

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

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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 (P<sub>NTD</sub>) binds L and its C-terminal domain (P<sub>CTD</sub>) 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 P<sub>NTD</sub> 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 replicate 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 (P<sub>NTD</sub>), a central domain (P<sub>CD</sub>), and a C-terminal domain (P<sub>CTD</sub>) (11, 14, 15). The roles of P<sub>NTD</sub> in binding L and of P<sub>CTD</sub> 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 (N<sup>0</sup>) via the extreme N terminus of P<sub>NTD</sub>, maintaining N<sup>0</sup> 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 P<sub>CTD</sub> has shown that P<sub>CTD</sub> makes direct contact with the C-terminal lobes of two adjacent N protomers (18). Structural and biophysical analyses have demonstrated that P<sub>CD</sub> 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 N<sup>0</sup> 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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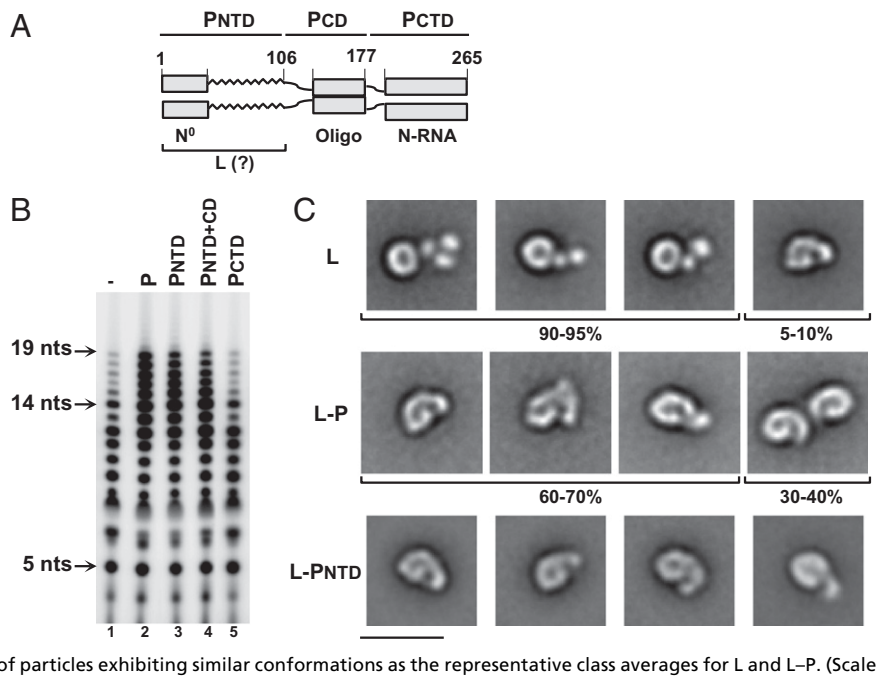
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**Fig. 1.** Effects of the N-terminal domain of P on the function and the structural organization of L. (A) Domain organization of VSV P. Schematic of a P homodimer showing the modular organization of P into three domains: N-terminal domain (P<sub>NTD</sub>, 1–106), central domain (P<sub>CD</sub>, 107–177), and C-terminal domain (P<sub>CTD</sub>, 178–265). Gray boxes depict regions of P with determined crystal structure, which were also shown to be involved in N<sup>o</sup> 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 nonencapsidated 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>CTD</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–P, and L–P<sub>NTD</sub> complexes showing 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.)



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 player in the RNA synthesis machine, influencing key aspects of polymerase structure and function.

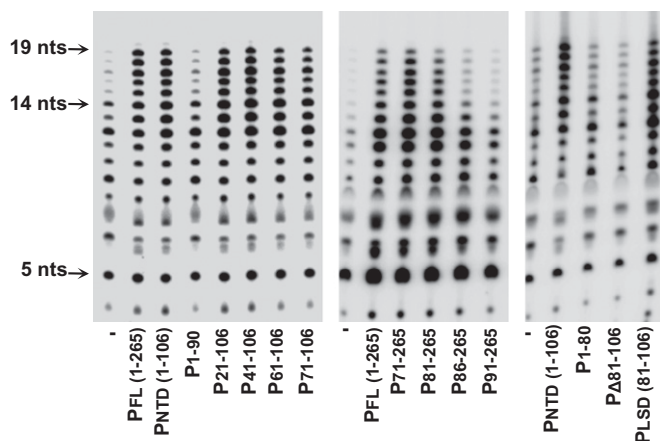
## Results

**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 3' 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>CTD</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

cap-forming activities (24). The flexibility of the appendage makes only two globular domains visible in some classes (24) (Fig. 1C, Top). In ~5–10% of particles, the appendage assumes a curved compact conformation (Fig. 1C, Top, rightmost). In the L–P complex, the flexible appendage is rearranged into a more compact conformation similar to that seen in the minor population of L alone (24) (Fig. 1C, Middle). Furthermore, a dimeric population constitutes 30–40% of the particles (Fig. 1C, Middle, rightmost). To examine the effect of P<sub>NTD</sub> on the molecular architecture of L, an L–P<sub>NTD</sub> complex was isolated using size-exclusion chromatography (Fig. S1B). EM of negatively stained L–P<sub>NTD</sub> revealed monodispersed particles (Fig. S1C); 5,842 particles were selected and classified into 20 classes (Fig. S1D). Four representative class averages (Fig. 1C, Bottom) illustrate that P<sub>NTD</sub> recapitulates the conformational rearrangement in L induced by P. The more compact structure resulting from the rearrangement of the flexible appendages of L is also reflected in the size-exclusion chromatography profile, in which L–P<sub>NTD</sub> elutes over a notably narrower range than L alone (Fig. S1B). However, in contrast to the L–P complex, the L–P<sub>NTD</sub> complex consists solely of monomers, providing evidence that the dimers observed with full-length P are mediated by its oligomerization domain. Therefore, P<sub>NTD</sub> is sufficient to alter the conformation of L.

**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. S2A 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 (PΔ81–106)



**Fig. 2.** Determining the region of  $P_{NTD}$  required for stimulation of L processivity on the Le19 template. Here  $0.2 \mu\text{M}$  P,  $P_{NTD}$ , or each of the deletion mutants of the  $P_{NTD}$  (Left) or N-terminal deletions of full-length P (Middle) were analyzed for stimulating L processivity as described in Fig. 1B. (Right) An internal deletion of residues 81–106 in full-length P ( $P_{\Delta 81-106}$ ) and a chemically synthesized peptide comprising residues 81–106 of P ( $P_{LSD}$ ) (both  $0.2 \mu\text{M}$ ) were tested as well. Quantitative analysis of two independent experiments is presented in Fig. S2B.

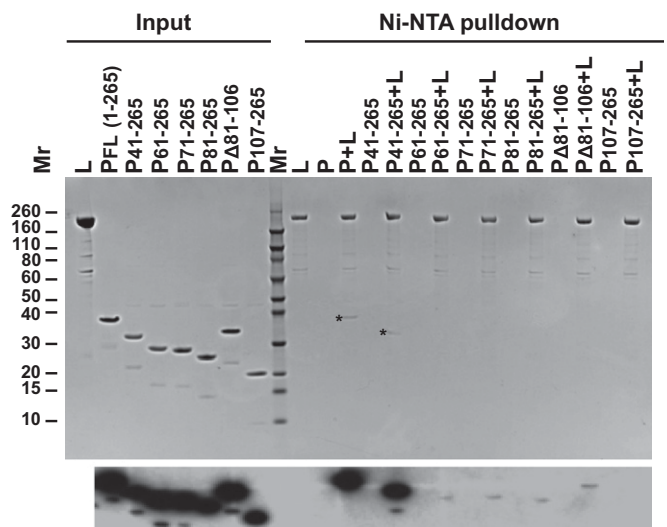
lacked any stimulatory activity, and a synthetic peptide consisting of residues 81–106 retained a stimulatory activity comparable to that of full-length P (Fig. 2, Right and Fig. S2B). We termed the P81–106 peptide  $P_{LSD}$  (for L stimulatory domain). Although  $P_{LSD}$  (81–106) stimulated the activity 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  $P_{LSD}$  to L (Fig. 3).

**L Binding Encompasses the Region Between the  $N^0$ -Binding Domain and the Oligomerization Domain of P.** Earlier work determined that  $P_{NTD}$  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  $P_{NTD}$  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  $P_{NTD}$  ( $P_{107-265}$ ) completely abolished binding. Deletion of the  $N^0$ -binding domain of P ( $P_{41-265}$ ) had no effect, whereas larger N-terminal deletions ( $P_{61-265}$ ,  $P_{71-265}$ , and  $P_{81-265}$ ) significantly decreased P binding to L. Consistent with the critical nature of the  $P_{LSD}$  in L stimulation, an internal deletion ( $P_{\Delta 81-106}$ ) also significantly decreased L binding. These results indicate that whereas  $P_{LSD}$  is sufficient for stimulation of L processivity on Le19, the L-binding region on P spans a larger interval between the  $N^0$ -binding domain and the oligomerization domain.

**$P_{NTD}$  Region Required to Induce Conformational Rearrangement in L.** We next used EM to examine the contribution of elements of  $P_{NTD}$  to the observed structural rearrangement in L. To do this, we mixed L with  $P_{NTD}$  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  $N^0$ -binding domain of  $P_{NTD}$  (P 41–106) retained the ability to induce the conformational rearrangement in L to a similar extent as  $P_{NTD}$ . Further deletions in the  $P_{NTD}$  ( $P_{61-106}$  and  $P_{81-106}$ ) decreased the population of L exhibiting

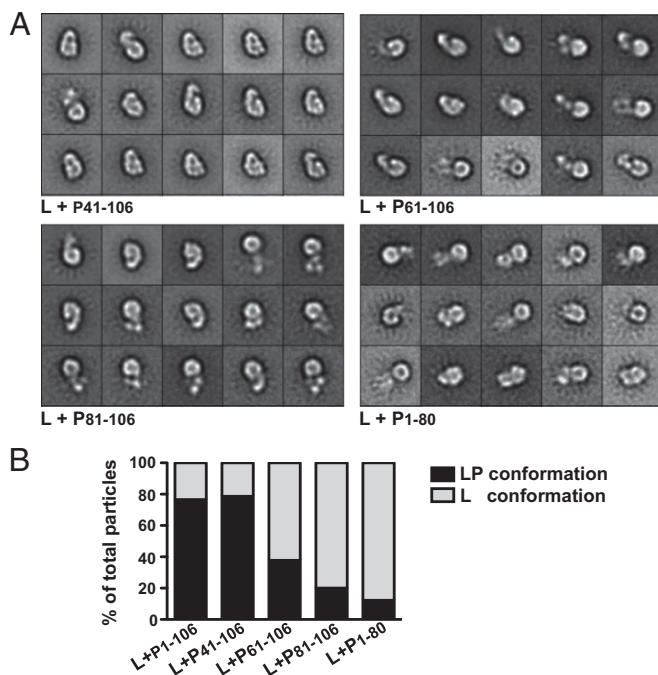
a conformational rearrangement. Similarly, deletion of  $P_{LSD}$  (P1–80) inhibited the conformational rearrangement of L, and this inhibition could not be rescued by supplementation of  $P_{LSD}$  (P1–80 + P81–106) (Fig. S4). Collectively, these results indicate that the entire L-binding region of 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  $P_{NTD}$ .** 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  $P_{NTD}$  in the context of full-length P, because  $P_{CTD}$  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 ( $P_R$ ) remained bound, which we estimated as  $\sim 16.67 \text{ nM}$  (Fig. S5A).  $P_R$  resulted in a basal level of transcription that was stimulated up to 8- to 10-fold by  $333.4 \text{ nM}$  exogenous purified P (Fig. S5B). We tested each of the P deletion mutants at a saturating concentration of  $1,667 \text{ nM}$  (Fig. 5A). Deletion of the  $N^0$ -binding domain ( $P_{41-265}$ ) 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 ( $P_{61-265}$ ,  $P_{71-265}$ , and  $P_{81-265}$ ) 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  $P_{LSD}$  ( $P_{\Delta 81-106}$ ) resulted in further decrease in stimulation, whereas deletion of the entire  $P_{NTD}$  ( $P_{107-265}$ ) served as a dominant negative, inhibiting the basal levels of transcription resulting from  $P_R$ . This likely reflects the ability of  $P_{107-265}$  to oligomerize with  $P_R$  or to compete it off the template. The



**Fig. 3.** Delineating the region of P involved in L binding. 6xHis-tagged L was mixed with a fourfold molar excess of full-length P or with each of the N-terminal or internal deletions of P. The complexes were precipitated using Ni-NTA agarose beads, separated on 4–12% SDS/PAGE, and visualized by Coomassie blue staining (precipitated P proteins indicated by \*) (Upper) or evaluated by Western blot analysis using a polyclonal antibody against P (Lower). For Western blot analysis, 20% of the input was loaded. FL, full-length. Quantitative analysis of this experiment is presented in Fig. S3B.





**Fig. 4.** EM characterization of the effect of  $P_{\text{NTD}}$  deletion mutants in inducing a conformational rearrangement in L. (A) L was mixed with each  $P_{\text{NTD}}$  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. 53) 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.

consequences of the deletion of elements of the  $P_{\text{NTD}}$  on stimulation of RNA synthesis are correlated with the effect of these deletions on L binding and their ability to induce a conformational rearrangement in L.

**P-Induced Conformational Change in L Stimulates Transcription Independent of P Oligomerization.** To further separate the impacts of conformational change and oligomerization status of P on RdRP function, we tested the effect of the isolated  $P_{\text{NTD}}$  (P1–106) on transcription levels (Fig. 5B and Fig. S5B). P1–106 stimulated the basal level of transcription resulting from  $P_{\text{R}}$  by fourfold to fivefold at concentrations ranging of 333.4–1,667 nM (Fig. S5B). This stimulatory effect is likely reflective of a dynamic exchange of L between  $P_{\text{NTD}}$  and full-length P, where high concentrations of  $P_{\text{NTD}}$  stimulate the RdRP that is already engaged with the *N*-RNA via full-length P. Consistent with this idea, no further stimulation by  $P_{\text{NTD}}$  was observed in the presence of optimal amounts of exogenous P (Fig. 5B). Furthermore,  $P_{\text{NTD}}$  stimulated transcription between fourfold and fivefold in the presence of 333.4 nM of P107–265. Because P107–265 contains the oligomerization domain but lacks the L-binding domain, the conformational change induced in L by the isolated  $P_{\text{NTD}}$  is sufficient to produce the enhanced activity. Fragments of P that were insufficient to induce the conformational change of L (P1–80,  $P_{\text{LSD}}$ , or a combination of the two) had no effect on transcription in the presence of  $P_{\text{R}}$ , exogenous P, or P107–265 (Fig. 5B). Furthermore, P1–80 and  $P_{\text{LSD}}$  failed to complement the corresponding deletion mutants of full-length P (P81–265 and PΔ81–106, respectively). These data indicate that the stimulatory effect of P on L function in transcription is independent of the oligomeric status of P. Consistent with this interpretation,  $P_{\text{NTD}+\text{CD}}$  stimulated basal transcription to a similar degree as  $P_{\text{NTD}}$ , and this stimulation was not enhanced by the addition

of  $P_{\text{CTD}}$  or  $P_{\text{CD}+\text{CTD}}$  (Fig. S6). Collectively, these results demonstrate that  $P_{\text{NTD}}$ , a domain that is sufficient for inducing a conformational rearrangement in L, can stimulate L-mediated transcription of an encapsidated RNA template.

## 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  $P_{\text{NTD}}$ . 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 ( $P_{\text{LSD}}$ , 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  $\alpha$ -helical region at residues 79–100. These analyses suggest that  $P_{\text{LSD}}$  (P81–106) is likely a structured domain of  $P_{\text{NTD}}$ .

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  $P_{\text{NTD}}$  as a chaperone for  $N^0$  and as a binding site for L. The atomic structure of the  $N^0$ -P1–60 complex reveals that P residues 6–40 form a molecular recognition element that engages  $N^0$ , 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  $N^0$ -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  $N^0$  during replication. Whether a single  $P_{\text{NTD}}$  can simultaneously bind  $N^0$  and L remains unknown; however, the close proximity of the two binding regions makes such a scenario unlikely. Perhaps this need for  $P_{\text{NTD}}$  to interact with two molecular partners explains one of the requirements for oligomerization of P, with one  $P_{\text{NTD}}$  binding L and a second  $P_{\text{NTD}}$  binding the  $N^0$  necessary for encapsidation of the nascent RNA during replication.

The prediction that P81–106 is a structured domain is supported by the demonstration that  $P_{\text{LSD}}$  (P81–106) as a discrete domain stimulates processivity of L on the naked RNA template. Independent folding of  $P_{\text{LSD}}$  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  $P_{\text{LSD}}$ , suggests that the binding of P41–106 to L is likely a cooperative mechanism involving the IDR (P41–80) and  $P_{\text{LSD}}$ . Such a mechanism is also consistent with the failure to restore the conformational change and the



Le19, the conformational rearrangement has no effect on RNA synthesis, because these levels of control are irrelevant. Therefore, this independent role of P<sub>LS</sub>D in stimulating RNA synthesis on Le19 might reflect an evolutionary pathway in which a processivity factor sufficient to stimulate polymerase activity on naked 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.** P<sub>LS</sub>D (AEQVEGFIQGLDDYADEVDVVFST) 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% (wt/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 RNeasy (Qiagen), separated by acid-agarose urea gel electrophoresis, and analyzed with a PhosphorImager.

**EM and Image Processing.** L (0.14 μM) was mixed with P<sub>NTD</sub> deletions (1.4 μM) in buffer containing 50 mM Tris-HCl (pH 7.4), 280 mM NaCl, 3% (wt/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% (wt/vol) uranyl formate as described previously (30). Images were collected and processed as described in detail in *SI Materials and Methods*.

**Ni-Nitrilotriacetic Acid Pulldown Assay.** For this assay, 3 μg (12.5 pmol) of His-tagged L was incubated with 50 pmol of untagged P or P deletions, and the formed complex was pulled down by Ni-nitrilotriacetic acid (NTA) agarose, as described in *SI Materials and Methods*. The precipitated proteins were separated on 4–12% (wt/vol) SDS/PAGE gels and visualized by Coomassie blue staining or transferred to a nitrocellulose membrane for Western blot analysis with an anti-P antibody, as described in *SI Materials and Methods*.

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