Critical phosphoprotein elements that regulate polymerase architecture and function in vesicular stomatitis virus

Amal A. Rahmeh, Benjamin Morin, Andreas D. Schenk, Bo Liang, Bianca S. Heinrich, Vesna Brusic, Thomas Walz, and Sean P. J. Whelan

The RNA-dependent RNA polymerase (RdRP) of nonsegmented negative-sense RNA viruses consists of a large catalytic protein (L) and a phosphoprotein cofactor (P). During infection, the RdRP replicates and transcribes the viral genome, which resides inside an oligomer of nucleocapsid protein (N-RNA). The classical view of P as a cofactor for L assigns a primary role of P as a bridge mediating the access of L to the RNA template, whereby its N-terminal domain (PNTD) binds L and its C-terminal domain (PCTD) binds N-RNA. Recent biochemical and structural studies of a prototype nonsegmented negative-sense RNA virus, vesicular stomatitis virus, suggest a role for P beyond that of a mere physical link: P induces a structural rearrangement in L and stimulates polymerase processivity. In this study, we investigated the critical requirements within P mediating the functional interaction with L to form a fully functional RdRP. We analyzed the correlation between the impact of P on the conformation of L and its activity in RNA synthesis and the consequences of these events on RdRP function. We identified three separable elements of the PNTD that are required for inducing the conformational rearrangement of L, stimulating polymerase processivity, and mediating transcription of the N-RNA. The functional interplay between these elements provides insight into the role of P as a dynamic player in the RNA synthesis machine, influencing essential aspects of polymerase structure and function.

large polymerase | replication and transcription | Mononegavirales | rhabdovirus

The functional unit necessary for transcription and replication of nonsegmented negative-sense (NNS) RNA viruses is a ribonucleoprotein (RNP) complex. The RNP complex comprises a genomic RNA encapsidated by a nucleocapsid protein oligomer (N-RNA), associated with the RNA-dependent RNA polymerase (RdRP) consisting of a complex of the large polymerase protein (L) and a phosphoprotein (P) (1–3). The template RNA is buried between the N- and C-terminal lobes of each N protomer; nevertheless, the overall integrity of the N-RNA structure is maintained during copying by the RdRP (4, 5). The L protein is the multifunctional catalytic core of the RNA synthesis machinery, harboring the RdRP as well as a capping enzyme and two methyltransferase activities that are required for mRNA synthesis (6–10). During RNA synthesis, L must gain access to the RNA, and its enzymatic activities must be regulated in accordance with a replicase or transcriptase mode of RNA synthesis. The access of L to the N-RNA is mediated by the noncatalytic cofactor P, which engages L and the N oligomer simultaneously (11, 12).

The functioning of an RNP as a highly regulated RNA synthesis machine requires an intricate, tight coordination of its individual components. The mechanisms that govern such functional coupling are largely unknown. Much of our understanding of the assembly, structure, and function of NNS RNA virus RNPs have come from studies of vesicular stomatitis virus (VSV). In part, this reflects the uniquely robust in vitro transcription that can be reconstituted from purified VSV N-RNA, P, and L (3, 13). The VSV P protein exhibits a modular organization (Fig. 1A), comprising an N-terminal domain (PNTD), a central domain (PCRD), and a C-terminal domain (PCTD) (11, 14, 15). The roles of PNTD in binding L and of PCTD in binding N-RNA were first recognized by biochemical and genetic studies, establishing a primary role of P as a mediator of L’s access to the N-RNA template (11). A second essential role of P involves chaperoning the free N protein (N0) via the extreme N terminus of PNTD, maintaining N0 solubility and regulating its assembly on the nascent genomic RNA during replication (16, 17).

An expanding structural map of P is beginning to provide additional functional insight into its role in RNA synthesis. Analysis of the crystal structure of the VSV PCTD has shown that PCTD makes direct contact with the N-terminal lobes of two adjacent N protomers (18). Structural and biophysical analyses have demonstrated that PCTD is a homodimerization domain (14, 19). The role of P dimerization remains uncertain; however, it has been proposed to play a role in mediating the progression of L along the N-RNA template (20). The structure of N0 lacking an N-terminal arm, in complex with a peptide corresponding to the first 60 residues of P, supports a model in which residues 6–35 of P block the polymerization of adjacent N molecules as well as access of RNA to the RNA-binding groove (21). Biophysical and bioinformatic analyses have indicated the presence of multiple intrinsically disordered regions (IDRs) separating the different structured domains of P (15). These disordered regions have been suggested to enhance the dynamic properties of P function. Furthermore, a role for phosphorylation of P by cellular kinases has been proposed to play a role in regulating the function of P in viral transcription and replication (22, 23).

Until recently, functional interactions between L and P were largely uncharacterized. Recent biochemical and structural studies point to additional roles of P beyond serving as a mere physical bridge between L and the N-RNA template. EM characterization of L revealed its organization into a ring domain harboring the RNA polymerase, linked to a flexible appendage of three globular domains containing the cap-forming activities (24). After complex formation with P, the appendage rearranges into a more compact structure. Furthermore, reconstitution of RNA synthesis using purified L and a short synthetic RNA template demonstrated that P stimulates the initiation and processivity of L, indicating a role of P in stimulating L function.

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independent of facilitation of access of L to the encapsidated RNA template (25).

In this study, we investigated the critical events resulting from the impact of P on the structure and function of L, and examined the contribution of these events to achieving a fully functional RdRP complex. We identified the elements of P necessary for inducing a conformational rearrangement in L and stimulating the processivity of L, and characterized the influence of these elements on RdRP function in copying the N-RNA template.

Here we propose a model for an emerging role of P as a dynamic influence of these key aspects of polymerase structure and function.

**Results**

**P<sub>NTD</sub> Is Sufficient to Induce Conformational Change in L and Stimulate L Processivity.** To probe the relationship between the conformational change in L on binding of P and processivity of the RdRP, we first defined the elements of P that mediate each of these functions. For the analysis of RdRP processivity, we used an RNA synthesis assay comprising a synthetic naked 19-nt template corresponding to the 5′ leader sequence of the VSV genome (Le19) (25). This assay specifically allowed us to study the function of P as a processivity factor independent of its role in facilitating L access to an encapsidated template. The L polymerase uses Le19 as a template to synthesize RNA products of 3–19 nt, and the addition of P resulted in a three- to fourfold increase in RNA synthesis and enhancement of the 13- to 19-nt products (Fig. 1B, lanes 1 and 2). To define the P domains that stimulate L activity in RNA synthesis, individual or combinations of the P domains were expressed in *Escherichia coli* and purified (Fig. S1A). We previously demonstrated that the phosphorylation of P did not influence polymerase activity on the Le19 template (25). P<sub>NTD</sub>(1–106) and P<sub>NTD+CD</sub>(1–177) stimulated L processivity to a similar extent as full-length P (1–265), whereas P<sub>CD</sub>(178–265) had no effect on RNA synthesis (Fig. 1B, lanes 3–5). These data demonstrate that the P<sub>NTD</sub> is sufficient for stimulation of L processivity independent of the oligomeric state of P.

EM analysis revealed the organization of L into a ring-like domain containing the RNA polymerase activity and a flexible appendage consisting of three globular domains containing the N0 binding, homo-oligomerization, and N-RNA binding. The zigzag line depicts the region of P of undetermined structure. P<sub>NTD</sub> has been shown to be involved in binding L; however, the precise binding site has not been determined. (B) P<sub>NTD</sub> is sufficient to stimulate the processivity of L on a non-encapsidated RNA template. RNA synthesis reactions were carried out with 0.2 μM Le19 template and 0.2 μM L, with 0.2 μM of either full-length P or fragments comprising the P<sub>NTD</sub>, P<sub>NTD+CD</sub>, or P<sub>CD</sub> added when indicated. Reaction products were analyzed on a 20% polyacrylamide/7 M urea gel. (C) P<sub>NTD</sub> recapitulates the structural rearrangement in L induced by full-length P. EM characterization of L, L<sub>NTD</sub>, and L<sub>NTD+CD</sub> complexes shows five representative class averages of each, illustrating the structural similarity of the L-P<sub>NTD</sub> complex to the monomer population of the L-P complex. Shown is the percentage of the population of particles exhibiting similar conformations as the representative class averages for L and L-P. (Scale bar: 20 nm.)

**P<sub>NTD</sub> Residues 81–106 Are Sufficient to Stimulate L Processivity on a Nonencapsidated RNA Template.** To further define the elements of P<sub>NTD</sub> that stimulate the processivity of L on the Le19 template, a series of sequential deletions of P<sub>NTD</sub> were expressed in *E. coli* and purified (Fig. S2A). Whereas P1–90 was inactive in stimulating L processivity, P21–106 and P41–106 retained full activity, and P61–106 and P71–106 exhibited a modest decrease in activity (Fig. 2, Left and Fig. S2B). To further refine the N-terminal boundary, a series of N-terminal truncations of P were expressed and purified (Fig. S2 A and B). Whereas P81–265 stimulated L processivity comparably to full-length P (1–265), P86–265 exhibited decreased L stimulation and P91–265 failed to stimulate L (Fig. 2, Middle). Collectively, these results indicate that P residues 81–106 constitute the element necessary for stimulation of L processivity. Consistent with this conclusion, P1–80 as well as an internal deletion in full-length P (P481–106)
that deletions up to the N0-binding domain of PNTD (P41–265) were analyzed for stimulating L processivity as described in Fig 1B. Right) An internal deletion of residues 81–106 in full-length P (P81–106) and a chemically synthesized peptide comprising residues 81–106 of P (PPLSD) (both 0.2 μM) were tested as well. Quantitative analysis of two independent experiments is presented in Fig S2B.

Lacking any stimulatory activity, and a synthetic peptide consisting of residues 81–106 retained a stimulatory activity comparable to that of full-length P (Fig. 2, Right and Fig. S2B). We termed the P81–106 peptide PPLSD (for L stimulatory domain). Although PPLSD (81–106) stimulated the activity of L similarly to full-length P, we noted the appearance of slightly shorter products (12–14 nt), which might reflect decreased binding efficiency of PPLSD to L (Fig. 3).

L Binding Encompasses the Region Between the N0-Binding Domain and the Oligomerization Domain of P. Earlier work determined that PNTD binds L (11); however, the region within P that binds L was not mapped precisely. Given that single amino acid changes in P that disrupt L binding have not been identified, we elected to use deletion mutants in P to map the L-binding domain. To delineate the region within the PNTD that binds L, we used His-tagged L as bait to precipitate untagged deletion mutants of full-length P (Fig. 3 and Fig. S3). As expected, deletion of the entire PNTD (P107–265) completely abolished binding. Deletion of the N0-binding domain of P (P41–265) had no effect, whereas larger N-terminal deletions (P61–265, P71–265, and P81–265) significantly decreased P binding to L. Consistent with the critical nature of the PPLSD in L stimulation, an internal deletion (P81–106) also significantly decreased L binding. These results indicate that whereas PPLSD is sufficient for stimulation of L processivity on Le19, the L-binding region on P spans a larger interval between the N0-binding domain and the oligomerization domain.

PNTD Region Required to Induce Conformational Rearrangement in L. We next used EM to examine the contribution of elements of PNTD to the observed structural rearrangement in L. To do this, we mixed L with PNTD mutants harboring the indicated deletions and calculated 20 class averages for each sample (Fig. 4A and Fig. S4). In Fig. 4A, the 15 classes shown for each sample are arranged in order of decreasing abundance from left to right and top to bottom. Individual class averages from each sample were visually scored as L conformation or L–P conformation, and the percentage of each conformation relative to the total number of particles was calculated (Fig. 4B). The class averages illustrate that deletions up to the N0-binding domain of PNTD (P41–106) retained the ability to induce the conformational rearrangement in L to a similar extent as PNTD. Further deletions in the PNTD (P61–106 and P81–106) decreased the population of L exhibiting a conformational rearrangement. Similarly, deletion of PPLSD (P1–80) inhibited the conformational rearrangement of L, and this inhibition could not be rescued by supplementation of PPLSD (P1–80 + P81–106) (Fig. S4). Collectively, these results indicate that the entire L-binding region of P (41–106) appears to be necessary to elicit a conformational rearrangement in L.

Level of Stimulation of L Activity on N-RNA Correlates with Extent of Conformational Rearrangement in L Induced by Elements of PNTD. To determine the impact of altering the conformational state of L on its RdRP function during RNA synthesis, we used an in vitro transcription assay reconstituted with individually purified N-RNA, L, and P or sequential N-terminal deletions of full-length P. We analyzed deletions of the PNTD in the context of full-length P, because PPLSD is required to bind the template-associated N. We isolated N-RNA from detergent-disrupted purified virus using a standard protocol of high-salt treatment followed by double isolation on a CsCl gradient (26). This purification removes virtually all of the endogenous P protein; nevertheless, trace amounts of P (PPLSD) remained bound, which we estimated as ~16.67 nM (Fig. S5A). PPLSD resulted in a basal level of transcription that was stimulated up to 8- to 10-fold by 333.4 nM exogenous purified P (Fig. S5B). We tested each of the P deletion mutants at a saturating concentration of 1,667 nM (Fig. 5A). Deletion of the N0-binding domain (P41–265) resulted in transcription levels indistinguishable from those achieved by full-length P, indicating that the role of this domain is restricted to genome replication. Larger N-terminal deletions (P61–265, P71–265, and P81–265) resulted in a gradual decrease in stimulation of transcription relative to full-length P. The N-terminal deletions in P resulted in a more pronounced reduction of RNA synthesis on N-RNA than on Le19. This indicates that the conformational change in L mediated by P41–106 is critical for efficient use of the N-RNA template. Internal deletion of the PPLSD (P81–106) resulted in further decrease in stimulation, whereas deletion of the entire PNTD (P107–265) served as a dominant negative, inhibiting the basal levels of transcription resulting from PPLSD. This likely reflects the ability of P107–265 to oligomerize with PPLSD or to compete it off the template. The
Fig. 4. EM characterization of the effect of PNTD deletion mutants in inducing a conformational rearrangement in L. (A) L was mixed with each PNTD deletion mutant at a 1:10 molar ratio, and the resulting complexes were analyzed by EM. The averages of the 15 most populous classes obtained by classification of the particles into 20 classes are shown. The side length of the individual panels is 29 nm. (B) Individual class averages from two independent experiments (Fig. S3) were scored as L conformation or LP conformation. The average percentage of each conformation relative to the total number of particles in each sample is plotted.

Discussion

This study provides a detailed analysis of the elements of P that are involved in the functional interaction with L and their effect on the architectural organization and RNA synthesis activity of the RdRP. The region of P that mediates the functional interaction with L spans residues 41–106 of the PNTD. Whereas this entire region appears to be necessary for inducing a conformational rearrangement in L and stimulating RNA synthesis on the viral encapsidated N-RNA template, a subdomain of this region (P_{LSD}, residues 81–106) is sufficient for stimulating L processivity on a short naked RNA template. We posit a model in which the effects of P on RdRP are separated into (i) stimulation of L processivity in directly copying an RNA substrate that is likely mediated through a local effect on the polymerase active site and (ii) stimulation of L activity on the encapsidated template, which requires a global rearrangement in the architecture of L that may be necessary for correct positioning of L in coordination with the N molecules in its vicinity and/or for coordination of the various enzymatic activities of L during viral RNA synthesis.

L-Binding Region of P. The results of this study demonstrate that the L-binding region of P resides within P residues 41–106. There are no available structural data for this region. Bioinformatic analysis predicts that residues 40–90 constitute an IDR and that residues 91–106 constitute a structured domain speculated to be the binding site for L (15). Further analysis using the PHYRE protein homology/analogy recognition engine (http://www.sbg.bio.ic.ac.uk/~phyre2) predicted an α-helical region at residues 79–100. These analyses suggest that P_{LSD} (P81–106) is likely a structured domain of P_{NTD}.

Recent reports have described a disorder-to-order transition governing the binding of N and P in paramyxoviruses, suggesting that IDRs play a role in establishing multiple molecular partnerships (27). These mechanisms are proposed to be advantageous for achieving pleiotropy and genetic compaction for the virus. Such molecular flexibility also might be required to support the roles of P_{NTD} as a chaperone for N and as a binding site for L. The atomic structure of the N0–P1–60 complex reveals that P residues 6–40 form a molecular recognition element that engages N0, with amino acids 1–5 and 41–60 remaining flexible. Those flexible regions of P were suggested to act as “entropic bristles” that repel incoming RNA or N molecules or mask their binding interfaces (21). In the present study, the dispensability of the N0-binding molecular recognition element during transcription (P41–265) demonstrates the lack of effect of this region on polymerase function and is consistent with a primary role in the chaperoning N0 during replication. Whether a single P_{NTD} can simultaneously bind N0 and L remains unknown; however, the close proximity of the two binding regions makes such a scenario unlikely. Perhaps this need for P_{NTD} to interact with two molecular partners explains one of the requirements for oligomerization of P, with one P_{NTD} binding L and a second P_{NTD} binding the N0 necessary for encapsidation of the nascent RNA during replication.

The prediction that P81–106 is a structured domain is supported by the demonstration that P_{LSD} (P81–106) as a discrete domain stimulates processivity of L on the naked RNA template. Independent folding of P_{LSD} would be consistent with the retention of its functionality when separated from the rest of the L-binding region. The significant decrease in L binding resulting from N-terminal deletions downstream of amino acid 41, as well as from an internal deletion of P_{LSD}, suggests that the binding of P41–106 to L is likely a cooperative mechanism involving the IDR (P41–80) and P_{LSD}. Such a mechanism is also consistent with the failure to restore the conformational change and the
Functionality analysis of PNTD in transcription of an encapsidated N-RNA template. (A) 50 μL in vitro transcription reactions were reconstituted with 5 μg of N-RNA, 1 μg (4.16 pmol) of L, and 83.2 pmol P or each of the N-terminal or internal deletions of P. The N-RNA template was isolated from purified virus and retained 0.22 pmol of residual P (PΔ) μg of purified N-RNA, resulting in a final concentration of 16.67 nM per reaction (Fig S5A). Transcription reactions were reconstituted in the presence of [α-32P] GTP. The products were separated by electrophoresis on acid-agarose gels and analyzed with a PhosphorImager. The five VSV mRNAs, P, M (matrix), N, G (glycoprotein), and L indicate the right. The total amount of synthesis was quantified by summing the band intensities, normalized to levels of RNA synthesis produced by PΔ and graphed. Error bars represent the SD from the mean of three independent experiments. (B) In vitro transcription reactions were reconstituted with 5 μg of N-RNA, 1 μg (4.16 pmol) of L, and 16.64 pmol of P, P107–265, P81–265, or PΔ81–106, with 83.2 pmol of P1–80, P1–106, or P81–106 added to reactions when indicated. RNA synthesis was analyzed as in A.

Stimulation of L Processivity by PNTD. P3SD is sufficient for stimulating L processivity on a short naked RNA template, independent of the rest of the L-binding region. The mechanism by which P3SD stimulates L processivity is not clear. EM analysis of L in the presence of P3SD showed no significant change in the conformation of L, suggesting a lack of correlation between stimulation of L processivity by P3SD and the global rearrangement in L conformation. However, the possibility of a more subtle or local alteration of L conformation that is not discernible by EM at the current resolution cannot be ruled out. For a mechanistic understanding of the effect of P3SD on polymerase processivity, kinetic analysis of the effects of P and P3SD on the affinity of the polymerase for the template and on the rate of polymerase elongation is needed. However, the retained ability of P3SD to stimulate L processivity on Le19 indicates that such a mechanism does not require a tight association between L and P. In contrast, the entire L-binding region is required for stimulation of polymerase activity on N-RNA, indicating a requirement for a tighter binding in this latter case.

Role of PNTD in Stimulating L Activity on the Encapsidated N-RNA Template. Our results point to a correlation between the conformational rearrangement in L and stimulation of its activity on N-RNA. Specifically we found that (i) N-terminal deletions in full-length P downstream of the N3-binding site resulted in a gradual decrease in stimulation of the RNA synthesis activity on the N-RNA template; (ii) an internal deletion of P3SD also significantly reduced synthesis; and (iii) similar deletions in PNTD resulted in a gradual decrease in the population of L exhibiting a conformational rearrangement similar to that induced by PNTD. However, a conformational rearrangement in L per se is not necessary for stimulation of processivity, given that L processivity on an non-encapsidated 19-nt RNA was dependent only on the P3SD, which did not induce a significant alteration in the molecular architecture of L.

Collectively, our data support a model in which the conformational rearrangement in L is necessary for stimulation of polymerase activity on the encapsidated RNA. A notable feature of NNS virus genomic RNA is its resistance to digestion by nucleases even during the process of RNA synthesis (28), indicating a tight spatial regulation coordinating the release of a stretch of RNA from N to be copied by L. Thus, a global change in the conformation of L controlling the flexibility of the appendage of L might be required for precise positioning of L on the template in coordination with the nucleocapsid. The conformational rearrangement also could be necessary for spatial and temporal control of the cap-modifying activities during replication and transcription. On a short naked RNA such as
Le19, the conformational rearrangement has no effect on RNA synthesis, because these levels of control are irrelevant. Therefore, this independent role of P in stimulating RNA synthesis on L might reflect an evolutionary pathway in which a processivity factor sufficient to stimulate polymerase activity on linked RNA is concatenated to additional regions that mediate RdRP activity on an encapsidated template. Finally, although the phosphorylation status of P does not impact polymerase activity in the in vitro assays used in this study, phosphorylation of P is essential for virus recovery from infected cells (29), indicating a key role for phosphorylation in regulating P function.

In summary, our work provides insight into the mechanisms by which the VSV P protein facilitates RdRP function of the L protein. A full understanding of the dynamic role of P in RNA synthesis will require knowledge not only of the atomic-level structure of L, but also of how the L-P complex accesses the N-RNA. This work provides important tools for examining these issues by providing a better molecular understanding of the function of specific regions of P and how they influence the architecture of L. Because the process of RNA synthesis involves dynamic interactions between multiple molecular partners, further studies of the spatial and temporal regulation of this complex RNA synthesis machinery also will likely benefit from the application of single-molecule enzymology approaches.

Materials and Methods

Protein Expression and Purification. Proteins were expressed and purified as detailed in SI Materials and Methods.

Peptide Synthesis. PNTD (AEQVFGIQDPLYADGGVDFV) was synthesized with standard Fmoc chemistry on ABI 431 Peptide Synthesizers at the Tufts University Core Facility, purified using reverse-phase HPLC, and analyzed by MS.

RNA Synthesis Assays. RNA synthesis on Le19. Le19 was chemically synthesized and PAGE-purified (Integrated DNA Technologies). Polymerase reactions were performed as described previously (25) using 0.2 μM Le19 and 0.2 μM L, with 0.2 μM P or P deletions added when indicated. Reactions were incubated for 3 h, and products were resolved by 20% (w/vol) polyacrylamide/7 M urea gel electrophoresis and analyzed with a PhosphorImager (GE Healthcare).

In vitro transcription of N-RNA. Standard in vitro transcription reactions were carried out as described previously (24) using 5 μg of N-RNA, 1 μg (4.16 pmol) of L, and the indicated concentrations of P or P deletions. The products were purified by RNasey (Qiagen), separated by acid-agarose urea gel electrophoresis, and analyzed with a PhosphorImager.

EM and Image Processing. L (0.14 μM) was mixed with PNTD deletions (1.4 μM) in buffer containing 50 mM Tris-HCl (pH 7.4), 280 mM NaCl, 3% (w/vol) glycerol, and 1 mM DTT and incubated on ice for 1 h. Samples were diluted 1:30–1:50 in binding buffer without glycerol and adsorbed to glow-discharged, carbon-coated EM grids, then stained with 0.75% (w/vol) uranyl formate as described previously (30). Images were collected and processed as described in detail in SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Protein Expression and Purification. L was expressed in Sf21 cells with an N-terminal 6xHis tag and purified by Ni- nitritoltriacetic acid (NTA) affinity, followed by MonoS chromatography as described previously (1). N-RNA was isolated from purified vesicular stomatitis virus (VSV) as described previously (2), with an additional passage on the CsCl gradient. P, P N-terminal domain (PNTD), and PNTD deletions were cloned with N-terminal 6xHis-ENLYFQSNV in a modified pET16 vector. [The underlined residues indicate the tobacco etch protein (TEV) protease recognition motif; cleavage by TEV occurs between Q and S.] P and N-terminal P deletions were cloned with an N-terminal 6xHis-GSS-(maltose binding protein)-ENLYFQSGG. The plasmids were transformed in BL21 (DE3) Escherichia coli. The cells were grown in LB containing 100 μg/mL of ampicillin and induced at an A600 of 0.6 with 1 mM isopropyl β-D-thiogalactopyranoside for 4 h at 30 °C. All fragments were first purified by Ni-NTA agarose chromatography (Qiagen) following the manufacturer’s standard protocol with gradient elution. To remove the N-terminal tags, proteins were dialyzed in 20 mM Tris-HCl (pH 7.4), 250 mM NaCl, and 25 mM imidazole and removed the N-terminal tags, proteins were dialyzed in 20 mM Tris (pH 7.4), 150 mM NaCl, and 1 mM DTT.

Size-Exclusion Chromatography. Here 120 μg of L and 24.5 μg of PNTD were individually passed through a Superdex 200 HR 10/30 column (GE Healthcare) or, alternatively, first mixed together for 1 h on ice before column passage. The column was run at 0.25 mL/min, and 250-gL fractions were collected. Apparent molecular weights were extrapolated from a standard curve calculated from the elution volumes of a gel filtration standard (BioRad).

EM and Image Processing. Micrographs were collected using a Tecnai T12 electron microscope (FEI) equipped with an LaB6 filament and operated at an acceleration voltage of 120 kV. Micrographs were recorded on imaging plates at a magnification of 67,000× and a defocus of approximately –1.5 μm using low-dose procedures. For the L–PNTD deletion complexes, two independent datasets were recorded. Imaging plates were read with a scanner (DITABIS) using a step size of 15 μm, a gain setting of 20,000, and a laser power setting of 30%; 2 × 2 pixels were averaged to yield a pixel size of 4.5 Å at the specimen level (3). BOXER, part of the EMAN software package (4), was used to interactively select particles. For the L + PNTD sample, 5,842 particles were selected from 54 images. For the L + P41–106 sample, 4,890 particles were selected from 28 images for the first dataset and 3,950 particles were selected from 36 images for the second dataset. For the L + P61–106 sample, 4,028 particles were selected from 39 images for the first dataset and 3,826 particles were selected from 39 images for the second dataset. For the L + P81–106 sample, 3,615 particles were selected from 26 images for the first dataset and 2,541 particles were selected from 39 images for the second dataset. For the L + P1–80 sample, 2,150 particles were selected from 21 images for the first dataset and 2,325 particles were selected from 37 images for the second dataset. For the L + P1–80 + P81–106 sample, 3,985 particles were selected from 38 images for the first dataset and 2,465 particles were selected from 39 images for the second dataset. All particles were windowed into 64 × 64-pixel images and classified using the SPIDER software package (5). The particles were rotationally and translationally aligned and subjected to 10 cycles of multireference alignment. Each round of multireference alignment was followed by k-means classification into 20 classes. The references used for the first multireference alignment were chosen at random from the particle images.

Ni-NTA Pulldown Assay. Here 3 μg (12.5 pmol) of 6xHis-tagged L was incubated with 50 pmol of untagged P or P deletions in 300 μL of buffer containing 50 mM NaH2PO4 (pH 7.4), 250 mM NaCl, and 10 mM imidazole for 1 h on ice, followed by the addition of 15 μL of Ni-NTA agarose beads (Qiagen) with end-to-end rotation for 1 h at 4 °C. The beads were precipitated by centrifugation at 2,000 × g for 3 min and then washed five times with 500 μL of binding buffer containing 30 mM imidazole. The beads were boiled in 2× SDS/PAGE loading buffer, and the proteins were separated by 4–12% SDS/PAGE. The precipitated bands were quantitated using ImageJ software.

Western Blot Analysis. A polyclonal antibody against P was generated by immunization of rabbits with His-P purified from Escherichia coli (Covance). Nitrocellulose membranes were probed using a 1:20,000 dilution of anti-P, followed by a 1:5,000 dilution of anti-rabbit HRP (Santa Cruz Biotechnology). Bands were visualized by ECL (Pierce) and quantified using an AlphaImager (Alpha Innotech).

Fig. S1. Purification and EM characterization of an L–PNTD complex. (A) Full-length P or fragments comprising PNTD (1–106), PNTD+CD (1–177), or PCTD (178–265) were expressed in E. coli with a 6xHis-MBP tag. Proteins were purified, and the tag was cleaved off with TEV and separated by Ni-NTA chromatography. The purified proteins were analyzed by 4–12% SDS/PAGE and visualized with Coomassie blue staining. (B) Isolation of an L–PNTD complex by size-exclusion chromatography. L (Top), PNTD (Middle), and L + PNTD (Bottom) were passed through a Superdex 200 column. The eluted fractions were analyzed by 4–12% SDS/PAGE and visualized with Coomassie blue staining. The elution of molecular weight standards is indicated by arrows. BC, before column; Mr, molecular weight marker. (C) Representative EM image of L + PNTD in a negative stain. (Scale bar: 50 nm.) (D) Class averages of single particles of the L–PNTD complex obtained after classification of 5,842 particles into 20 classes. The side length of the individual panels is 29 nm.
Fig. S2. Effects of P_NTD deletion mutants and N-terminal deletions of full-length P (PFL) on stimulation of L processivity on the Le19 template. (A) Series of deletion mutants spanning P_NTD (Left) and N-terminal deletions of PFL (Right) were expressed and purified as in Fig. S1A. The purified proteins were separated by 4–12% SDS/PAGE and visualized with Coomassie blue staining. (B) Total amount of RNA synthesis on Le19 in the presence of each of the P_NTD deletion mutants and the N-terminal deletions of PFL were quantified by summing the band intensities, normalized to levels of RNA synthesis produced in the absence of P, and graphed. Error bars represent the SD from the mean of two independent experiments.
Fig. S3. Quantitation of the fraction of precipitated P deletions by L. The input and the precipitated bands of PFL and P deletions in Fig. 3 were quantitated, and the fraction of each precipitated band/input was calculated. The precipitated fractions of each deletion were normalized relative to that of PFL and graphed.
Fig. S5. Optimization of in vitro transcription conditions using an encapsidated N-RNA template. (A) Estimation of the amount of residual P remaining on the purified N-RNA. (Left) N-RNA was isolated from purified virus and visualized by Coomassie blue staining on low-Bis (0.13% Bis) 10% SDS/PAGE. (Right) The presence of residual P (P<sub>R</sub>) on N-RNA was detected by Western blot analysis using a polyclonal antibody against P, where 0.22 pmol P<sub>R</sub>/μg of purified N-RNA were estimated by quantification of the band intensity of 4 μg of N-RNA relative to the band intensity of 0.8335 pmol of purified recombinant P using an Alphalmager. (B) Measurement of the stimulation of transcription as a function of increasing concentrations of P and the effect of increasing concentrations of P<sub>N<sub>TD</sub></sub> in a range of P concentrations. (Left) Here 50-μL in vitro transcription reactions were reconstituted with 5 μg of N-RNA, 1 μg (4.16 pmol) of L, and increasing concentrations of P (lanes 1–7). The products were separated by electrophoresis on acid-agarose gels and analyzed with a PhosphorImager. The five VSV mRNAs P, M (matrix), N, G (glycoprotein), and L are shown to the right. (Right) (Upper) The total amount of synthesis was quantified by summing the band intensities, normalized to levels of RNA synthesis produced by P<sub>R</sub>, and graphed. (Lower) Increasing concentrations of P<sub>N<sub>TD</sub></sub> were added to in vitro transcription reaction in the absence of exogenous P (lanes 8–10), or in the presence of suboptimal (lanes 11–13) or optimal (lanes 13–16) concentrations of P. RNA synthesis was analyzed as in A and graphed.

Fig. S6. Complementation analysis of the domains of P in transcription of an encapsidated N-RNA template. Here 50-μL in vitro transcription reactions were reconstituted with 5 μg of N-RNA, 1 μg (4.16 pmol) of L, and 41.6 pmol (833.5 nM) each of P<sub>N<sub>TD</sub></sub>, P<sub>N<sub>TD</sub> + CTD</sub>, or P<sub>CD</sub> + CTD</sub>, or a combination of P<sub>N<sub>TD</sub></sub> + P<sub>CD</sub> + CTD</sub>. RNA synthesis was analyzed as in Fig. S5 and graphed.