Published in final edited form as:

J Mol Biol. 2014 July 15; 426(14): 2580–2593. doi:10.1016/j.jmb.2014.04.028.

Cystoviral Polymerase Complex Protein P7 Uses its Acidic C-terminal Tail to Regulate the RNA-directed RNA Polymerase P2

Sébastien Alphonse¹, Jamie J. Arnold², Shibani Bhattacharya³, Hsin Wang¹, Brian Kloss³, Craig E. Cameron², and Ranajeet Ghose^{1,4}

¹Department of Chemistry, The City College of New York, 160 Convent Avenue, New York, NY 10031

²Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA 16802

³The New York Structural Biology Center, 89 Convent Avenue, New York, NY 10027

⁴The Graduate Center of the City University of New York, 365 Fifth Avenue, New York, NY 10016

Abstract

In bacteriophages of the cystovirus family, the polymerase complex (PX) encodes a 75-kDa RNA-directed RNA polymerase (P2) that transcribes the double-stranded RNA genome. Also a constituent of the PX is the essential protein P7 that, in addition to accelerating PX assembly and facilitating genome packaging, plays a regulatory role in transcription. Deletion of P7 from the PX leads to aberrant plus-strand synthesis suggesting its influence on the transcriptase activity of P2. Here, using solution NMR techniques and the P2 and P7 proteins from cystovirus ϕ 12, we demonstrate their largely electrostatic interaction *in vitro*. Chemical shift perturbations on P7 in the presence of P2 suggest that this interaction involves the dynamic C-terminal tail of P7, more specifically an acidic cluster therein. Patterns of chemical shift changes induced on P2 by the P7 C-terminus resemble those seen in the presence of single-stranded RNA suggesting similarities in binding. This association between P2 and P7 reduces the affinity of the former towards template RNA and results in its decreased activity both in *de novo* RNA synthesis and in extending a short primer. Given the presence of C-terminal acidic tracts on all cystoviral P7 proteins, the electrostatic nature of the P2/P7 interaction is likely conserved within the family and could constitute a mechanism through which P7 regulates transcription in cystoviruses.

Keywords

NMR; polymerase complex; RNA directed RNA polymerase; cystovirus; transcription.

Address correspondence to: Ranajeet Ghose Department of Chemistry, The City College of New York, 160 Convent Avenue, New York, NY 10031. Phone: (212) 650-6049; Fax: (212) 650-6107; rghose@sci.cuny.cuny.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://>.

Introduction

The *cystoviridae* ($\phi 6$ - $\phi 14$, $\phi 2954$) [1-6] constitute a class of double-stranded RNA (dsRNA) viruses that infect various species of Gram-negative bacteria, most notably the plant pathogen *Pseudomonas syringae*. In spite of some key differences, various members of the cystovirus family share a common overall structure for their respective virions that are characterized by a double protein capsid surrounded by lipid [7-9]. 120-copies of the major capsid protein (P1) arranged as asymmetric dimers on a T=1 lattice forms the structural skeleton of the inner protein capsid known as the polymerase complex (PX) which encloses the three segments (L, large, ~6.4 kb; M, medium, ~4 kb; S, small, ~2.9 kb) of the dsRNA genome [10]. In addition to P1, the other PX proteins include an RNA directed RNA polymerase (RdRp, P2), that carries all its replicase and transcriptase activities [11, 12], a hexameric NTPase of the RecA family (P4) [13] and an essential protein (P7) [14].

After invasion, PX particles carrying the three segments of the dsRNA genome enter the host cell. The PX then uses P2 to perform semi-conservative transcription [12] with the plus-strands being extruded into the host cytoplasm through the central cavity of a P4 hexamer located at the six 5-fold axes [15]. The plus-strands are then utilized by cellular ribosomes and translated into the PX proteins. The P1, P2, P4, P7 proteins, all encoded by the L segment, self-assemble into an empty PX or procapsid (PC). The plus-strands are then packaged into the PCs utilizing the hydrolysis of NTPs by P4. The packaging occurs in a specific order - the S+ segment is packaged first, followed by the M+ segment and finally the L+ segment. The PC undergoes expansion at each stage of packaging [16, 17]. After the L+ segment is fully packaged, the PC is competent to commence replication (minus-strand synthesis using P2) to recapitulate the dsRNA genome. At this stage the daughter PX holding its entire dsRNA genome is fully expanded and capable of efficient transcription [18]. This new PX can either perform additional rounds of plus-strand synthesis ultimately leading to the generation of new PC particles or acquire other proteins (encoded by the genomic M and S segments) that make up the intact virus and finally the lipid coat from the host cell membrane resulting in a daughter virion that is shed after cell lysis [12].

The structures of virions from the $\phi 6$ [7], $\phi 8$ [8] and $\phi 12$ [9] cystoviruses have been determined by cryo-electron microscopy (cryo-EM). The atomic structures of the individual PX proteins are known, P2 from $\phi 6$ [19] and $\phi 12$ [20]; P4 from $\phi 6$, $\phi 8$, $\phi 12$, $\phi 13$ [21, 22], P7 from $\phi 12$ [23] and recently, P1 from $\phi 6$ [24] and $\phi 8$ [25]. In spite of this extensive structural data on the constituent proteins of the PX backed by biochemical and functional studies (largely on $\phi 6$), the precise roles of the individual PX proteins at various stages of the cystoviral life cycle and the underlying molecular mechanisms that regulate their functions are only beginning to be fully understood. Of all the PX proteins, P7 that is essential for virus viability [14, 26] is the most intriguing. P7 contains a C-terminal tail that has been shown by solution NMR studies to be highly dynamic [23]. This tail is indispensable for the formation of infectious particles and $\phi 6$ nucleocapsids (NC) assembled using a P7 construct missing the C-terminal tail, are unable to infect their hosts [26]. P7 plays multiple roles in the context of the PX at various stages of the cystovirus life cycle with the C-terminal tail appearing to hold pole position in each of these events. P7 has been shown to accelerate the *in vitro* assembly of PCs from recombinant PX proteins [26, 27].

The P7 C-terminal tail, that stabilizes a nucleation intermediate in the PC assembly pathway, also enables the efficient packaging of the viral genomic segments into the empty PC [26]. In addition to its role in PC assembly and genome packaging, earlier studies have indicated that P7 plays a regulatory role in transcription [14]. It was shown that PX particles that lacked P7 (P124) not only appeared to have an enhanced transcriptase activity i.e. synthesis of plus-strands in the presence of Mn^{2+} , in contrast to intact PX particles, but produced transcripts of the incorrect size [14]. This suggests that P7 either directly or indirectly influences the enzymatic activity of P2 in the fully assembled, dsRNA containing, transcriptionally competent PX.

CryoEM studies on the ϕ 12 virion suggest that P2 and P7 are located in spatial proximity to one other in the intact core particle at the 5-fold axis [9]. Two separate electron microscopy based single particle reconstructions of the ϕ 6 empty pre-expanded PCs suggest that P2 and P7 occupy spatially proximate positions at the 3-fold axes [28, 29]; one of these studies suggests a close association between P2 and P7 [28]. Given this body of evidence that hints at the strong interdependency between P2 and P7, we explored the possibility that P7 could directly interact with P2 *in trans*. The availability of complete resonance assignments of full-length ϕ 12 P7 [23] and a significant number backbone amide ^{15}N , 1H and methyl ^{13}C , 1H resonance assignments for ϕ 12 P2, obtained in the course of this study, enabled us to test the nature of interactions between the P2 and P7 using solution NMR methodology. Our results show that P7 utilizes its C-terminal tail to bind P2 and reduces the affinity of the latter towards ssRNA. This interaction also leads to a modest decrease in the ability of P2 to initiate RNA synthesis *de novo* and to extend short ssRNA primers.

Results

P2/P7 interactions observed from the P7 standpoint

Complete ^{15}N , 1H resonance assignments are available for full-length ϕ 12 P7 (169 residues) which forms an elongated dimer in solution (~37 kDa) with an effective correlation time of ~20 ns. The C-terminal tail of full-length P7 (129-169) is highly mobile on the fast, ps-ns, timescale while the N-terminal core of this dimeric protein is quite rigid [23]. Thus, in fully-protonated, ^{15}N -labeled full-length P7, an appropriate contouring choice in a ^{15}N , 1H HSQC spectrum allows the sharp resonances corresponding to this C-terminus to be visualized independent of the broad resonances of the rigid core (e.g. Fig. S1) and protein-protein interactions involving the C-terminal tail, that has been deemed essential for virus viability as mentioned previously [26], may be analyzed independent of the N-terminal core in the context of full-length protein. Using this approach and a series of ^{15}N , 1H HSQC spectra, we found that under low salt conditions (0 mM NaCl), resonances corresponding to the entire C-terminal tail displayed large spectral perturbations (Fig. S1A) in the presence of an excess of P2. The largest changes were seen for the segment between L156 and E169, with all corresponding resonances, with the exception of L159 and D160, being broadened to below the threshold of detection (Fig. 1A). Resonances corresponding to L159 and D160, though substantially broadened, could still be detected, and displayed the largest chemical shift perturbations with δ_{amide} values (calculated using Eq. (3A)) of 0.039 and 0.027 ppm, respectively. More modest changes were seen in 50 mM NaCl (Fig. S1B), with resonances

corresponding to H161 (0.028 ppm), D165 (0.013 ppm), D166 (0.021 ppm), A167 (0.021 ppm), D168 (0.021 ppm) and E169 (0.018 ppm) reappearing and now showing significant δ_{amide} values (> 0.013 ppm; Fig. 1A). No significant perturbations were seen for any of resonances at higher ionic strength (150 mM NaCl, Fig. S1C) suggesting a lack of interaction at high salt (Fig. 1A). Based on these results, it is apparent that the P2/P7 interactions are at least partly mediated by the P7 C-terminal tail (encompassed by residues L156 and E169) and this interaction is electrostatic in nature. However, the overall pattern of spectral perturbations indicates that the extreme C-terminus of P7 bearing the acidic cluster that includes residues D165, D166, D168 and E169 plays a more dominant role in defining these interactions. In order to further test the role of this segment, the ability of two point mutants P7^{D160N} and P7^{D168N} to interact with P2 was tested in 50 mM NaCl (all subsequent experiments were performed in 50 mM NaCl, which provides the best compromise between detectable perturbations and long-term sample stability). These mutants were chosen based on the fact that in 50 mM NaCl, D160 did not display any significant perturbations in the presence of P2 while the perturbations seen for D168, that lies in the acidic cluster at the extreme C-terminus, were among the largest (Fig. 1A). The perturbations seen for the P7^{D160N} mutant in the presence of P2 were largely comparable to those seen for wild-type P7, while they were significantly reduced in P7^{D168N} (Fig. 1B; Fig. S2) suggesting a more substantial role for the extreme C-terminus of P7 in its interactions with P2.

We then constructed two sets of P7 mutants - in the first, termed P7⁰, all the acidic residues in the L156-E169 segment were replaced by corresponding neutral residues (D158N/D160N/D165N/D166N/D168N/E169Q); in the second, termed P7⁺, the C-terminal acidic residues were replaced by corresponding basic residues (D158K/D160K/D165K/D166K/D168K/E169K). No significant spectral perturbations were seen for either of these mutants in the presence of P2 (Fig. S3), confirming the role of the acidic cluster on the C-terminal end of P7 in facilitating its interactions with P2. Resonances corresponding to residues of the N-terminal core (1-129) did not display any significant perturbations in ¹⁵N, ¹H TROSY spectra of full-length, uniformly ¹⁵N, ²H-labeled P7 (and in ¹⁵N, ²H-labeled P7 C that is missing 41 C-terminal residues) recorded in the presence of P2 (data not shown). These assays confirmed that the C-terminal tail of P7 is sufficient in mediating its interactions with P2. Additionally, the behavior of the C-terminal tail residues was similar in ¹⁵N, ²H-labeled P7 and ¹⁵N-labeled P7 suggesting that the broadening seen (under low salt conditions) is due to intermediate exchange rather than due to the highly efficient relaxation expected for a rigid 112 KDa species tumbling as a single unit. Given the fact that the C-termini of all cystoviral P7 proteins with known sequences ($\phi 6$, $\phi 8$, $\phi 12$, $\phi 13$ and $\phi 2954$, see Fig. 1C) contain clusters of acidic residues, this electrostatic interaction between P2 and P7 could represent a common mode of interaction in cystoviruses. Additionally, given the low sequence conservation in this region, the precise amino-acid sequence of the acidic cluster is likely not important (see Fig. 1C), the acidic nature is all that is necessary to mediate the interaction. This hints towards a role for the C-terminal acidic patch in cystoviral P7 proteins as a non-sequence-specific electrostatic modulator that could potentially interact with the positively charged template entry portal of P2 thus regulating template binding and therefore RNA synthesis. This possibility was further investigated (see below) especially since such

mimicry would not be unprecedented in bacteriophages. For example, in T7, the single-stranded DNA (ssDNA) binding protein gp2.5 contains an acidic C-terminal tail that associates with the same positively charged surface that binds ssDNA [30]. This C-terminal tail, that is critical for proper spatiotemporal co-ordination of the four-protein complex at the replication fork during viral DNA synthesis, also interacts with the positively charged thumb domain of T7 DNA polymerase [31].

Mapping the P7 interaction site on P2

Next we proceeded to determine the site/s utilized by P2 in the electrostatic interaction determined above. For this, substantial resonance assignments with sufficient and uniform coverage of the P2 surface were required. However, for a protein the size of $\phi 12$ P2 (~75 kDa, 659 residues) with an effective correlation time ~41 ns, backbone directed resonance assignment strategies become especially inefficient. This problem is exacerbated by the fact the P2 is not stable in solution beyond concentrations of 200-250 μ M and at temperatures higher than 25° C for the extended periods required for the NMR experiments. In spite of these limitations, we used a variety of approaches (see Supplementary Data) to obtain unambiguous ^{15}N , ^1H assignments (Fig. S4B) for 245 of 619 (40%) non-Pro ^{15}N , ^1H resonances of P2. Additionally, we were able to obtain ^{13}C , ^1H resonance assignments for 26 of 32 Ile $\delta 1$ positions (81%), 17 of 47 Leu $\delta 1/2$ positions (36%), 26 of 44 Val $\gamma 1/2$ (59%) and 17 of 18 Met (excluding M1) ϵ positions (94%) (Fig. S4C). While the resonance assignment procedure is an ongoing process, the currently available amide ^{15}N , ^1H and methyl ^{13}C , ^1H assignments that are distributed uniformly over the structural domains of P2 (see Fig. S4) and all conserved sequence motifs (A-F) [32] provide ~400 probes of P2/P7 interactions. Most of these resonances display well-resolved chemical shifts (i.e. not random coil values) suggesting that there is no inherent bias towards more disordered parts of the protein.

As demonstrated above, the C-terminal tail of P7 is sufficient for interaction with P2, and the core domain of P7 plays no role in P2/P7 interactions. Thus, to ensure that saturation conditions could be achieved while limiting the total mass of protein in solution and to improve the long-term sample stability for the NMR experiments, a 15-residue peptide (Fig. 1C) corresponding to the C-terminal tail of P7 (155-169, P7pep) was synthesized and used for all NMR titrations with labeled P2. In a series of ^{15}N , ^1H TROSY spectra of U- ^{15}N , ^2H] (or ILVM, U- ^{15}N , ^2H) P2 acquired in the presence of various concentrations of P7pep, large chemical shift perturbations (Fig. S5A) were seen at the top of the thumb and fingers domain surrounding the template entry portal including some residues that line the template tunnel (Fig. 2A). Significant perturbations (δ_{amide} values for a P2:P7pep molar ratio of 1:10) were seen for R248 (0.1 ppm) and W249 (0.1 ppm; W249 ϵ – 0.16 ppm) that lie inside the template tunnel. Large perturbations were also seen for V26 (0.13 ppm) and D28 (0.15 ppm) that lie at the N-terminus. Fitting the δ_{amide} values with increasing P7pep concentration to Eq. (4) for selected well-resolved resonances yielded K_d values $74 \pm 14 \mu\text{M}$ (the standard deviation is over the values for fitted resonances not errors for individual fits; representative fits are shown in Fig. S5B). ^{13}C , ^1H HMQC spectra of ILVM, U- ^{15}N , ^2H] P2 displayed a similar distribution of chemical shift changes in the presence of P7pep, with the largest perturbations (δ_{met}) seen at the top of the fingers and thumb domains and in the

template tunnel (Fig. 2B). Not surprisingly, the magnitudes of perturbations seen for the methyl resonances were smaller than those for the backbone (Fig. S5C). Nevertheless, these perturbations provide outstanding probes of conformational or environmental changes within the hydrophobic core. The largest of these chemical shift changes were seen for V26 ($\delta_{\text{met}} = 0.02$ ppm) and L30 (0.026 ppm) at the N-terminus; M48 (0.036 ppm), M190 (0.025 ppm), V199 (0.022 ppm), V247 (0.023 ppm) and I266 (0.022 ppm) on the fingers domain; and I591 (0.030 ppm) on the thumb domain. Additionally, several significant perturbations were seen near the catalytic site of P2 in the presence of P7pep, the largest being displayed V350 (0.037 ppm) located in motif A and V299 (0.023 ppm) located on motif F. Significant perturbations were also seen in ^{15}N , ^1H TROSY spectra for the resonances of the motif B residues R419 (0.035 ppm), S420 (0.028 ppm) and in the motif C residue G468 (0.026 ppm). The last of these residues belongs to the so-called catalytic triad ($^{468}\text{GDD}^{470}$) conserved in DNA and RNA polymerases. Given the pattern of chemical shifts including the fact that several residues that showed the largest perturbations lie within the template tunnel (e.g. V247, R248, W249) together with some significant perturbations seen at the active site (e.g. V350 and G468) seems to suggest that extreme C-terminus of P7pep enters the template tunnel. The perturbations seen in the thumb and fingers domain could indicate limited conformational changes of these domains to alter the geometry of the template tunnel to accommodate the C-terminal end of P7pep. Alternatively, they could also indicate local contacts between the remainder of the peptide at the top of the fingers and thumb domains. A potential local contact could involve the sidechains of H161 on P7pep (a significant $\delta_{\text{amide}} = 0.028$ ppm was seen for this residue of full-length P7 in the presence of P2 while the δ_{amide} values for the flanking D160 and G162 were much smaller; see above) and D28 ($\delta_{\text{amide}} = 0.15$ ppm; see above) that lies close to the template tunnel of P2 (see Fig. 2A).

Influence of P7 on the P2/RNA interactions

P2 binds ssRNA with micromolar affinity (see Table S3, Fig. S6A) in 50 mM NaCl that appears to be marginally weaker than that seen for $\phi 6$ P2 (extrapolating to high salt; the measurements for $\phi 6$ P2 were made in a buffer containing 400 mM NaCl [33]). We acquired ^{15}N , ^1H TROSY and ^{13}C , ^1H HMQC spectra of ILVM, U- ^{15}N , ^2H P2 in the presence of increasing amounts of the 5'FL-UUUUC-3', the tightest binding 5-nt ssRNA construct tested ($K_d = 0.2 \pm 0.1$ μM ; see Table S3). The ^{15}N , ^1H TROSY spectra revealed substantial perturbations in the presence of ssRNA (data not shown), far larger than the corresponding perturbations seen in the presence of P7pep discussed in the previous section. The magnitudes of these chemical shift changes were significant enough to prevent a detailed analysis without large-scale reassignment of the resonances of RNA-bound P2. The chemical shift perturbations seen in the ^{13}C , ^1H HMQC spectra were smaller though slightly larger in magnitude than those seen in the presence of P7pep (Fig. S6B). These perturbations could nevertheless be analyzed in a reliable manner without the need for reassignment. The relatively modest perturbations seen for the P2 methyl resonances in the presence of RNA indicate the lack of significant rearrangement of the hydrophobic core usually induced by large conformational changes e.g. large-scale domain reorientation, upon RNA binding. This is in line with that expected upon comparing the crystal structures of $\phi 6$ P2 in the absence or the presence of nucleic acids [19, 34], though a high-resolution structure of $\phi 12$ P2 bound to

RNA is not yet available. As in the case of P7pep, the largest perturbations (P2:ssRNA ratio of 1:4) were seen at the top of the fingers and thumb domains, inside the template tunnel and near the catalytic site (Fig. S6C). Very significant changes (δ_{met} values > 0.03) were seen for I83 (thumb, 0.031 ppm), V199 (fingers, 0.042 ppm), V299 (fingers, motif F within the template tunnel, 0.031 ppm), M318 (palm, 0.031 ppm), V438 (palm, near motif B, 0.064 ppm) and I591 (thumb, 0.049 ppm).

A comparison between Figs. 2B and S6C reveals that the overall distribution of spectral perturbations are quite similar for P7pep and the ssRNA oligo. This is especially apparent within the template tunnel (Fig. 2C) with L74 (P7pep – 0.021 ppm, RNA – 0.024 ppm; recall that the P2:P7pep and P2:ssRNA ratios were 1:10 and 1:4, respectively), V247 (P7pep – 0.023 ppm, RNA – 0.022 ppm) and V299 (P7pep – 0.023 ppm, RNA – 0.031 ppm) being significantly perturbed in both cases. Based on these results, it appears that a possible mode of interaction could involve the acidic cluster of the C-terminal tail of P7 occupying the positively charged template tunnel of P2 (Fig. 2D) in a manner reminiscent of the 3'-end of the ssRNA template.

Next we tested the ability of P7pep to interfere with the ability of P2 to bind ssRNA templates. The affinity of 5'-FL-UUUUC-3', the tightest binding 5-nt ssRNA construct tested, was reduced 6-fold from a K_d of $0.2 \pm 0.1 \mu\text{M}$ in the absence of P7pep to a K_d of $1.3 \pm 0.1 \mu\text{M}$ in the presence of $600 \mu\text{M}$ P7pep (see Fig. 3A). In case of the weakest binding construct tested, 5'-AAGCC-3' (corresponding to the 3'-end of the template used in the polymerase assays described below), the $K_d = 5.6 \pm 0.1 \mu\text{M}$ in the absence of P7pep was reduced almost 8-fold ($K_d = 44.1 \pm 4.1 \mu\text{M}$) in the presence of $700 \mu\text{M}$ P7pep. It has been noted that the C-terminus of P7 in the context of full-length P7 has a weak affinity ($\sim\text{mM}$) for ssRNA in solution [23] and this has been suggested to play a role in facilitating genome packaging into the PC [29]. However our fluorescence anisotropy titration data indicates that the C-terminal tail of P7 in isolation i.e. P7pep has no ability to bind ssRNA in the absence of the N-terminal core (Fig. 3A).

In order to further test the ability of P7pep to inhibit RNA binding by P2, we collected ^{13}C , ^1H HMQC spectra using ILV, U- ^{15}N , ^2H] P2 containing saturating amounts of 5'-UUUUC-3' (P2:ssRNA molar ratio of 1:2) in the absence or the presence of a large excess of P7pep (P2:ssRNA:P7pep molar ratio 1:2:12). Addition of an excess of P7pep to the P2:ssRNA complex shifted resonances to positions that were closer to those seen for P2+P7pep alone (Fig. 3B) confirming that P7pep indeed interferes with the ability of P2 to bind ssRNA.

Influence of P7 on RNA synthesis by P2

Based on the NMR results above, we surmised that the altered ability of P2 to interact with ssRNA in the presence of P7pep should also affect its ability to form a stable initiation complex and the *de novo* synthesis of a daughter chain. We first tested the ability of P2 to synthesize the dinucleotide pppGpGp on the template 5'-AAACUGAGAAGGAGAAAGCC-3' (U-template) using only GTP ($[\gamma\text{-}^{32}\text{P}]\text{-GTP}$) (Fig. S7A). The choice was based on the fact that $\phi 12$ P2 [35], like the RdRps of $\phi 6$, $\phi 8$ and $\phi 13$, prefers pyrimidine bases, especially cytosines, at the template 3'-termini [36]. Optimal *de*

de novo synthesis of pppGpG was seen at 30° C, using 2 mM Mn²⁺. These conditions are similar to those noted previously [35]. Using 5 μM U-template (100 μM GTP) we found that the pppGpG synthesis was linear throughout the 0-60 minute range probed with a slope of 0.67 ± 0.13 μM/minute (not shown). We then repeated the experiment in the presence of varying concentrations (0-1000 μM) of P7pep (or P7pep⁰, a peptide corresponding to residues 155-169 of the P7⁰ mutant described above, as control) using an incubation time of 30 minutes. The quantity of pppGpG product produced in the presence of increasing concentrations of P7pep (or P7pep⁰) was quantified and fitted to Eq. (5) to obtain IC₅₀ values. P7pep inhibited pppGpG synthesis about 2.6-fold more efficiently (Fig. 4A, IC₅₀ = 181 ± 41 μM) than P7pep⁰ (IC₅₀ = 468 ± 60 μM).

Additionally, we tested the ability of P7pep (and P7pep⁰) to inhibit the extension of a short pGpG primer (P2 utilizes pppGpG and pGpG with equal efficiency; data not shown), in the presence of CTP on the U-template (5 μM) (Fig. S7B). Extension of this short dinucleotide primer to generate pGpGpC, using 10 μM end-labeled pGpG and 100 μM CTP, was linear during the entire range of incubation times tested (up to 120 minutes) with a slope of 3.3 ± 0.2 nM/minute (data not shown). As in the case of *de novo* synthesis, P7pep inhibited extension of the pGpG primer with about 2.5-times higher efficiency than P7pep⁰ (IC₅₀ = 287 ± 28 μM compared with IC₅₀ = 729 ± 134 μM; Fig. 4B).

Discussion

Using solution NMR spectroscopy as the primary tool to probe structural changes, we have demonstrated that the P2 and P7 proteins of cystovirus φ12 are capable of a physical interaction *in vitro*. The P2/P7 association relies purely on the dynamic C-terminal tail of P7 and more specifically the acidic cluster on its extreme C-terminus. P7-induced chemical shift perturbations in the NMR spectra of P2 are distributed on the top of the thumb and fingers domains near the template tunnel, within the template tunnel and in specific catalytic motifs that are conserved in viral RdRps. Given that the structural distribution of these perturbations is similar to those induced on P2 upon binding ssRNA, together with the fact that the presence of P7 reduces the ability of P2 to bind RNA, is consistent with a scenario in which the acidic cluster on the C-terminal tail of P7 enters the P2 template tunnel and is stabilized by the largely positively charged environment provided by it. If this is indeed true then in a sense the C-terminal acidic cluster on P7 mimics 3'-end of the template ssRNA. This behavior is somewhat reminiscent of that seen in the structure of the foot-and-mouth disease virus (FMDV) RdRp (3Dpol) in complex with the primer protein VPg, the uridylation of the latter initiates replication [37, 38]. VPg binds 3Dpol in a fashion that is highly analogous to that seen for the RNA primer and the duplex RNA product of replication [39], being stabilized by several basic residues in the nucleic acid binding cleft. While the interaction between P2 and P7 appears to be weak and the inhibitory effect on RNA synthesis is modest in solution, it should be remembered that both the interaction strength and consequently the inhibitory effect are likely enhanced several-fold if P2 and P7 are assembled onto the PX in the fully expanded dsRNA containing PC. Under such circumstances the P2/P7 interaction would no longer be diffusion-controlled as in solution.

As mentioned previously, the structures of the $\phi 6$ [7], $\phi 8$ [8] and $\phi 12$ [9] virions have been solved using cryo-EM. While the positions of the P2 and P7 components could not be assigned in the $\phi 6$ and $\phi 8$ virions, in the structure of the $\phi 12$ virion, density at the 5-fold axis that intercalates between the gaps in the incomplete T=13 shell formed by the outer capsid protein P8, was attributed to P7 dimers [9]. In this same structure, P2 was assigned to positions below the 5-fold axes. It is to be noted that the $\lambda 3$ or VP1 polymerases in the homologous reovirus [40] or rotavirus [41], respectively, are also situated near the 5-fold axes of the core particles. Two independent sets of cryo-EM reconstructions of the structures of empty $\phi 6$ PCs suggest that P2 is located at the 3-fold axis as is P7 [28, 29]. Both these studies suggest that in the empty PC, P7 occurs as an association of three monomers or a trimer in contrast to the dimeric state assigned to P7 in the mature $\phi 12$ virion. In the study by Nemecek *et al.*, P2 and P7 are suggested to occupy overlapping positions but the same particles in their preparations do not appear to contain P2 and P7 simultaneously [29]. In contrast, in the study by Katz *et al.* several lines of evidence are cited to suggest that P2 and P7 do exist on the same particle with P7 trimers surrounding each P2 at the 3-fold axes [28]. The authors of this study suggest a role of P7 in “holding” P2 to the P1 shell in an empty PC. This ability of P2 and P7 to co-exist on a single empty PC is also borne out by biochemical studies that indicate that at very high-concentrations of P7, the *in vitro* assembled PCs contained around 60 copies of P7 (around 40 in an intact $\phi 6$ virion), and the P2 content did not decrease to 0 but remained around 8 (an intact $\phi 6$ virion contains around 10 copies of P2) [27]. All of these studies indicate a similar spatial localization of the P2 and P7 proteins and thus a physical interaction between them would not be out of the ordinary.

As has been stated earlier, RNA synthesis by replication-competent (containing just the positive strands of the dsRNA genome) and transcription-competent (containing dsRNA) for complete $\phi 6$ core particles containing P1, P2, P4 and P7 has been compared to corresponding PX particles that lack P7 (P124 particles) [14]. While P124 particles replicated normally producing minus-strands of the correct size, plus-strand synthesis was aberrant and transcripts of the incorrect size were produced. Notably, while replacement of catalytic Mg^{2+} with Mn^{2+} lead to a decrease in transcriptase activity by normal core particles, an increase in activity was seen in the P124 particles suggesting P7 induced inhibition of P2. The exact origins of this aberrant behavior or of the differential effects [14] during plus-strand synthesis (transcription) and minus-strand synthesis (replication) are not immediately clear. However, the latter may simply be due to the fact that transcription occurs efficiently only after replication is complete and the fully expanded particles containing dsRNA is conformationally distinct from those that contain only the genomic plus strands at the start of replication. It has been noted that $\phi 6$ core particles that are generated using P7 that lacks the C-terminal tail do not form infectious centers [26]. This was attributed in part due to a reduction in genome packaging and instabilities in the polymerase complex, however the role of aberrant transcription and the exact effects of the P7 C-terminal tail (or lack thereof), if any, in this context needs to be ascertained.

Finally, most of the understanding of the behavior of the assembly processes of cystoviral PCs from constituent proteins, packaging of genomic segments, replication and transcription are based on the results obtained for $\phi 6$. Similar protocols need to be developed and studies

performed in $\phi 12$, not only to allow direct comparison with our present results since the behavior of the isolated RdRps may bear differences with those in the context of an intact viral capsid [42], but also to ascertain species-specific variations. The latter is also important given that while cystoviruses share gross similarities, differences have been noted amongst their host specificities and mechanisms of infection [3], the structures of their virions [8] and the structures and behavior of individual PX proteins [22]

Materials and Methods

Protein expression and purification

P7—Full-length (P7, 1-169) and truncated (P7 C, 1-129) constructs of $\phi 12$ P7 were cloned into a pET28b vector containing an N-terminal His₆Smt3-tag (a kind gift from the Shuman laboratory, Sloan-Kettering Institute). The pET28b-SUMO-P7 vector was used as DNA template for all the *p7* mutant alleles. Specific *p7* mutants were generated using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent) as described in detail in the Supplementary Data. Mutations were confirmed by sequencing (Genewiz). The expression and purification protocol for uniformly ¹⁵N-labeled P7, ¹⁵N,²H-labeled P7 or P7 C is detailed in the Supplementary Data. The purified P7 proteins exchanged into a buffer containing 20 mM Bis-Tris at pH 6.5, 7.5 mM MgCl₂ and the NaCl composition was modified as needed (0 mM, 50 mM or 150 mM NaCl). This buffer referred to as the P7 buffer from hereon.

Peptides (see Fig. 1C) derived from the C-terminal tail of wild-type P7 (P7_{pep}, ¹⁵⁵PLVDFDHGAIDDADE¹⁶⁹ and the P7⁰ mutant (P7_{pep}⁰, ¹⁵⁵PLVNFNHGAINNANQ¹⁶⁹) were custom synthesized by Peptide 2.0 and obtained HPLC-purified.

P2—The plasmid pPG24 [20, 35] (a kind gift from the Gottlieb laboratory, CCNY) containing the gene coding for $\phi 12$ *p2* was transformed into *E. coli* BL21-CodonPlus(DE3)-RIL cells (Agilent). Mutations used for the Met assignments were performed using plasmid pPG24 as template and a pair of specific primers carrying the desired mutation. Mutations were confirmed by DNA sequencing (Beckman Coulter Genomics). Several $\phi 12$ P2 samples with various patterns of labeling with NMR-active stable isotopes were required to complete different aspects of the work presented here. The nomenclature used for these various samples are as follows:

1. P2: Unlabeled P2.
2. U-[¹⁵N,²H] P2: Uniformly ¹⁵N, ²H-labeled P2.
3. U-[¹⁵N,¹³C,²H] P2: Uniformly ¹⁵N, ¹³C, ²H-labeled P2.
4. ¹³C'-F, U-[¹⁵N,²H] or ¹³C'-Y, U-[¹⁵N,²H] or ¹³C'-M, U-[¹⁵N,²H] P2: P2 that was ¹³C-labeled at the carbonyl carbon (C') for each of Phe, Tyr or Met residues, in addition to being uniformly ¹⁵N, ²H-labeled.

5. ILV, U- $[^{15}\text{N}, ^2\text{H}]$ P2: P2 that was labeled in the following pattern – for Ile $\delta 1$ positions ($^{13}\text{CH}_3$), for Leu δ positions ($^{13}\text{CH}_3, ^{12}\text{CD}_3$) and for Val γ positions ($^{13}\text{CH}_3, ^{12}\text{CD}_3$). All nitrogens were ^{15}N ; all non-exchangeable protons were ^2H except those specifically mentioned.
6. ILVM, U- $[^{15}\text{N}, ^2\text{H}]$ P2: Met residues were protonated and the ϵ position was ^{13}C -labeled in addition to the pattern described for ILV, U- $[^{15}\text{N}, ^2\text{H}]$ P2.
7. ILV, U- $[^{15}\text{N}, ^{13}\text{C}, ^2\text{H}]$ P2: All carbons were ^{13}C in addition to the patterns described for ILV, U- $[^{15}\text{N}, ^2\text{H}]$ P2.
8. M-labeled P2: The ϵ position of Met residues was ^{13}C -labeled.

The various P2 samples described above were expressed and purified using protocols that are detailed in the Supplementary Data. Purified P2 proteins were exchanged into a buffer that contained 20 mM Bis-Tris at pH 6.5, 7.5 mM MgCl_2 (P7 buffer as mentioned before) or 20 mM Tris at pH 8, 1 mM DTT (referred to as the P2 buffer from hereon). The salt concentration in the buffer was adjusted as required (0 mM NaCl, 50 mM NaCl or 150 mM NaCl).

Fluorescence anisotropy measurements

Fluorescence anisotropy measurements were performed using 6 different 5-nucleotide (5-nt) ssRNA oligos (see Table S3) that were tagged at their 5'-ends using 6-carboxyfluorescein (5'-FL). RNA constructs were purchased HPLC-purified from Dharmacon Research. All anisotropy measurements were performed at 25° C using a constant concentration of RNA ($C_{\text{RNA}} = 5 \text{ nM}$) in the presence of increasing amounts of P2 in P2 buffer containing 50 mM or 150 mM NaCl supplemented with 5 mM MgCl_2 and 1 unit/ μL of RNase Inhibitor (New England Biolabs). Additional fluorescence anisotropy measurements were carried out for the 5'-UUUUC-3' and 5'-AAGGC-3' oligos in the presence of 600 μM and 700 μM of P7pep respectively. All experiments were performed on a Panvera Beacon 2000 using an excitation filter centered at 490 nm, a transmission filter centered at 520 nm and using an integration time of 10 seconds [33, 43]. Fluorescence anisotropy values were calculated from the intensities with parallel (I_{VV}) and perpendicular (I_{VH}) settings of the polarizers using the following equation:

$$A = \frac{I_{\text{VV}} - I_{\text{VH}}}{I_{\text{VV}} + 2I_{\text{VH}}} \quad (1)$$

The apparent dissociation constant (K_d) was obtained by measuring the anisotropy values (A_C) in the presence of varying concentrations of P2 (C_{P2}) using the following equation:

$$A_C - A_0 = (A_\infty - A_0) \frac{(K_d + C_{\text{P2}} + C_{\text{RNA}}) - \sqrt{(K_d + C_{\text{P2}} + C_{\text{RNA}})^2 - 4C_{\text{RNA}}C_{\text{P2}}}}{2C_{\text{RNA}}} \quad (2)$$

where A_0 and A_∞ are the values of the anisotropy in the absence of P2 and in the steady-state, respectively. Experimental data were fit to theoretical curves described by Eq. (2) using in-house software that utilized the ODRPACK subroutines [44].

NMR spectroscopy

NMR experiments were recorded at 25° C using either Bruker Avance spectrometers operating at ^1H frequencies of 700, 800 or 900 MHz or a Varian Inova spectrometer operating at a ^1H frequency of 600 MHz. All spectrometers were equipped with cryogenic probes capable of applying pulsed field-gradients along the z-axis. NMR data were processed using the NMRPipe/NMRDraw package [45] and analyzed using the NMRViewJ software suite [46].

Resonance assignment

All experiments for P2 assignments were carried out in P2 buffer containing 150 mM NaCl. Amide (^{15}N , ^1H) resonances were assigned using a standard suite of TROSY-based triple-resonance experiments [47] complemented by the analysis of the amide-amide cross-peaks in a ^{15}N , ^1H NOESY-TROSY experiment [48]. Additionally, two-dimensional HNC0 experiments separately utilizing $^{13}\text{C}'\text{-F}$, U- ^{15}N , ^2H], $^{13}\text{C}'\text{-Y}$, U- ^{15}N , ^2H] and $^{13}\text{C}'\text{-M}$, U- ^{15}N , ^2H] samples were used to confirm assignments.

For assignment of methyl resonances of Ile, Leu and Val residues, cross-peak patterns in a 3-dimensional ^1H , ^{13}C , ^1H NOESY-HMQC [49] and a three-dimensional ^1H , ^{13}C , ^{13}C HMQC-NOESY-HMQC [50] were analyzed in the context of the high-resolution structure of P2 [20]. Both these experiments were modified to selectively edit for the methyl resonances. Leu and Val-edited 2-dimensional ^{13}C , ^1H HSQC experiments [51] were analyzed together with a 3-dimensional CT-HmCmC experiment for unambiguous side-chain correlations. Additional assignments were obtained using a 3-dimensional HMCM(CG)CBCA together with a HMCM(CG)CBCA)CO (separately optimized for Val and Leu residues) [52]. These experiments provided correlations between the ^1H , ^{13}C chemical shifts of the methyl groups with ^{13}C chemical shifts for the C α /C β (and C γ for Leu and Ile residues) or CO resonances, connecting these data with those from HNCACB/ HN(CO)CACB and HNC0 experiments used for backbone resonance assignment. The ϵ -positions of Met residues were assigned by Met-to-Leu mutations (except for M48I). Experimental parameters and samples used for obtaining resonance assignments are summarized in Table S2.

Interactions between P7 and P2

^{15}N , ^1H HSQC spectra for 50 μM samples of U- ^{15}N] P7 (or mutants) in P7 buffer (see above) were acquired in the presence of P2 under the following conditions:

1. P7 against P2 at three different ionic strengths (0 mM, 50 mM and 150 mM NaCl) using P7:P2 ratios of 1:0 and 1:2. For 50 mM NaCl, an additional point with a P7:P2 ratio of 1:1 was also acquired.
2. Each of P7 $^{\text{D160N}}$, P7 $^{\text{D168N}}$, P7 0 and P7 $^+$ in the presence of 50 mM NaCl using P7:P2 ratios of 1:0 and 1:2.

All spectra were acquired at 25° C at 600 MHz using 1024 and 512 points in the direct and indirect dimension respectively, with spectral windows of 13 ppm (^1H) and 36 ppm (^{15}N).

Interactions between P2 and P7pep

A series of ^{15}N , ^1H TROSY spectra were acquired using samples containing 100 μM of U- ^{15}N , ^2H] P2 and the following concentrations of P7pep: 0, 16.7, 25, 50, 100, 200, 400 and 1000 μM , corresponding to the following P2:P7pep ratios of 1:0, 1:0.17, 1:0.25, 1:0.5, 1:1, 1:2, 1:4, 1:10. Experiments were performed in P7 buffer containing 50 mM NaCl. All spectra were acquired at 25° C at 600 MHz using spectral windows of 15 ppm (1024 complex points) and 37 ppm (128 complex points) for the ^1H and ^{15}N dimensions, respectively. In addition, ^{15}N , ^1H TROSY spectra were acquired in P2 buffer containing 50 mM NaCl at P2:P7pep ratios of 1:0 and 1:10 (700 MHz) using sweep-widths of 15 ppm (1024 complex points) and 37 ppm (128 complex points) in the ^1H and ^{15}N dimensions respectively. The residues that displayed significant perturbations were identical to those in P7 buffer (described above) though the overall magnitude of the perturbations was slightly different for a few residues.

^{13}C , ^1H HMQC spectra of ILVM, U- ^{15}N , ^2H] P2 (100 μM ; in P2 buffer containing 50 mM NaCl) were acquired in absence or presence of P7pep using P2:P7pep ratios of 1:0.5, 1:1, 1:2, 1:4 and 1:8. Spectra were acquired at 600 MHz using sweep-widths of 12 ppm and 20 ppm in the direct ^1H (512 points) and the indirect ^{13}C (128 points) dimensions, respectively.

Interactions between P2 and 5'-UUUUC-3'

To analyze the interaction between P2 and the high-affinity ssRNA oligo (5'-UUUUC-3', see Table S3), ^{13}C , ^1H HMQC spectra (700 MHz) of ILVM, U- ^{15}N , ^2H] P2 (100 μM ; in P2 buffer with 50 mM NaCl) containing 0.8 unit/ μL of RNase inhibitor (New England Biolabs) were acquired in the presence of 5'FL-UUUUC-3' at concentrations of 0, 50, 100, 200 and 400 μM . The spectral parameters were identical to those in the previous section.

P2/RNA interactions in the presence of P7pep

A 100 μM sample of ILV, U- ^{15}N , ^2H] sample of P2 was used for titrations with the high-affinity RNA construct, 5'FL-UUUUC-3', in the absence or the presence of a saturating amount of P7pep. Titrations were performed at 700 MHz (as above) in P2 buffer (50 mM NaCl) containing 0.8 units/ μL of RNase inhibitor (New England Biolabs). Data were acquired for P2 alone; P2:ssRNA ratios of 1:1 and 1:2; and a P2:ssRNA:P7pep ratio of 1:2:12.

Analysis of chemical shift changes

In all NMR-based titration experiments, the chemical shift changes (δ_{amide} or δ_{met}), where specifically calculated, were determined for each residue using the following equations (as applicable):

$$\Delta\delta_{amide} = \sqrt{(\delta_{H_0} - \delta_H)^2 + \left[\frac{1}{8.62} (\delta_{NO} - \delta_N)\right]^2} \quad (3A)$$

$$\Delta\delta_{met} = \sqrt{(\delta_{Hmet0} - \delta_{Hmet})^2 + \left[\frac{1}{3.94} (\delta_{Cmet0} - \delta_{Cmet})\right]^2} \quad (3B)$$

where δ_{H0} , δ_{N0} , δ_{Hmet0} and δ_{Cmet0} are the amide ^1H , ^{15}N or methyl ^{13}C , ^1H chemical shifts in the absence of titrant respectively, while δ_H , δ_N , δ_{Hmet} and δ_{Cmet} are the corresponding values for a particular concentration of titrant. The scaling coefficients were derived from the weighted standard deviations of amide $^{15}\text{N}/^1\text{H}$ and methyl $^{13}\text{C}/^1\text{H}$ chemical shifts in the Biological Magnetic Resonance Data Bank (BMRB). The restricted mean and restricted standard deviations used in Figs. 2 and S6, were calculated using a two-step process - the mean and standard deviations for the chemical shift perturbations for all residues (assigned or not, calculated separately for δ_{amide} and δ_{met}) were first determined. The mean and standard deviations were then recalculated after excluding values that exceeded the mean by one standard deviation. The mean and standard deviation values that were then obtained correspond to the restricted mean ($\delta_{amide/met,avg}$) and restricted standard deviation ($\sigma_{amide/met}$). Using these values, three classes of residues were determined –

$$1\sigma \text{ class: } \delta_{amide/met,avg} + \sigma_{amide/met} \quad \delta_{amide/met} < \delta_{amide/met,avg} + 2\sigma_{amide/met}$$

$$2\sigma \text{ class: } \delta_{amide/met,avg} + 2\sigma_{amide/met} \quad \delta_{amide/met} < \delta_{amide/met,avg} + 3\sigma_{amide/met}$$

$$3\sigma \text{ class: } \delta_{amide/met} \quad \delta_{amide/met,avg} + 3\sigma_{amide/met}$$

In addition, the apparent K_d for P7pep towards P2 was obtained utilizing the full ^{15}N , ^1H TROSY based titration and resultant δ_{amide} values, using the following equation:

$$\Delta\delta_{amide} = \Delta\delta_{amide,\infty} \frac{\left(K_d + C_{P2} + C_{P7pep}\right) - \sqrt{\left(K_d + C_{P2} + C_{P7pep}\right)^2 - 4C_{P7pep}C_{P2}}}{2C_{P2}} \quad (4)$$

Purification and 5'- ^{32}P labeling of RNA oligonucleotides

The RNA dinucleotide pGpG (Dharmacon Research), purified by denaturing PAGE, was 5'-end-labeled using $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ (MP Biomedical) and T4 polynucleotide kinase (USB) utilizing an exchange reaction. Briefly, reactions contained 1X exchange buffer (50 mM imidazole, 12 mM MgCl_2 , 1 mM 2-mercaptoethanol and 70 μM ADP at pH 6.4), 1 μM HPLC purified $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$, 100 μM pGpG and 0.4 units/ μL T4 polynucleotide kinase. Reactions were incubated at 37° C for 60 minutes, then placed at 65° C for 10 minutes to heat inactivate the T4 polynucleotide kinase.

P2 catalyzed nucleotide incorporation assays

Reactions were performed at 30° C in 50 mM Tris at pH 8.0, 5 mM MgCl_2 , 2 mM MnCl_2 , 1 mM DTT and 50 mM NaCl. For the *de novo* initiation assays, reactions contained 0.5 μM P2, 5 μM RNA template (U-template, 5'-AAACUGAGAAGGAGAAAGCC-3'), 100 μM GTP, 0.2 $\mu\text{Ci}/\mu\text{L}$ $[\gamma\text{-}^{32}\text{P}]\text{-GTP}$ (MP Biomedical) and P7pep (or P7pep⁰, 0 - 1000 μM). For primer extension assays using pGpG, reactions contained 0.5 μM P2, 5 μM U-template, 10 μM end-labeled pGpG, 100 μM CTP and P7pep (or P7pep⁰, 0 - 600 μM). All reactions were

initiated by the addition of P2 and quenched at various times (10, 30 and 60 minutes for *de novo* synthesis; 60, 90 and 120 minutes for primer extension) by the addition of 50 mM EDTA. Products were resolved by using 20% polyacrylamide gel containing 1X TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) and 7 M urea. Electrophoresis was performed in 1X TBE at 90 W. Bands were visualized by using a PhosphorImager and quantified by using ImageQuant software (Molecular Dynamics) to determine the amount of RNA product. Data analysis was performed by in-house software that utilized the MATLAB suite (MathWorks Inc.). All experiments shown are representative, single experiments that have been performed after at least three individual trials to define the concentration or time range shown. In all cases, values for parameters measured during individual trials were within the limits of the error reported for the final experiments.

To determine the IC_{50} value the amount of RNA product was plotted as a function of P7pep (or P7pep⁰) concentration and fit to a hyperbolic equation:

$$C_{RNA} = C_{RNA,0} - \frac{C_P C_{RNA,0}}{IC_{50} + C_P} \quad (5)$$

$C_{RNA,0}$ and C_{RNA} are the concentrations of the RNA product in the absence of the presence of a concentration C_P of P7pep (or P7pep⁰), respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Drs. Leonard Mindich (PHRI), David Jeruzalmi (CCNY) and Andrea Piserchio (CCNY) for useful discussions. Dr. Aneel K. Aggarwal (Mt. Sinai School of Medicine) is thanked for use of the Panvera Beacon 2000. Dr. Paul Gottlieb (City College of New York) is thanked for the kind gift of the pPG24 plasmid. This work is supported by NSF grant MCB 083141 (RG); JJA and CEC are supported by grant AI45818 from NIH/NIAID; the structural biology facilities at CCNY are partially supported by NIH grant 8G12MD007603.

References

1. Mindich, L.; Bamford, DH. Lipid-containing bacteriophages.. In: Calendar, R., editor. The Bacteriophages. Plenum Publishing Corporation; New York: 1988. p. 475-519.
2. Mindich L, Nemhauser I, Gottlieb P, Romantschuk M, Carton J, Frucht S, et al. Nucleotide sequence of the large double-stranded RNA segment of bacteriophage $\phi 6$: genes specifying the viral replicase and transcriptase. J Virol. 1988; 62:1180–5. [PubMed: 3346944]
3. Mindich L, Qiao X, Qiao J, Onodera S, Romantschuk M, Hoogstraten D. Isolation of additional bacteriophages with genomes of segmented double-stranded RNA. J Bacteriol. 1999; 181:4505–8. [PubMed: 10419946]
4. Hoogstraten D, Qiao X, Sun Y, Hu A, Onodera S, Mindich L. Characterization of $\phi 8$, a bacteriophage containing three double-stranded RNA genomic segments and distantly related to $\phi 6$. Virology. 2000; 272:218–24. [PubMed: 10873764]
5. Qiao X, Qiao J, Onodera S, Mindich L. Characterization of $\phi 13$, a bacteriophage related to $\phi 6$ and containing three dsRNA genomic segments. Virology. 2000; 275:218–24. [PubMed: 11017801]
6. Qiao X, Sun Y, Qiao J, Di Sanzo F, Mindich L. Characterization of $\phi 2954$, a newly isolated bacteriophage containing three dsRNA genomic segments. BMC Microbiol. 2010; 10:55. [PubMed: 20170499]

7. Huiskonen JT, de Haas F, Bubeck D, Bamford DH, Fuller SD, Butcher SJ. Structure of the bacteriophage $\phi 6$ nucleocapsid suggests a mechanism for sequential RNA packaging. *Structure*. 2006; 14:1039–48. [PubMed: 16765897]
8. Jäälinoja HT, Huiskonen JT, Butcher SJ. Electron cryomicroscopy comparison of the architectures of the enveloped bacteriophages $\phi 6$ and $\phi 8$. *Structure*. 2007; 15:157–67. [PubMed: 17292834]
9. Wei H, Cheng RH, Berriman J, Rice WJ, Stokes DL, Katz A, et al. Three-dimensional structure of the enveloped bacteriophage $\phi 12$: An incomplete T= 13 lattice is superposed on an enclosed T= 1 shell. *PLoS ONE*. 2009; 4:e6850. [PubMed: 19727406]
10. Poranen MM, Tuma R, Bamford DH. Assembly of double-stranded RNA bacteriophages. *Adv Virus Res*. 2005; 64:15–43. [PubMed: 16139591]
11. Makeyev EV, Bamford DH. Replicase activity of purified recombinant protein P2 of double-stranded RNA bacteriophage $\phi 6$. *EMBO J*. 2000; 19:124–33. [PubMed: 10619851]
12. Makeyev EV, Bamford DH. The polymerase subunit of a dsRNA virus plays a central role in the regulation of viral RNA metabolism. *EMBO J*. 2000; 19:6275–84. [PubMed: 11080173]
13. Juuti JT, Bamford DH, Tuma R, Thomas GJ. Structure and NTPase activity of the RNA-translocating protein (P4) of bacteriophage $\phi 6$. *J Mol Biol*. 1998; 279:347–59. [PubMed: 9642042]
14. Juuti JT, Bamford DH. Protein P7 of phage $\phi 6$ RNA polymerase complex, acquiring of RNA packaging activity by in vitro assembly of the purified protein onto deficient particles. *J Mol Biol*. 1997; 266:891–900. [PubMed: 9086268]
15. Kainov DE, Lísál J, Bamford DH, Tuma R. Packaging motor from double-stranded RNA bacteriophage $\phi 12$ acts as an obligatory passive conduit during transcription. *Nucleic Acids Res*. 2004; 32:3515–21. [PubMed: 15247341]
16. Mindich L. Packaging, replication and recombination of the segmented genome of bacteriophage $\phi 6$ and its relatives. *Virus Res*. 2004; 101:83–92. [PubMed: 15010219]
17. Mindich L. Packaging in dsRNA viruses. *Adv Exp Med Biol*. 2012; 726:601–8. [PubMed: 22297532]
18. Frilander M, Poranen M, Bamford DH. The large genome segment of dsRNA bacteriophage $\phi 6$ is the key regulator in the in vitro minus and plus strand synthesis. *RNA*. 1995; 1:510–8. [PubMed: 7489512]
19. Butcher SJ, Grimes JM, Makeyev EV, Bamford DH, Stuart DI. A mechanism for initiating RNA-dependent RNA polymerization. *Nature*. 2001; 410:235–40. [PubMed: 11242087]
20. Ren Z, M CF, Ghose R. Structure of the RNA-directed RNA Polymerase from the cystovirus $\phi 12$. *Proteins*. 2013; 81:1479–84. [PubMed: 23568335]
21. Mancini EJ, Kainov DE, Grimes JM, Tuma R, Bamford DH, Stuart DI. Atomic snapshots of an RNA packaging motor reveal conformational changes linking ATP hydrolysis to RNA translocation. *Cell*. 2004; 118:743–55. [PubMed: 15369673]
22. El Omari K, Meier C, Kainov D, Sutton G, Grimes JM, Poranen MM, et al. Tracking in atomic detail the functional specializations in viral RecA helicases that occur during evolution. *Nucleic Acids Res*. 2013; 41:9396–410. [PubMed: 23939620]
23. Eryilmaz E, Benach J, Su M, Seetharaman J, Dutta K, Wei H, et al. Structure and dynamics of the P7 protein from the bacteriophage $\phi 12$. *J Mol Biol*. 2008; 382:402–22. [PubMed: 18647606]
24. Nemecek D, Boura E, Wu W, Cheng N, Plevka P, Qiao J, et al. Subunit Folds and Maturation Pathway of a dsRNA Virus Capsid. *Structure*. 2013; 21:1374–83. [PubMed: 23891288]
25. El Omari K, Sutton G, Ravantti JJ, Zhang H, Walter TS, Grimes JM, et al. Plate tectonics of virus shell assembly and reorganization in phage $\phi 8$, a distant relative of Mammalian reoviruses. *Structure*. 2013; 21:1384–95. [PubMed: 23891291]
26. Poranen MM, Butcher SJ, Simonov VM, Laurinmäki P, Bamford DH. Roles of the minor capsid protein P7 in the assembly and replication of double-stranded RNA bacteriophage $\phi 6$. *J Mol Biol*. 2008; 383:529–38. [PubMed: 18793644]
27. Sun X, Bamford DH, Poranen MM. Probing, by self-assembly, the number of potential binding sites for minor protein subunits in the procapsid of double-stranded RNA bacteriophage $\phi 6$. *J Virol*. 2012; 86:12208–16. [PubMed: 22933292]

28. Katz G, Wei H, Alimova A, Katz A, Morgan DG, Gottlieb P. Protein P7 of the Cystovirus $\phi 6$ is located at the three-fold axis of the unexpanded procapsid. *PLoS ONE*. 2012; 7:e47489. [PubMed: 23077625]
29. Nemecek D, Qiao J, Mindich L, Steven AC, Heymann JB. Packaging accessory protein P7 and polymerase P2 have mutually occluding binding sites inside the bacteriophage $\phi 6$ procapsid. *J Virol*. 2012; 86:11616–24. [PubMed: 22896624]
30. Marintcheva B, Marintchev A, Wagner G, Richardson CC. Acidic C-terminal tail of the ssDNA-binding protein of bacteriophage T7 and ssDNA compete for the same binding surface. *Proc Natl Acad Sci USA*. 2008; 105:1855–60. [PubMed: 18238893]
31. Hamdan SM, Marintcheva B, Cook T, Lee S-J, Tabor S, Richardson CC. A unique loop in T7 DNA polymerase mediates the binding of helicase-primase, DNA binding protein, and processivity factor. *Proc Natl Acad Sci USA*. 2005; 102:5096–101. [PubMed: 15795374]
32. Bruenn JA. A structural and primary sequence comparison of the viral RNA-dependent RNA polymerases. *Nucleic Acids Res*. 2003; 31:1821–9. [PubMed: 12654997]
33. Ren Z, Ghose R. Slow conformational dynamics in the cystoviral RNA-directed RNA polymerase P2: influence of substrate nucleotides and template RNA. *Biochemistry*. 2011; 50:1875–84. [PubMed: 21244027]
34. Salgado PS, Makeyev EV, Butcher SJ, Bamford DH, Stuart DI, Grimes JM. The structural basis for RNA specificity and Ca^{2+} inhibition of an RNA-dependent RNA polymerase. *Structure*. 2004; 12:307–16. [PubMed: 14962391]
35. Yang H, Gottlieb P, Wei H, Bamford DH, Makeyev EV. Temperature requirements for initiation of RNA-dependent RNA polymerization. *Virology*. 2003; 314:706–15. [PubMed: 14554097]
36. Yang H, Makeyev EV, Bamford DH. Comparison of polymerase subunits from double-stranded RNA bacteriophages. *J Virol*. 2001; 75:11088–95. [PubMed: 11602748]
37. Ferrer-Orta C, Arias A, Agudo R, Pérez-Luque R, Escarmís C, Domingo E, et al. The structure of a protein primer-polymerase complex in the initiation of genome replication. *EMBO J*. 2006; 25:880–8. [PubMed: 16456546]
38. Ferrer-Orta C, Arias A, Escarmís C, Verdaguer N. A comparison of viral RNA-dependent RNA polymerases. *Current Opin Struct Biol*. 2006; 16:27–34.
39. Ferrer-Orta C, Arias A, Perez-Luque R, Escarmís C, Domingo E, Verdaguer N. Structure of foot-and-mouth disease virus RNA-dependent RNA polymerase and its complex with a template-primer RNA. *J Biol Chem*. 2004; 279:47212–21. [PubMed: 15294895]
40. Zhang X, Walker SB, Chipman PR, Nibert ML, Baker TS. Reovirus polymerase lambda 3 localized by cryo-electron microscopy of virions at a resolution of 7.6 Å. *Nature Struct Biol*. 2003; 10:1011–8. [PubMed: 14608373]
41. Estrozi LF, Settembre EC, Goret G, McClain B, Zhang X, Chen JZ, et al. Location of the dsRNA-dependent polymerase, VP1, in rotavirus particles. *J Mol Biol*. 2013; 425:124–32. [PubMed: 23089332]
42. Qiao J, Mindich L. The template specificity of bacteriophage $\phi 6$ RNA polymerase. *J Virol*. 2013; 87:10190–4. [PubMed: 23864621]
43. Ren Z, Wang H, Ghose R. Dynamics on multiple timescales in the RNA-directed RNA polymerase from the cystovirus $\phi 6$. *Nucleic Acids Res*. 2010; 38:5105–18. [PubMed: 20385578]
44. Boggs PT, Donaldson JR, Byrd RH, Schnabel RB. ODRPACK software for weighted orthogonal distance regression. *ACM Trans Math Software*. 1989; 15:348–64.
45. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR*. 1995; 6:277–93. [PubMed: 8520220]
46. Johnson BA. Using NMRView to visualize and analyze the NMR spectra of macromolecules. *Meth Mol Biol*. 2004; 278:313–52.
47. Salzmänn M, Pervushin K, Wider G, Senn H, Wüthrich K. TROSY in triple-resonance experiments: new perspectives for sequential NMR assignment of large proteins. *Proc Natl Acad Sci USA*. 1998; 95:13585–90. [PubMed: 9811843]
48. Zhu G, Xia Y, Sze KH, X Y. 2D and 3D TROSY-enhanced NOESY of ^{15}N labeled proteins. *J Biomol NMR*. 1999; 14:377–81.

49. Ikura M, Kay LE, Tschudin R, Bax A. 3-dimensional NOESY-HMQC spectroscopy of a ^{13}C -labeled protein. *J Magn Reson.* 1990; 86:204–9.
50. Vuister GW, Clore GM, Gronenborn AM, Powers R, Garrett DS, Tschudin R, et al. Increased Resolution and Improved Spectral Quality in Four-Dimensional ^{13}C , ^{13}C -Separated HMQC-NOESY-HMQC Spectra Using Pulsed Field Gradients. *J Magn Reson B.* 1993; 101:210–3.
51. Hu W, Namanja AT, Wong S, Chen Y. Selective editing of Val and Leu methyl groups in high molecular weight protein NMR. *J Biolmol NMR NMR.* 2012; 53:113–24.
52. Tugarinov V, Kay LE. Ile, Leu, and Val methyl assignments of the 723-residue malate synthase G using a new labeling strategy and novel NMR methods. *J Am Chem Soc.* 2003; 125:13868–78. [PubMed: 14599227]

Highlights

- P7 is known to regulate transcription by the RNA polymerase P2 in cystoviruses.
- The interaction between P2 and P7 *in vitro* is demonstrated using NMR.
- The P2/P7 interaction is mediated by the acidic C-terminal tail of P7.
- The P2/P7 interaction reduces the ability of P2 to initiate *de novo* RNA synthesis.

Fig. 1.

(A) Chemical shift perturbations for the C-terminal resonances of P7, determined from ^{15}N , ^1H HSQC spectra (600 MHz) of ^{15}N -labeled full-length P7 in the absence or the presence of P2 (P7:P2 molar ratio 1:2). δ_{amide} values, calculated using Eq. (3A), in 0 mM NaCl (green bars), 50 mM NaCl (black bars) and 150 mM NaCl (red bars) are shown. Negative bars (for 0 mM NaCl) represent residues for which the resonances are broadened below the threshold of detection. (B) δ_{amide} values for the C-terminal resonances of full-length wild-type P7 (black), P7^{D160N} (blue) and P7^{D168N} (yellow) in the presence of a 2-fold excess of P2. The buffer contained 50 mM NaCl in all cases. (C) The C-terminal sequences of the P7 proteins from the $\phi 6$, $\phi 8$, $\phi 12$, $\phi 13$ and $\phi 2954$ cystoviruses are shown. Sequence alignment was performed using the entire sequences of the five P7 proteins ($\phi 6$:1-161, ABF19537.1; $\phi 8$:1-175, NP_652745.1; $\phi 12$:1-169, NP_690822.1; $\phi 13$:1-156, AF261668_1; $\phi 2954$:1-177, YP_002600762.1) though only the C-terminal sequences are depicted. While the overall conservation is low, all the sequences are characterized by a large concentration of acidic residues (shown in red letters; basic residues are shown in blue letters) at the C-terminal ends. The P7⁰ and P7⁺ mutants (see inset) were generated by replacing all the acidic residues of C-terminus of $\phi 12$ P7 by corresponding neutral residues

or by positively charged residues, respectively. In addition, peptides encompassing residues 155 through 169 of P7 (P7_{pep}) and P7⁰ (P7_{pep}⁰) were synthesized.

Fig. 2.

(A) Residues for which the δ_{amide} values (for a P2:P7pep molar ratio 1:10) belong to the 1 σ class ($0.024 \text{ ppm} > \delta_{\text{amide}} \geq 0.016 \text{ ppm}$), the 2 σ class ($0.031 \text{ ppm} > \delta_{\text{amide}} \geq 0.024 \text{ ppm}$) and the 3 σ class ($\delta_{\text{amide}} > 0.031 \text{ ppm}$) are depicted on the structure of P2 and colored yellow, orange and red respectively. The arrow indicates the mouth of the template tunnel in all cases. (B) Residues for which δ_{met} values (for a P2:P7pep molar ratio 1:8) belong to the 2 σ class ($0.017 \text{ ppm} > \delta_{\text{met}} \geq 0.013 \text{ ppm}$) or the 3 σ class ($\delta_{\text{met}} > 0.017 \text{ ppm}$) are depicted as in (A). (C) Surface representation of P2 showing the 2 σ and 3 σ class

perturbations in the presence of P7pep (P2:P7pep molar ratio 1:8 as in **B**, left) or 5'FL-UUUUC-3' (P2:ssRNA molar ratio 1:4; right). A portion of the front surface has been removed to show the inside of the template tunnel. Note the similarities in the overall pattern of perturbations seen in the two cases. Key residues discussed in the text are labeled. **(D)** Charge distribution within the template tunnel. The negatively charged surface at the base of the tunnel is created by the catalytic aspartates (D349, D469 and D470).

Fig. 3.

(A) Binding isotherms determined by measuring the change in fluorescence anisotropy for two 5-nt non-genomic ssRNA constructs (5'FL-AAGCC-3', black; 5'FL-UUUUC-3', red) in the absence (solid lines, filled circles) or the presence (dotted lines, open circles) of P7pep (also see Table S3). Circles represent the experimental data and the lines represent fits to Eq. (2). Also shown as blue circles are the anisotropy values measured for increasing concentrations of P7pep in the absence of P2. (B) ^{13}C , ^1H HMQC spectra (600 MHz) of ILV, U- ^{15}N , ^2H P2 (black) in the presence of 5'-UUUUC-3' (P2:ssRNA molar ratio 1:2; green) and after the addition of P7pep (P2:ssRNA:P7pep molar ratio 1:2:12; orange). As shown in the insets, positions of the resonances in the case of P2+ssRNA+P7pep seem to shift towards those for P2+P7pep.

Fig. 4.

(A) Inhibition of *de novo* RNA synthesis on the U-template using only GTP in the presence of increasing concentrations of either P7pep or P7pep⁰. (B) Inhibition of the elongation of a pGpG primer on the U-template using CTP in the presence of increasing concentrations of either P7pep or P7pep⁰. Circles represent the experimental data and the lines represent fits to Eq. (5).