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An *In Vitro* RNA Synthesis Assay for Rabies Virus Defines Ribonucleoprotein Interactions Critical for Polymerase Activity

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ABSTRACT We report an *in vitro* RNA synthesis assay for the RNA-dependent RNA polymerase (RdRP) of rabies virus (RABV). We expressed RABV large polymerase protein (L) in insect cells from a recombinant baculovirus vector and the phosphoprotein cofactor (P) in *Escherichia coli* and purified the resulting proteins by affinity and size exclusion chromatography. Using chemically synthesized short RNA corresponding to the first 19 nucleotides (nt) of the rabies virus genome, we demonstrate that L alone initiates synthesis on naked RNA and that P serves to enhance the initiation and processivity of the RdRP. The L-P complex lacks full processivity, which we interpret to reflect the lack of the viral nucleocapsid protein (N) on the template. Using this assay, we define the requirements in P for stimulation of RdRP activity as residues 11 to 50 of P and formally demonstrate that ribavirin triphosphate (RTP) inhibits the RdRP. By comparing the properties of RABV RdRP with those of the related rhabdovirus, vesicular stomatitis virus (VSV), we demonstrate that both polymerases can copy the heterologous promoter sequence. The requirements for engagement of the N-RNA template of VSV by its polymerase are provided by the C-terminal domain (CTD) of P. A chimeric RABV P protein in which the oligomerization domain (OD) and the CTD were replaced by those of VSV P stimulated RABV RdRP activity on naked RNA but was insufficient to permit initiation on the VSV N-RNA template. This result implies that interactions between L and the template N are also required for initiation of RNA synthesis, extending our knowledge of ribonucleoprotein interactions that are critical for gene expression.

IMPORTANCE The current understanding of the structural and functional significance of the components of the rabies virus replication machinery is incomplete. Although structures are available for the nucleocapsid protein in complex with RNA, and also for portions of P, information on both the structure and function of the L protein is lacking. This study reports the expression and purification of the full-length L protein of RABV and the characterization of its RdRP activity *in vitro*. The study provides a new assay that has utility for screening inhibitors and understanding their mechanisms of action, as well as defining new interactions that are required for RdRP activity.

KEYWORDS *in vitro*, phosphoprotein, polymerase, rabies, ribavirin

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Rabies virus (RABV), a nonsegmented negative-strand (NNS) RNA virus in the family *Rhabdoviridae*, causes lethal infection of humans and mammals resulting in an estimated 55,000 deaths per year (1, 2). Vaccination protects against infection, even when administered up to 48 h postexposure, but costs limit access, particularly in Asia

and Africa. Antiviral therapy may complement existing vaccination strategies, although at present there are no RABV-specific antivirals.

The replicative machinery of NNS viruses comprises a ribonucleoprotein (RNP) template of the genomic RNA completely coated by the viral nucleoprotein (N). This RNP serves as the template for transcription and replication by the polymerase complex (3–7). For RABV, each molecule of N encapsidates 9 nucleotides (nt) of RNA, and each N molecule tightly interacts with its neighbors to provide a sheath of protein around the RNA (3). During copying of this template, the polymerase complex must transiently release the RNA. For RABV, the viral components of the polymerase complex are a 242-kDa large protein (L) and a 33-kDa phosphoprotein cofactor (P). L contains all the enzymatic activities necessary for transcription, including an RNA-dependent RNA polymerase (RdRP) and an unconventional set of mRNA capping enzymes—a GDP polyribonucleotidyltransferase (PRNTase) and a dual-specificity mRNA cap methylase (MTase) (8–13). This replication machinery is replete with possible targets for antiviral intervention.

Structural studies of vesicular stomatitis virus (VSV) L show that its RdRP domain is intimately associated with the PRNTase domain, which is separated from the MTase domain and a further structural C-terminal domain (CTD) by a connector domain (14, 15). Complex formation between VSV L and P compacts the connector, MTase, and CTD of L and enhances the activity of the RdRP domain (14–16). Like VSV P, RABV P comprises three domains: a C-terminal domain (P_{CTD}) that binds the genomic N-RNA, a central oligomerization domain (P_{OD}), and an N-terminal domain (P_{NTD}) that binds L (5, 17–19). During replication of the template, the P_{NTD} also engages N to keep it soluble (N^0) for loading onto the nascent RNA chain concomitant with its synthesis by L (18, 20–27).

Approaches to studying NNS RNA virus polymerase activity *in vitro* were established by the discovery of an RdRP in VSV particles (28). Subsequent reconstitution of RNA synthesis with N-RNA and separately isolated L and P from purified particles defined L as the RdRP and revealed a role for P in polymerase processivity (29). The adaptation of this approach to other NNS RNA viruses was restricted by the difficulties in obtaining sufficient quantities of N-RNA templates and purified polymerase. We therefore established a different RdRP assay for VSV that overcomes those limitations by reconstituting synthesis *in vitro* with recombinant L and a chemically synthesized RNA corresponding to the first 19 nt of the VSV genome (16). Using that assay, we defined a stimulatory role for P in RNA synthesis (16, 30) and demonstrated that the template N is required for RdRP processivity and correct recognition of transcription termination signals (16). We also demonstrated that 2′-substituted nucleoside triphosphates can inhibit RNA synthesis *in vitro* (16, 30, 31). The versatility of this approach was further exemplified by its adaptation to the study of the paramyxovirus human respiratory syncytial virus and identification of the mechanism by which a CTP analog blocks viral replication (32).

In the present study, we adapted this *in vitro* RNA synthesis assay to RABV. We demonstrate that, like VSV, RABV L copies naked RNA and that RABV P stimulates RdRP activity. The stimulatory activity of P depends upon specific sequences within its NTD that cannot be provided by the equivalent region of VSV P. Both VSV and RABV L, however, can initiate and copy the heterologous RNA templates, demonstrating the importance of the cognate proteins in regulating template function. We further tested the importance of the requirements for homologous proteins by generating a chimeric P protein that contains the L binding domain (P_{NTD}) of RABV and the oligomerization and N-RNA binding domains (P_{OD} and P_{CTD}) of VSV. This chimeric P provides the stimulatory activity to RABV L in copying naked RNA *in vitro* but is insufficient to allow copying of the VSV N-RNA. This work extends to the study of RABV polymerase a powerful *in vitro* assay that may aid in the development of inhibitors that target polymerase and uncovers a previously unappreciated interaction between the polymerase and the template-associated N protein required for initiation.

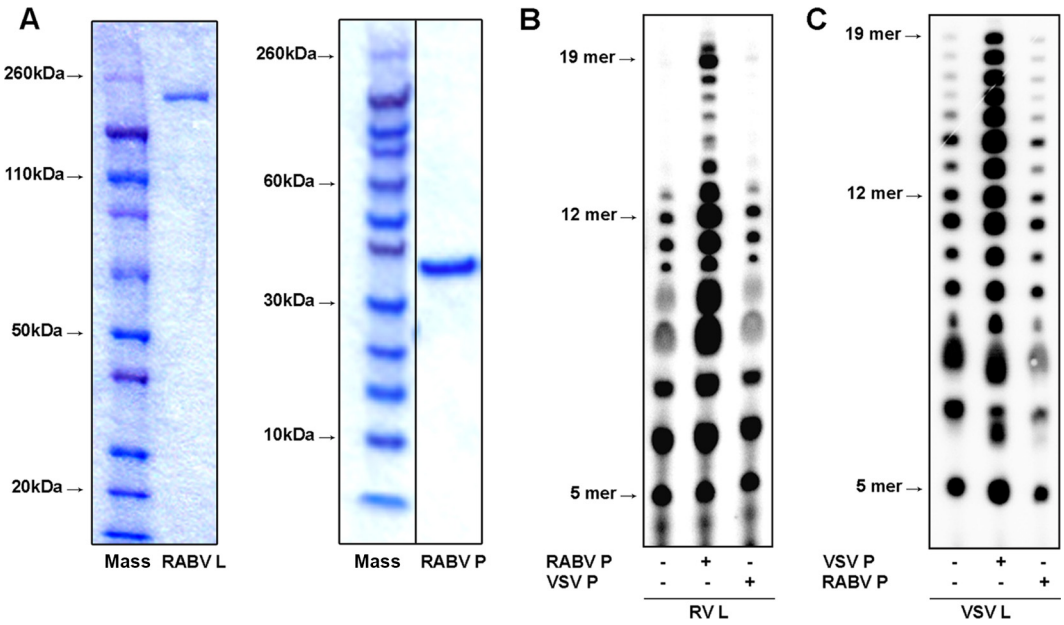

FIG 1 Production and activity of RABV polymerase complex. (A) Purification of RABV polymerase complex elements. L (left) and P (right) were individually expressed in insect cells and bacteria, respectively. Both proteins were purified as described in Materials and Methods and analyzed by SDS-PAGE. Intervening lanes were removed from the gel, as indicated by the solid black line. (B) *De novo* RNA synthesis by RABV L on a RABV Le19 RNA template in the absence or presence of either RABV P or VSV P. The reactions were set up in the presence of [α-^{32}P]GTP, quenched by the addition of EDTA/formamide, and analyzed on a 20% polyacrylamide-7 M urea gel. *De novo* initiation product sizes are indicated on the left. (C) *De novo* RNA synthesis by VSV L on a VSV Le19 RNA template in the absence or presence of either VSV P or RABV P; the products were analyzed as for panel B.

RESULTS

Establishment and characterization of a minimal *in vitro* RNA synthesis assay for RABV. To establish a minimal *in vitro* RNA synthesis assay for RABV, we expressed RABV L and P independently in insect cells and bacteria, respectively, and purified them by Ni-nitrilotriacetic acid (NTA) affinity, followed by size exclusion chromatography (Fig. 1A). Using an approach that we first developed for VSV (16), we showed that RABV L has RdRP activity on a nonencapsidated RNA corresponding to the first 19 nt of the 3′ end of the RABV genome (RABV Le19) (Fig. 1B). This result demonstrates that RABV L synthesizes RNA *in vitro*, although the RdRP is poorly processive. In the presence of RABV P, the RNA polymerase activity and processivity of L are stimulated (Fig. 1B), consistent with our earlier findings for VSV (16). To determine whether the stimulation of the RdRP by P requires specific interactions between homologous L and P, we compared the abilities of VSV P to stimulate RABV L and *vice versa*. Only homologous combinations enhance the RdRP activity of L (Fig. 1B and C), consistent with the need for specific protein-protein interactions between P and L.

Identification of a minimal RdRP-stimulating portion of P. Earlier work demonstrated that residues 1 to 19 of RABV P were sufficient to detect L binding but residues 1 to 52 were required to stabilize the association (21). For VSV P, the L binding domain resides within the P_{NTD}, and full binding and stimulation map to residues 41 to 106 (30). To further delineate the requirements within RABV P for L binding and stimulation of RdRP activity, we generated 6 deletion mutants within the NTD: P_{1-91}, P_{21-91}, P_{11-91}, P_{1-40}, P_{1-50}, and P_{11-50} (Fig. 2A and B). In addition to including the known L binding region, some of the mutants of P extend up to the oligomerization domain and thus encompass the equivalent region from VSV P required to stimulate the RdRP activity of VSV L. RABV P_{1-91} and P_{11-91} stimulate the RdRP activity of RABV L almost as efficiently as full-length P, whereas P_{21-91} had no detectable stimulatory effect (Fig. 2C). This result suggests that residues 11 to 21 of RABV P play a critical role in L binding. Truncating the C terminus of the P fragment demonstrated that P_{1-40} failed to stimulate the RdRP

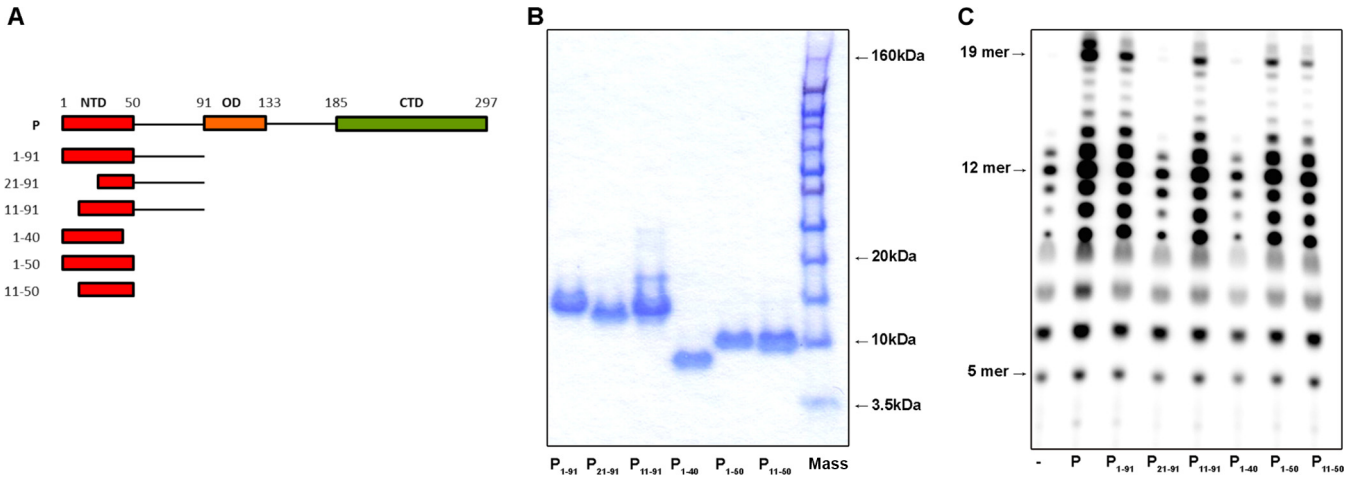

FIG 2 Identification of the minimal P L-enhancing domain. (A) Schematic diagram of the various deletions in the rabies virus P protein to indicate which portions of the molecule remain in the various constructs. (B) SDS-PAGE showing the purification of RABV $P_{1–91}$, $P_{21–91}$, $P_{11–91}$, $P_{1–40}$, $P_{1–50}$, and $P_{11–50}$. All the P proteins were individually expressed in bacteria and purified as described in Materials and Methods. (C) *De novo* RNA synthesis by RABV L on a RABV Le19 RNA template in the absence or presence of either RABV P or RABV $P_{1–91}$, $P_{21–91}$, $P_{11–91}$, $P_{1–40}$, $P_{1–50}$, or $P_{11–50}$. The products were resolved on 20% polyacrylamide-7 M urea gels and detected with a phosphorimager.

activity of L, whereas $P_{1–50}$ stimulated RdRP activity (Fig. 2). This result suggests that residues 40 to 50 are critical for RdRP stimulation. We therefore generated $P_{11–50}$, which enhances L polymerase activity and defines a 40-residue sequence of P that is sufficient for L binding and RdRP stimulation. This result extends earlier work that defined residues 1 to 52 of P as required for L binding (21) by demonstrating that residues 11 to 50 are sufficient for both binding and stimulation of RdRP activity.

Template specificity of the RABV polymerase complex. We next investigated the sequence properties of the template in the RNA synthesis activity of L. To do this, we compared the activities of RABV L in copying chemically synthesized RNA corresponding in sequence to the first 19 nt of the RABV and VSV genomes. We reported previously that the first 3 nt of the VSV template are essential for VSV L to initiate RNA synthesis (16). Those 3 nt are conserved between the RABV and VSV templates. Consistent with the conservation of this critical initiation sequence, RABV L copies the VSV template (Fig. 3A). This result highlights the importance of the sequence of the first 3 nt in RNA synthesis and shows that sequence differences beyond position 3 reduce RNA synthesis by 50%. The products of RNA synthesis from each template reveal a specific pattern (Fig. 3B) showing an accumulation of products predominantly around positions 5 and 6, 12, and 19 in copying the RABV template and 5, 11, and 17 to 19 in copying the VSV template. Those positions in the respective templates likely correspond to residues at which incorporation of the relevant NTP or subsequent elongation are less efficient. The VSV L-P complex exhibits a similar 50% reduction during synthesis from a RABV RNA template (Fig. 4A) and exhibits a similar accumulation of products from the RABV template (Fig. 4B).

Critical role of homologous template N in RNA synthesis. During transcription, VSV P binds L via the P_{NTD} and the N-RNA via the P_{CTD} to bridge the interaction between the polymerase and its template. We therefore designed a chimeric RABV P comprising the N-terminal 91 amino acids (aa) of RABV P together with the oligomerization domain and CTD of VSV P, residues 107 to 265 (Fig. 5A). This chimeric P should contain the L-RNA binding and *in vitro* RdRP-stimulating activities of RABV L and the N-RNA template binding activities of VSV. Although this chimeric P stimulates full RABV L RdRP activity on naked RNA *in vitro* (Fig. 5B), we could not detect any synthesis from the VSV N-RNA template despite the presence of sequence elements at the 3′ end of the genome that can serve as the template for RABV L. This result suggests that further protein-protein recognition events, likely involving the template N and the P_{NTD} and/or L, are required to permit the RdRP domain to access the 3′ end of the template.

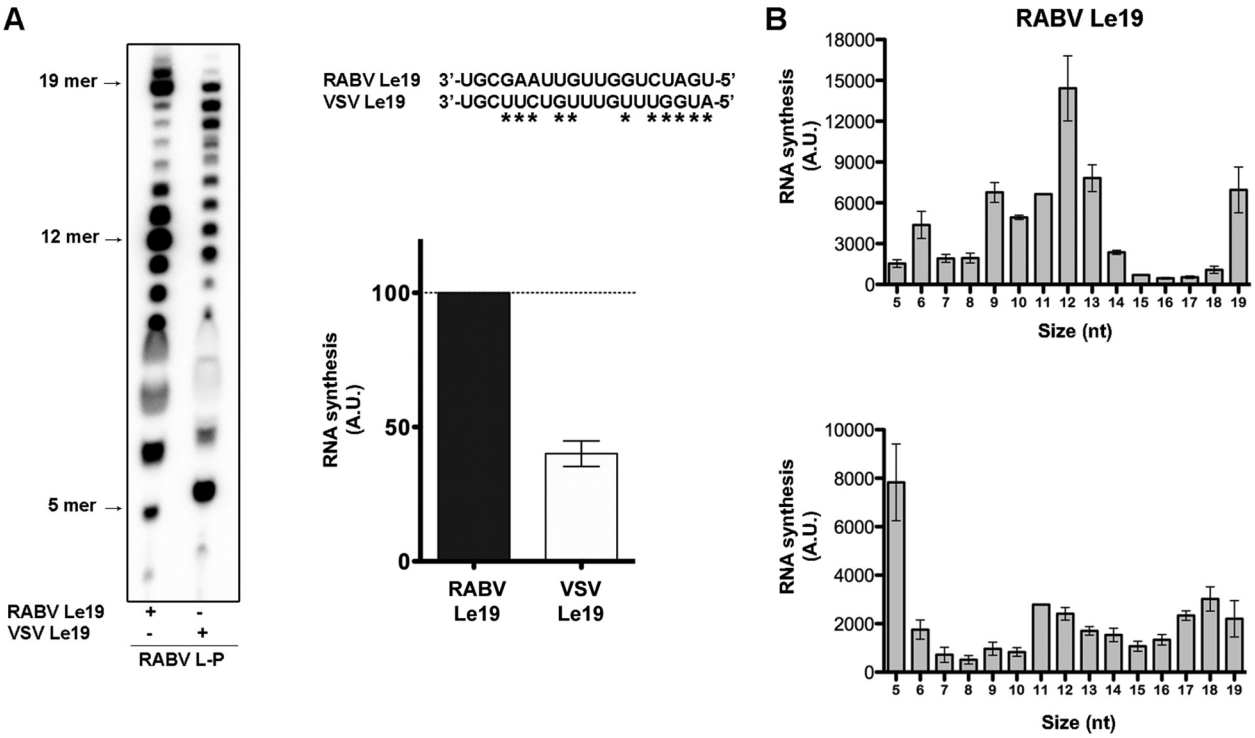

FIG 3 Template specificity for RNA synthesis by RABV L-P. (A) *De novo* RNA synthesis by RABV L-P on RABV Le19 and VSV Le19 RNA templates. (Left) The products were resolved on a 20% polyacrylamide-7 M urea gel and detected with a phosphorimager, and a representative gel is shown. (Top right) Sequences of both RNAs, with the asterisks indicating nucleotides not conserved between the two templates. (Bottom right) Comparison of total RNA synthesis by RABV L-P on RABV Le19 and VSV Le19. Total RNA synthesis was quantified, normalized to levels of RNA synthesis on RABV Le19, and graphed. The error bars represent the standard deviations of the mean of the results of three independent experiments. A.U., arbitrary units. (B) Individual reaction products of different sizes synthesized for panel A for RABV Le19 (top) and VSV Le19 (bottom) were quantified and normalized to the size of the 11-mer. The error bars represent the standard deviations of the mean of the results of three independent experiments.

Effect of RTP on RABV L RNA synthesis activity. The broad-spectrum RNA virus inhibitor ribavirin has multiple mechanisms of inhibition, including suppression of polymerase activity, conversion into ribavirin triphosphate (RTP) and subsequent incorporation (33), and suppression of intracellular GTP pools through targeting of IMPDH (34). Using our *in vitro* assay, we tested the effect of RTP on RABV RdRP. In the presence of RTP, RABV L-P show inhibition of RdRP activity (Fig. 6A). The approximate 50% inhibitory concentration (IC_{50}) for RTP was 1 mM (Fig. 6B), suggesting that direct inhibition of polymerase activity would be difficult to achieve *in vivo*. Nevertheless, these data provide proof of principle that this assay can be used for the discovery and analysis of the effects of new inhibitors of the RABV polymerase.

DISCUSSION

The principal breakthrough reported here is the establishment of an *in vitro* RNA synthesis assay for RABV. This *in vitro* assay for RABV RdRP extends previous work showing that a minimal RNA synthesis functional unit for NNS RNA viruses comprises a genomic RNA and L (16). A similar assay was subsequently reported for human respiratory syncytial virus (hRSV) (35). The abilities of the respective polymerases to copy naked RNA are consistent with the expectation that the N protein must be displaced during copying of the N-RNA template. For VSV, available evidence supports a model in which that displacement is transient because the RNA template remains resistant to nuclease attack during polymerase copying. Structural data on N-RNA from VSV, RABV, and RSV are also consistent with the need to dissociate the RNA from N during copying (3, 4, 7). The extensive nature of N-N interactions in the N-RNA are also incompatible with a model in which the N protein would completely dissociate during template copying.

In contrast to the assays established for RABV and VSV, hRSV L production depends

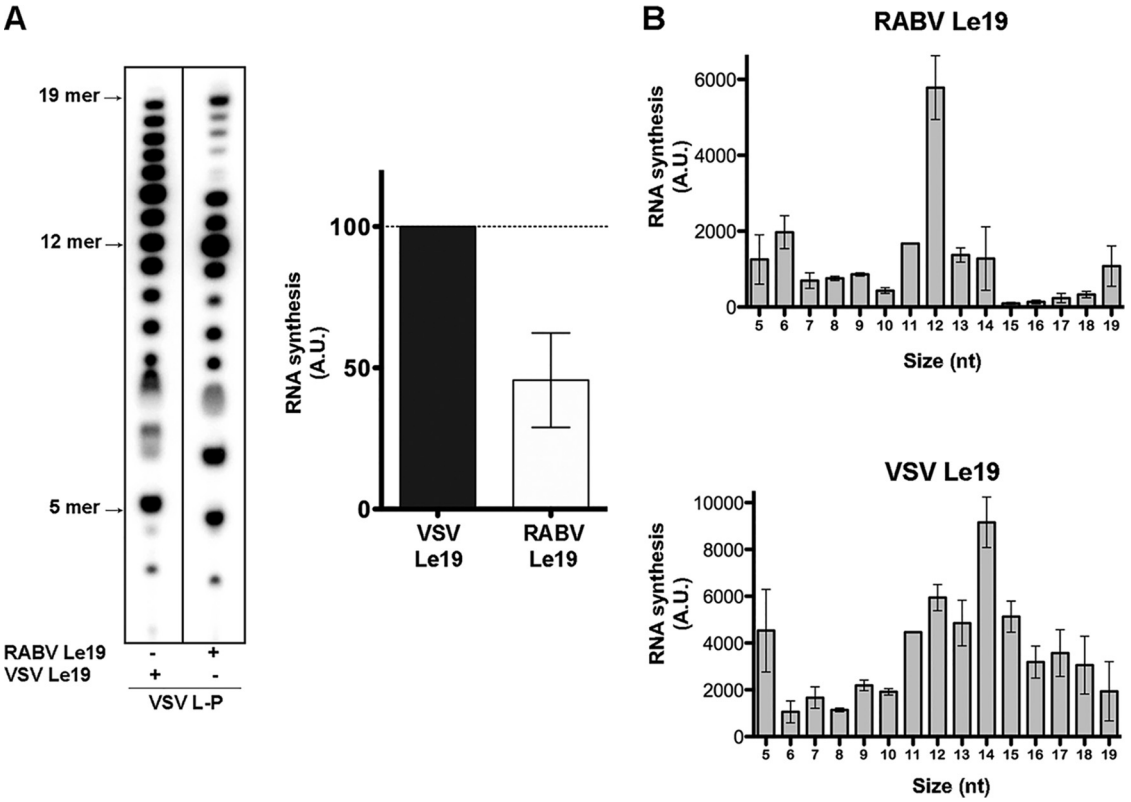

FIG 4 Template specificity for RNA synthesis by VSV L-P. (A) *De novo* RNA synthesis by VSV L-P on VSV Le19 and RABV Le19 RNA templates. (Left) The products were resolved on a 20% polyacrylamide-7 M urea gel and detected with a phosphorimager, and a representative gel is shown. Intervening lanes were removed from the gel, as indicated by the solid black line. (Right) Comparison of total RNA synthesis by VSV L-P on VSV Le19 and RABV Le19. Total RNA synthesis was quantified, normalized to levels of RNA synthesis on VSV Le19, and graphed. The error bars represent the standard deviations of the mean of the results of three independent experiments. (B) Individual reaction products of different sizes synthesized for panel A for RABV Le19 (top) and VSV Le19 (bottom) were quantified and normalized to the size of the 11-mer. The error bars represent the standard deviations of the mean of the results of three independent experiments.

on coexpression of its cofactor, P (35). For some NNS RNA viruses, including VSV, P stabilizes L (36). Perhaps the requirement for coexpression of hRSV P and L reflects reduced stability of hRSV L alone compared to RABV and VSV L proteins. One advantage of the production of L and P separately is the ability to definitively measure the effects of P on the RNA synthesis activity of L. We previously reported that VSV P enhances RdRP activity independently of its function in recruiting L to the N-RNA template (30). Here, we show that RABV P has a similar stimulating effect on RABV L, suggesting that this stimulatory function of P is conserved—at least among rhabdoviruses (Fig. 1). The residues required for this stimulation are not conserved between RABV and VSV P, and the two P molecules cannot substitute for each other to stimulate L (Fig. 1B and C), underscoring the specificity of this interaction.

We recently reported the 3.8-Å structure of VSV L in complex with a portion of its P protein, residues 35 to 106 (14). In that structure, the density of P was not clearly resolved. Density that likely corresponds to P was, however, observed in portions of the molecule between the connector domain and the capping domain, as well as between the connector and the L_{CTD} and MTase domains. Earlier negative-stain electron microscopy (EM) studies also demonstrated that $P_{41–106}$ compacts those globular domains onto the RdRP domain (30). This observation further supports the notion that the density seen between the domains likely corresponds to P or a P-mediated arrangement of flexible linkers between the domains of L. The lack of obvious sequence conservation in the RABV and VSV P_{NTD} and the corresponding lack of obvious sequence conservation in the linkers that connect the various domains of L are consistent with the inability of the two P fragments to functionally replace one another.

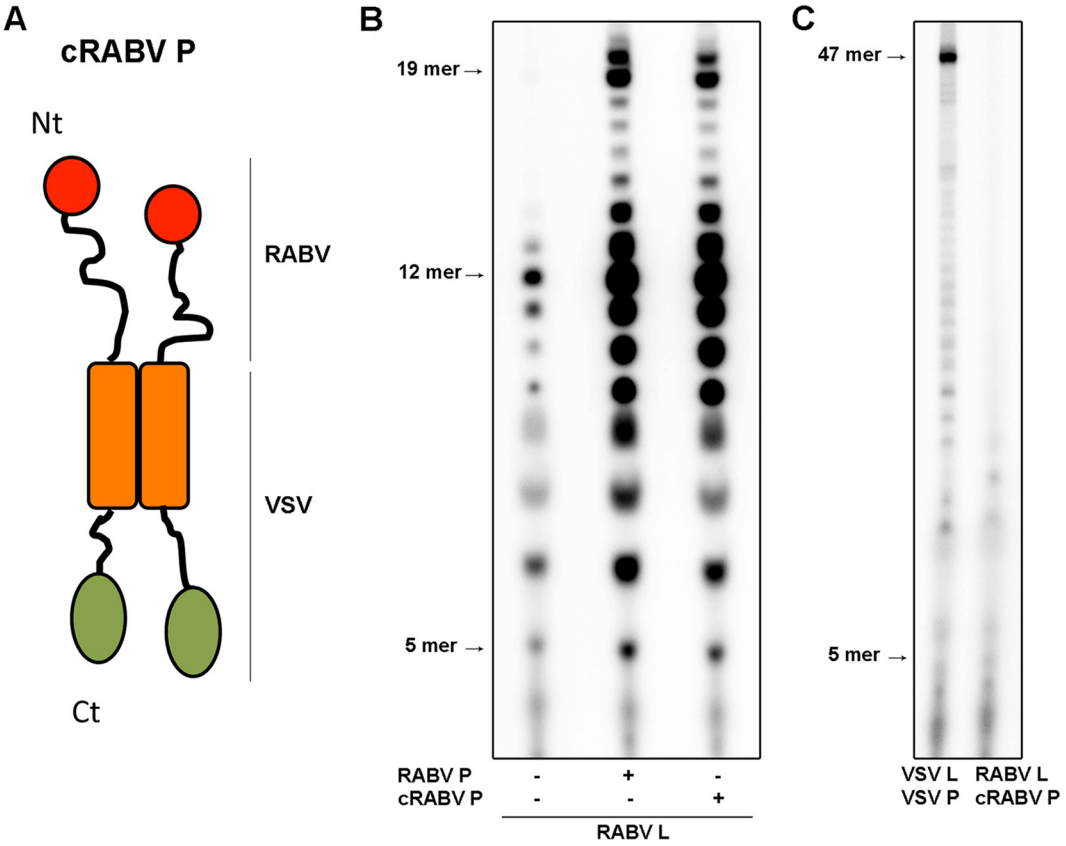

FIG 5 Activity of RABV L on VSV L N-RNA. (A) Schematic representation showing the structure of chimeric cRABV P composed of three major domains separated by flexible linkers (lines). The cRABV P contains the N^0- and L-binding regions of the RABV PNTD (red), and the VSV POD (orange) and PCTD (green) required for binding the VSV N-RNA. The viruses from which the domains come are indicated on the right. (B) *De novo* RNA synthesis by RABV L on a RABV Le19 RNA template in the absence or presence of either RABV P or cRABV P. The products were resolved on a 20% polyacrylamide-7 M urea gel and detected with a phosphorimager, and a representative gel is shown. (C) RNA synthesis by either VSV L-P or RABV L/cRABV P on the genomic VSV N-RNA analyzed as for panel B.

Using sequential deletions at the N- and C-terminal ends of the N-terminal domain of RABV P (aa 1 to 91), a minimal L-stimulating domain of P was defined as residues 11 to 50 (Fig. 2B). This result extends an earlier study that mapped a major L binding domain of P to residues 1 to 19, with residues 19 to 52 stabilizing the complex (21). Although the main L binding region of P resides within the first 19 residues, our results show that the domain itself is not sufficient to stimulate L, as P_{1-40} does not enhance RdRP activity (Fig. 2B). Collectively, these data suggest that the L binding and L-stimulating domains of RABV P require different but closely positioned regions of P, as RABV L required P_{1-19} for binding and P_{11-50} for stimulation. This situation is similar to VSV L, where P_{41-106} binds and induces a structural rearrangement and full stimulation of RdRP activity, whereas shorter fragments, including P_{81-106} still retain some stimulatory activity (30). In both VSV and RABV, the P_{NTD} thus plays multiple roles during RNA synthesis; it binds L (30), stimulates RdRP activity (16), and also engages N^0 for encapsidation (19). The oligomeric nature of P likely aids in this multifunctional role of the P_{NTD} by obviating the need for any single molecule of P to simultaneously accomplish all 3 tasks. Additional structural studies will likely be required to resolve the precise role of the P_{NTD} in RNA synthesis.

The development of the *in vitro* RNA synthesis assay for RABV provides insights into the functional interaction of the L protein with promoter sequences. RABV L-P can initiate RNA synthesis on the VSV Le19 template, reinforcing our previous work on VSV showing that the first 3 nucleotides at the 3′ end of the template are essential for correct initiation of RNA synthesis (Fig. 3A) (16). Sequences from position 4 clearly also

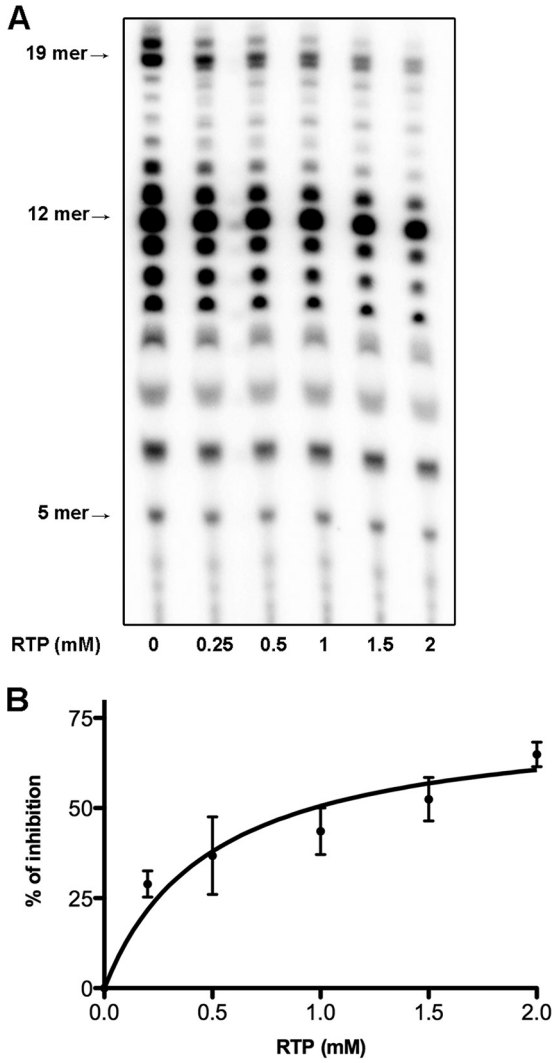

FIG 6 Effects of RTP on RABV L-P RNA synthesis. (A) Inhibitory effects of increasing RTP concentrations on *de novo* RABV L-P RNA synthesis. The products of RNA synthesis reactions were resolved on a 20% polyacrylamide-7 M urea gel and detected with a phosphorimager, and a representative gel is shown. (B) Total amounts of products were quantified, and the percentages of RABV L-P RNA synthesis inhibition by the different RTP concentrations are shown. The error bars represent the standard deviations of the mean of the results of independent experiments.

influence RdRP activity, since RABV L exhibits only 50% activity on the VSV template, and *vice versa*. We do not know the roles of the specific nucleotides in the template in affecting initiation or whether they are required in the template strand, nascent strand, or both. A detailed understanding will likely await further structural studies of L in complex with the naked RNA template.

The development of a fully processive RdRP assay has proved challenging because of the difficulties in obtaining significant quantities of pure N-RNA templates of RABV. Combined with the fact that RABV L can initiate on the sequence 3′-UGC, we examined whether RABV L could use the VSV N-RNA template by testing a chimeric P protein (cRABV P) that harbors the binding domain of RABV L and VSV N-RNA to test RABV RNA synthesis on an encapsidated template. The inability of RABV L to copy the VSV N-RNA (Fig. 5) showed that providing a simple bridge for the L-P complex to access a noncognate N-RNA template is not sufficient to allow the polymerase to copy the encapsidated genome, suggesting that another interaction is required. There are two possible explanations for the inability of the chimeric P to allow copying of the VSV N-RNA by RABV L. Direct interactions between the template N and L proteins may be

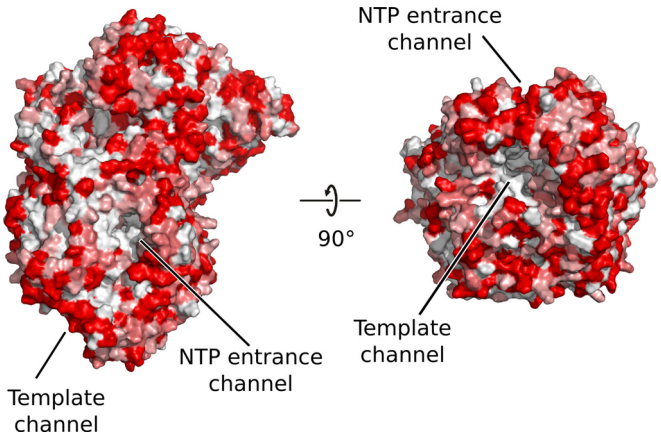

FIG 7 Comparison of VSV and RABV L proteins. The differences between VSV and RABV L sequences were mapped on the surface representation of VSV L structure (14). The sequences of VSV strain Indiana and RABV L proteins were aligned with the MAFFT algorithm (38). Identical residues (white), residues with similar chemical properties (pink), and different residues (red) are shown on the VSV L surface representations.

required to facilitate initiation and to maintain the L protein on the N-RNA in a "transcription bubble" in which the chain of N passes around L. Specific interactions between the P_{NTD} and the template N protein may also be required to allow initiation by L. Perhaps the same region of the P_{NTD} that maintains N in the soluble state for encapsidation (19) could play a role in facilitating the transient release of the RNA from N for copying. Comparing the sequences of RABV and VSV L proteins and highlighting the differences on the VSV L structure show high conservation of the RdRp catalytic sites and of the template and nucleotide channels, whereas the surfaces of the proteins have low homology (Fig. 7). The conservation of the template channel is in accordance with the capacity of RV L to initiate RNA synthesis on the naked VSV L leader, and the high variability of the surface surrounding the entrance of the template channel, i.e., where L may contact the N-RNA, could explain the disruption of an interaction between L and the N-RNA.

The assay developed here also has some utility for understanding how potential inhibitors of the RABV polymerase function. Although we demonstrate that RTP can inhibit RABV RdRP activity, the 1 mM IC_{50} reflects a concentration that could not be achieved *in vivo*. Although ribavirin can inhibit virus replication by other mechanisms, including through its principal cellular target, IMPDH, and through incorporation of RTP, leading to error catastrophe, our data are compatible with a reported lack of efficacy of ribavirin against rabies *in vivo* (37). We should also caution that while this assay can inform about the mechanism of polymerase inhibition, different 2′-substituted nucleotides have differing levels of inhibition on an N-RNA versus an unencapsidated RNA with VSV L (31).

In summary, we have developed a new *in vitro* assay to study the RdRP activity of rabies virus, further extending the utility of an approach we began with the closely related rhabdovirus VSV. This assay allowed us to obtain evidence of additional interactions between the constituents of the RNP complex required for RNA synthesis and to delineate the requirements in P for L binding and stimulation of RdRP activity. We also present evidence of the utility of the assay for understanding how polymerase inhibitors function.

MATERIALS AND METHODS

Protein expression and purification. Recombinant L and P of rabies virus strain SADB19 (UniProtKB/Swiss-Prot accession no.: L, P16289.1; P, P16286.1) were expressed as described previously (15) with some modifications. Briefly, 6×His-tagged L was expressed in *Spodoptera frugiperda* 21 (*Sf*21) cells, and 6×His-tagged P was expressed in *Escherichia coli* BL21(DE3) cells. Both were affinity purified with HisTrap HP (GE Healthcare), followed by gel filtration (Superdex 200 HR 10/30; GE Healthcare).

***In vitro* polymerase assay.** Polymerase assays were carried out as described in reference 16 using 0.4 μM template with 0.1 μM RABV L or VSV L and 0.15 μM RABV P, RABV P mutants, cRABV P, or VSV P in a reaction mixture containing 20 mM Tris base, pH 8, 10 mM NaCl, 6 mM $MgCl_2$, 200 μM UTP, 1.5 mM ATP, 1.5 mM CTP, and 165 nM [α-^{32}P]GTP (3,000 Ci mmol^{-1}). The standard polymerase assay mixture was supplemented with 250 μM GTP when N-RNA was used as a template. Reaction mixtures were incubated at 30°C for 3 h, and the reactions were stopped by addition of EDTA/formamide. The reaction products were resolved using denaturing 20% polyacrylamide-7 M urea gel electrophoresis in Tris-borate-EDTA (TBE) buffer and analyzed by autoradiography. The sizes of the products were determined by comparison to a 19-nt marker RNA labeled with T4 polynucleotide kinase (New England BioLabs) using [γ-^{32}P]ATP (3,000 Ci mmol^{-1}). Assays studying the effects of RTP concentrations were carried out by adding the indicated concentrations of RTP in the standard polymerase assay. [α-^{32}P]GTP was purchased from PerkinElmer.

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