Tris–HCl, pH 7.8, 10 mM MgCl₂, and 100 mM NaCl), DNA-gp3 that had been dialyzed against TMS for 40 min at ambient temperature, and DNA-gp3 packaging protein gp16 that had been dialyzed against...
0.01 M Tris–Cl, pH 7.5, and 0.04 M KCl for 40 min on ice (Guo et al., 1986). The final mixture was then incubated for 30 min at ambient temperature.

To convert the DNA-filled capsids into an infectious phage (Lee and Guo, 1995a, b), the DNA-filled procapsids were incubated with 18 μl of gp8.5-9 extract from E. coli HMS174 (DE3)/pLysS containing plasmid pAR8.5-9 and 20 μl of gp11-12-13-14 extract from E. coli HMS174 (DE3)/pLysS containing plasmid pAR11-12-13-14 for 2 hr at ambient temperature.

**Assay for the binding of oligos to pRNAs**

Oligos P4, P10, and P11 were end-labeled with [γ-32P]ATP by T4 polynucleotide kinase and purified by MERmaid (Bio 101). A binding assay was performed with both unlabeled oligos and 32P-labeled oligos.

With the unlabeled oligos, 2 μl pRNA 7/4, pRNA 7/10, and pRNA 7/11 (2.5 μM or 100 ng/ml) was mixed with 3 μl oligos (100 μM) F2, P3, P4, P6, P10, P11, P13, P15, and P19, respectively, in 15 μl of TMS buffer at room temperature for 10 min. The samples were loaded to a 12% native polyacrylamide gel. The gel was stained with ethidium bromide and photographed.

To test for pRNA-oligo interaction, 2 μl of 32P-labeled oligo P4, P10, or P11 (100 μM) was incubated with 1 μl of pRNA 7/4, 7/10, or 7/11 (3.7 μM) in 15 μl of TMS buffer at room temperature for 10 min and loaded onto a 10% native polyacrylamide gel. The gel was autoradiographed.

**Assay for the binding of pRNA to procapsids**

3H-labeled pRNA 7/11 was used in the procapsid binding assay in the presence or absence of oligo P6, P11, or P15. Two microliters of [3H]pRNA 7/11 (3.7 μM) was mixed with 3 μl of oligo P6, P11, or P15 (100 μM), respectively, and dialyzed on a 0.025-μm type VS filter membrane (Millipore Corp.) against TBE buffer for 10 min. Purified procapsid from E. coli HMS174 (DE3) harboring plasmid pAR7-8-10/Nde was added to the mixture of [3H]-pRNA 7/11 and P6, P11, or P15. After further dialysis against TBE for 15 min the membranes containing the mixture of procapsid, [3H]pRNA, and P6, P11, or P15 were then transferred to TMS buffer for 30 min. The reaction mixture was brought up to a final volume of 100 μl with TMS and loaded onto a 5–20% sucrose gradient and then centrifuged in a SW25 rotor at 35,000 rpm for 30 min. Fractions were collected from the bottom and counted in a β-scintillation counter.

**Computer prediction of pRNA secondary structure**

Both GCG (Genetics Computer Group, Wisconsin) and GeneWorks (IntelliGenetics, Inc.) programs were used for RNA and DNA analysis (Devereaux et al., 1984; Glynies, 1991; Guo et al., 1987c). Secondary structures of pRNAs were predicted by the method of Zuker (1989) with the GCG program, and only those structures with the lowest predicted energy were selected.

**RESULTS**

The system for the assay of antisense nucleotides

The entire system consisted of: (a) recombinant procapsid overproduced in and purified from E. coli; (b) purified φ29 genomic DNA-gp3; (c) purified gp16, which is a DNA-packaging ATPase; (d) pRNA that was transcribed in vitro with T7 RNA polymerase; (e) tail proteins overproduced in and purified from E. coli; (f) extracts containing the coexpressed lower collar protein gp11, appendage protein gp12, and morphogenic factor gp13; and (g) reaction buffer containing ATP and Mg2+.

To assay the effect of oligos on phage assembly, pRNA was mixed with individual oligos and dialyzed. The pRNA/oligo complexes were incubated with purified procapsids. The pRNA-enriched procapsids were then mixed with purified DNA-gp3, the DNA packaging protein gp16, and reaction buffer containing ATP to package the φ29 genome into the procapsid. The DNA-filled procapsids were subsequently converted into infectious virions after the addition of the neck (gp11 and gp12) and tail (gp9) proteins and morphogenic factor gp13. Typically, 105 plaque-forming units (pfu)/ml were obtained in the absence of an antisense oligo (Table 2).

**Sequence and secondary structure of the target pRNAs**

Three pRNAs (Fig. 1) pRNAs 7/4, 7/10, and 7/11, were transcribed in vitro with T7 RNA polymerase on DNA templates synthesized with PCR primer pairs P7/P4, P7/P10, and P7/P11, respectively (Zhang et al., 1995). Mutant pRNAs 7/4 and 7/11 were 120 bases, and pRNA 7/10 was 117 bases, having a deletion of the 3 overhanging bases at the 3'-end, U116A117A120. In all three pRNAs, the first base U1 at the 5'-end was changed to G1 due to the requirement for transcription initiation with T7 RNA polymerase (Rosenberg et al., 1987). In pRNAs 7/10 and 7/11, the base A117, at the 3'-end was changed to C117, in order to maintain the secondary structure of the pRNA (Reid et al., 1994b; Zhang et al., 1994). The secondary structure of pRNAs 7/10 and 7/11 was identical with wild-type pRNA as predicted by computer folding (Zuker, 1989). In pRNA 7/4, the first base U1 at the 5'-end was changed to G1, as described above, while base A117 at the 3'-end was not modified, resulting in 2 unpaired bases that change the pRNA secondary structure. Nevertheless, all three pRNAs were biologically active as wild-type pRNA in φ29 DNA packaging and viral assembly as reported previously (Zhang et al., 1994, 1995). These three pRNAs were chosen in this study due to their
TABLE 2

Inhibition of Viral Assembly by Oligomers

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>Sense (+) or Antisense (−)</th>
<th>pRNA7/4</th>
<th>pRNA7/10</th>
<th>pRNA7/11</th>
</tr>
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<tr>
<td>None</td>
<td>−</td>
<td>5 × 10^9</td>
<td>3.7 × 10^9</td>
<td>2.4 × 10^9</td>
</tr>
<tr>
<td>P2</td>
<td>−</td>
<td>0</td>
<td>4 × 10^6</td>
<td>1.1 × 10^6</td>
</tr>
<tr>
<td>P3</td>
<td>−</td>
<td>0</td>
<td>1.14 × 10^6</td>
<td>2.5 × 10^6</td>
</tr>
<tr>
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<td>2.96 × 10^6</td>
<td>5 × 10^6</td>
</tr>
<tr>
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<td>6.8 × 10^6</td>
<td>1.86 × 10^6</td>
</tr>
<tr>
<td>P10</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>2.9 × 10^9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P13</td>
<td>−</td>
<td>1.88 × 10^9</td>
<td>4.26 × 10^6</td>
<td>1.7 × 10^6</td>
</tr>
<tr>
<td>P15</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P17</td>
<td>−</td>
<td>7 × 10^9</td>
<td>4.9 × 10^6</td>
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</tr>
<tr>
<td>P19</td>
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<tr>
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</tr>
<tr>
<td>P8</td>
<td>+</td>
<td>4.4 × 10^9</td>
<td>2.8 × 10^6</td>
<td>7.8 × 10^6</td>
</tr>
</tbody>
</table>

difference in sequences, variation in computer predicted secondary structure, and similarity in DNA packaging activity.

Strong inhibition of viral assembly by oligos targeting either the 5' or 3'-ends of the pRNA

It has been previously reported that the 5'- and 3'-ends of the 120-base viral pRNA are paired, and the paired ends are crucial for the packaging of bacteriophage φ29 DNA (Zhang et al., 1994). However, the 3 overhanging bases at the 3'-end are not essential for pRNA activity (Zhang et al., 1995). To study the inhibitory effect of antisense oligos on viral assembly, 15 oligos were investigated with pRNAs 7/4, 7/10, and 7/11 as targets (Table 1). The sequence of pRNA 7/10 was identical with that of pRNA 7/11 with the exception of a 3-base deletion extending from the 3'-end of the proximate region (Fig. 1), and pRNA 7/4 was similar to pRNA 7/11, although residue A₁₁₇ is replaced by C₁₁₇ in pRNA 7/11 (Fig. 1). Six of the 15 oligos were complementary (antisense) to the paired 5'/3'-ends. Oligo P15 targeted the 5'-end and the others (P4, P9, P10, P11, and P13) targeted the 3'-end (Fig. 1). Sense oligos P7 and P8 were used as controls. Five of the 6 oligos exhibited a strong inhibitory effect on viral assembly (Table 2). Oligos P10 and P15 completely inhibited the biological activity of all three pRNAs. P15 contains 17 bases complementary to residues 1 through 17 at the 5'-end of the pRNAs. P10 contains 21 bases complementary to the 3'-end of the pRNAs, from residues 96 to 117. P11 and P4 do not inhibit the activity of all the pRNAs (see below). Oligo P9 contains 17 bases complementary to residues 104–120 and has a 4-base mismatch at residues 114–117 (Table 2). P9 did not inhibit viral assembly, possibly due to this 4-base mismatch. An interesting finding is that, even with perfect complementation, P13 had a very weak inhibitory effect on viral assembly. This oligo complemented the 3'-end of the pRNA at residues 99–115, only 2 bases away from the essential 3'-end residue 117.

Influence on inhibitory effect by 1-base mismatch of the antisense oligos

Oligo P11 blocked the activity of pRNAs 7/10 and 7/11 completely, while only partially blocking the activity of pRNA 7/4 (Table 2). Oligo P4, a 19-mer oligo perfectly complementary to the 3'-end of pRNA 7/4 from residues 101 to 120, blocked the activity of pRNA 7/4 completely, while partially inhibiting the activities of pRNAs 7/10 and 7/11 (Table 2). It is important to note that there is a 1-base alteration between pRNAs 7/4 and 7/11, i.e., residue 117 in pRNA 7/11 was a C while in pRNA 7/4 residue 117 is an A. P11 was perfectly complementary to the 3'-end of pRNA 7/11, while being complementary to pRNA 7/4 with one mismatch. P4 was complementary to the 3'-end of pRNA 7/4, while complementary to pRNA 7/11 with one mismatch. These results suggest that a 1-base mismatch could affect the inhibitory effect of antisense oligos on pRNA activity. However, this 1-base mismatch was not important when the mismatch was placed at the end of the 21-base oligo. This was demonstrated with the 21-base oligo P10, which inhibited pRNA 7/4 completely even with a 1-base mismatch at the far 3'-end of the targeted region (residue 117 in pRNA 7/4 was "A" instead of "C").

Inhibition of viral assembly by oligos targeting internal regions of the pRNA

Oligo P6, which was complementary to pRNA residues 75 through 91, completely inhibited the activity of three
different pRNAs (Table 2). Oligo P19, which paired to pRNA residues 61 to 77, partially inhibited the activity of three pRNAs. Oligos P17, P21, and P23, which were complementary to pRNA residues 21 to 37, 38 to 54, and 65 to 81, respectively, did not affect the biological activity of the three pRNAs.

Oligos P2 and P3 were complementary to the three pRNAs from residues 88 to 106 and from 83 to 99, respectively (Table 1), and both oligos inhibited the activity of pRNA 7/4 completely. However, P2 did not affect the biological activity of pRNAs 7/10 and 7/11, while P3 partially inhibited the activities of pRNAs 7/10 and 7/11 (Table 2). The secondary structure of pRNAs 7/10 and 7/11 differed from that of pRNA 7/4 in that the 5'- and 3'-ends were unpaired in pRNA 7/4 (Fig. 1). The unpaired base at the 5'- and 3'-ends might play a role in the inhibitory effect of oligos P2 and P3.

Binding of antisense oligos to pRNA

To confirm the binding of oligos to pRNA, 32P-labeled oligos P4, P10, and P11 were mixed with unlabeled pRNAs 7/4, 7/10, and 7/11 and loaded to a 12% native polyacrylamide gel (Fig. 2A). With UV shadowing or ethidium bromide staining, a slower migration band was observed for each sample when both the pRNA and the oligo were present (Fig. 2B, lanes 4, 7, and 10). On the autoradiogram, only the slower migration band was observed when both the pRNA and the oligo were present (Fig. 2A, lanes 4, 7, and 10). This band was not observed.
polyacrylamide gel, was altered when the pRNA was mixed with an antisense oligo.

Although all three oligos blocked DNA packaging activity, internal oligo P6 blocked the binding of pRNA to the procapsid while terminal oligos P11 and P15 did not.

The activity of pRNA 7/11 was tested with the highly sensitive system (Lee and Guo, 1994) and shown to be functional in φ29 DNA packaging. A titer of $3.7 \times 10^8$ pfu/ml was obtained when $[^{3}H]$pRNA 7/11 was used. Viral assembly in vitro was completely inhibited by oligo P11, and the inhibitory effect was shown to be at the DNA packaging step (data not shown). Procapsid binding assays were further performed to understand the mecha-
nism of inhibition. In this assay, \(^{3}H\)-labeled pRNA 7/11 was incubated with purified procapsids in the presence or absence of oligos. The mixture was then subjected to sucrose gradient sedimentation (Fig. 4). The \(^{3}H\)-pRNA bound to procapsids and the pRNA/procapsid complexes migrated to fractions 24 to 28 in the gradient, while the \(^{3}H\)-pRNA 7/11 alone remained near the top of the gradient (Fig. 4A). With the addition of oligo P6 to the procapsid/\(^{3}H\)-pRNA mixture, the pRNA/procapsid complexes at fractions 24 to 28 were not detected (Fig. 4B), indicating that P6 blocked the binding of pRNA to the procapsid. Conversely, when oligo P15 (Fig. 4C) or P11 (Fig. 4D) was added to the procapsid/\(^{3}H\)-pRNA mixture, the pRNA/procapsid complexes at fractions 24 to 28 were not affected, suggesting that P11 or P15 did not block the binding of pRNA to the procapsid but inhibited other functions related to the packaging of DNA. These results strongly indicate that the pRNA contained two domains: an internal domain that is involved in procapsid binding and a paired 5'- and 3'-proximal domain that is involved in DNA packaging.

**DISCUSSION**

The mobility of pRNA, on a native polyacrylamide gel, was altered when the pRNA was mixed with an antisense oligo. The binding of an oligo to pRNA resulted in the formation of a pRNA/oligo complex with a slower migration rate in native polyacrylamide gels and is therefore detected by a band shift (Fig. 3 and see above). As mentioned above, oligos P6, P10, P11, and P15 completely inhibited the activity of pRNA 7/10, while oligos P4 and
P13 caused partial inhibition. A pronounced band shift was clearly detected when pRNA 7/10 was mixed with P6, P10, P11, or P15 (Fig. 3, upper panel, lanes 1, 3, 4, and 5). In the absence of an oligo, or in the presence  of P4 and P13, band shifting was detected but was not as significant as that produced by P6, P10, P11, or P15 (Fig. 3, upper panel, lanes 2, 6, and 7). Oligo sequence lengths (17 to 24 bases) were only 14 to 20% of the length of the pRNAs. The substantial change of migration rate due to the addition of a small oligo is speculated to be due to a conformational change of pRNA by oligo binding. In addition, alignment of bands showed that different mobility existed in the same pRNA bound by different oligos. This suggested that antisense oligos targeting different residues of the pRNA may cause different conformational changes and thus differ in migration rate.

Using antisense oligos in conjunction with a pRNA binding assay and a highly sensitive viral assembly assay allowed further characterization of the functional domains of pRNAs. It was shown that while some antisense oligos completely inhibited pRNA activity, other antisense oligos had partial or no inhibitory effect (Table 2). Such a range of inhibitory effects on pRNA activity is speculated to be due to the binding of antisense oligos to proposed essential or nonessential domains of pRNAs. Our results suggest that the pRNA contains at least two domains: an internal procapsid binding domain and a paired 5′- and 3′-proximal domain involved in DNA packaging. It has been shown, by RNA footprinting and mutagenesis studies, that the pRNA contains a procapsid binding region at nucleotides 22–64 (Reid et al., 1994). Our results showed that oligo P6, targeting residues 74 through 91, inhibits prohead binding and therefore inhibits DNA packaging, although oligos P17, P21, and P23, targeting residues 21–37, 38–54, and 65–81, respectively, did not inhibit DNA packaging.

Inhibition of DNA packaging by the various antisense oligos provides valuable information on the structure/function of pRNA. Oligos P9, P17, P19, P21, and P23 did not bind while P13 partially bound to pRNA 7/10. The results of binding were correlated to those of the inhibition assay presented in Table 2. The lack of binding and inhibition suggests the possibility that the pRNA sequences complementary to these oligos are buried internally in the RNA. It further suggests that antisense oligos could be used to probe RNA tertiary structure.

In summary, we have constructed a system that is highly efficient and sensitive for the assay of antisense oligos. This system will be very useful for those who are interested in investigating the effect of the chemical modification of RNA related to antisense RNA stability and binding affinity. For example, chemically modified bases can be incorporated into P6, P11, and P15. Their inhibitory effect could be analyzed by the titration of plaques produced from this in vitro viral assembly system.

ACKNOWLEDGMENTS

We thank Drs. Donald Bergstrom and Jufang Shi for inspiring discussions and Mr. Timothy Tellinghuisen for preparation of tables and figures. This work was supported by NIH Grants GM40480 and GM48159 to P.G.

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