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# The L–L oligomerization domain resides at the very N-terminus of the Sendai virus L RNA polymerase protein

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Received 2 December 2002; returned to author for revision 12 January 2003; accepted 4 April 2003

## Abstract

The Sendai virus RNA-dependent RNA polymerase is composed of the L and P proteins. We previously showed that the L protein gives intragenic complementation and forms an oligomer where the L–L interaction site mapped to the N-terminal half of the protein (S. Smallwood et al., 2002, Virology, 00, 000-000). We now show that L oligomerization does not depend on P protein and progressively smaller N-terminal fragments of L from amino acids (aa) 1-1146 through aa 1-174 all bind wild-type L. C-terminal truncations up to aa 424, which bind L, can complement the transcription defect in an L mutant altered at aa 379, although these L truncation mutants do not bind P. The fragment of L comprising aa 1-895, furthermore, acts as a dominant-negative mutant to inhibit transcription of wild-type L. N-terminal deletions of aa 1-189 and aa 1-734 have lost the ability to form the L–L complex as well as the L–P complex, although they still bind C protein. These data are consistent with the L–L interaction site residing in aa 1-174. Site-directed mutations in the N-terminal 347 aa, of L which abolish P binding, do not affect L–L complex formation, so while the L and P binding sites on L are overlapping they are mediated by different amino acids. The N-terminal portions of L with aa 1-424, aa 1-381, and to a lesser extent aa 1-174, can complement the transcription defect in an L mutant altered at aa 77-81, showing their L–L interaction is functional.
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## Introduction

The viruses belonging to the *Paramyxovirinae*, for which Sendai virus has served as one model system, contain a negative-sense (−), unsegmented RNA genome of about 15 kb. The genome is encapsidated by the nucleocapsid protein (NP) in a helical nucleocapsid which serves as the template for all viral RNA synthesis (Lamb and Kolakofsky, 2001). The virion-associated phosphoprotein [P, 528 amino acids (aa)] and the L protein (2228 aa) are the two subunits of the viral RNA-dependent RNA polymerase. For Sendai virus formation of the RNA polymerase requires the cotranslation of the P and L proteins where P binding stabilizes the L protein, probably by facilitating the correct folding of L (Horikami et al., 1992, 1997). The Sendai P protein is a

tetramer forming a coiled coil (Tarbouriech et al., 2000a,b). The L binding site on P was mapped to aa 412-479 (Curran et al., 1994; Smallwood et al., 1994). The L proteins of paramyxoviruses are all over 2000 aa and studies to begin mapping the P binding site on L showed a general location in the N-terminal quarter or half of the protein (Chandrika et al., 1995; Horikami et al., 1994; Parks, 1994). These data suggested that the P binding site resides from aa 1-400 of L and site-directed mutagenesis identified specific residues within the N-terminal 350 aa of the L protein of Sendai virus that mediate binding to P protein and the activity of the viral RNA polymerase (Holmes and Moyer, 2002). We recently demonstrated that the Sendai L protein forms an oligomer in the polymerase complex and showed that the L–L interaction was mediated by the N-terminal half of the protein (Smallwood et al., 2002a).

The L protein of the polymerase complex is thought to contain all the catalytic functions required for RNA synthesis. Transcription by the polymerase initiates at the 3′ end of the genome RNA giving the sequential synthesis of (+)-strand leader (*le+*) RNA and then the NP, P/C/V, M, F, HN,

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and L mRNAs (Lamb and Kolakofsky, 2001). During genome replication the synthesis of full-length viral RNA is coupled to its encapsidation by NP protein, utilizing an $NP_0$–P protein complex as the source of NP. Alignment of the L proteins of viruses of the order Mononegevirales showed six domains of relatively high conservation, designated I to VI, from the N- to C-terminus of the protein, which were proposed to specify the essential activities common to all L proteins (Poch et al., 1990; Sidhu et al., 1993). Recent characterization of Sendai L mutants in each of the six domains suggests that multiple domains contribute to the different steps in viral RNA synthesis, since mutants in different domains gave the same defective phenotypes (Chandrika et al., 1995; Cortese et al., 2000; Feller et al., 2000; Horikami and Moyer, 1995; Smallwood et al., 1999, 2002b). Nevertheless these domains can function in *trans* since we recently demonstrated that intragenic complementation between pairs of coexpressed inactive L mutants can restore viral RNA synthesis on an added template, consistent with L being an oligomer (Smallwood et al., 2002a). Viral RNA synthesis is downregulated by the C proteins encoded from the P gene (Curran et al., 1992). The Sendai C proteins were shown to bind to the L polymerase subunit to inhibit RNA synthesis (Horikami et al., 1997; Grogan and Moyer, 2001). In this study we have further mapped the L oligomerization site to the N-terminal 174 aa of L protein.

## Results

### L–L complex formation does not require P protein

We previously demonstrated oligomerization of the Sendai L protein by coimmunoprecipitation experiments with differentially epitope-tagged L proteins (Smallwood et al., 2002a). These experiments were done in the presence of P protein, so it was formally possible either that L oligomerized first and then bound P or alternatively that L might bind P and the L coimmunoprecipitation might occur through a P–P interaction. To address this question, we determined if differentially tagged full-length L and the truncated L BaK which contains the N-terminal half of L (aa 1-1146) can interact in the absence of P protein. The tagged proteins were expressed in VVT7-infected cells by transfection of the FLAG-L and HA-L BaK plasmids alone or together in the absence or presence of P plasmid. The cells were pulse-labeled with Express-$^{35}$S, since L protein is unstable in the absence of P (Horikami et al., 1997). Analysis of the total cytoplasmic cell extracts showed that all the proteins were synthesized (Fig. 1A). When the samples expressing the individual proteins were immunoprecipitated with α-FLAG antibody, FLAG-L was detected, but HA-L BaK was not (Fig. 1B, Lanes 3 and 4). There is a band found in mock-transfected cells at the position of HA-L BaK (Lane 1) which gives some background at that position in each lane. A very low amount of background P was detected with P
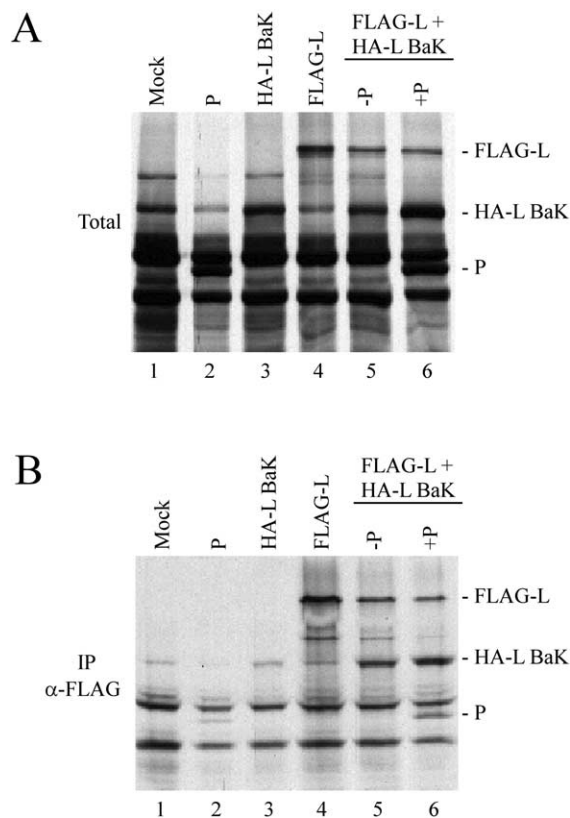


Fig. 1. L–L complex formation occurs in the absence of P protein. VVT7-infected cells were either not transfected (Mock) or transfected with the indicated FLAG-L and/or HA-L BaK tagged plasmids in the presence or absence of P plasmid. The cells were radiolabeled with Express-$^{35}$S for 1 h at 14 h posttransfection (pt). Cytoplasmic cell extracts were prepared and separated either by SDS–PAGE directly (Total, A) or after immunoprecipitation (IP) with α-FLAG antibody (B). The positions of the tagged L and L BaK proteins are indicated.

expressed alone (Lane 2). These data show that the FLAG antibody is specific for the appropriately tagged protein. Immunoprecipitation with α-FLAG antibody of an extract expressing both FLAG-L and HA-L BaK showed that HA-L BaK coimmunoprecipitated in both the absence and the presence of P indicative of a FLAG-L/HA-L BaK complex (Fig. 1B, Lanes 5 and 6). Furthermore in the latter case the P protein was also pulled down with the L complex (Lane 6). These data show that L–L binding does not require P protein.

### C-terminal truncations of L can form an oligomer with wild-type (wt) L

We previously showed that C-terminal truncations of L deleting up to half of the protein can still form an oligomer with wt L and can complement to restore transcription when coexpressed with a defective L mutated near the N-terminus (Smallwood et al., 2002a). To further delineate the region of L required for L–L complex formation, progressively shorter C-terminal truncations of tagged L were constructed

as described under Materials and methods and shown in Fig. 2A. The tagged proteins were expressed in VVT7-infected cells by transfection of the P plasmid and full-length HA-L without or with the FLAG-L truncation plasmids. Immunoblot analysis of cytoplasmic cell extracts with α-HA and α-FLAG antibodies showed that HA-L was detected when either expressed alone or together with truncated proteins and all of the C-terminally truncated proteins were expressed, although some were at lower levels (Fig. 2B, top). Immunoprecipitation of extracts expressing both wt HA-L and the FLAG-L truncations with α-FLAG antibody followed by immunoblotting with α-HA antibody showed that full-length HA-L coimmunoprecipitated with all of the L truncations (Fig. 2B, bottom). Similar analysis of FLAG-L and HA-L expressed alone showed only a small background cross-reactivity (Fig. 2B bottom, Lanes 2 and 3). This assay is semiquantitative and does not allow a determination of relative degrees of binding, but clearly some interaction occurred with all the L truncations. Complex formation was also demonstrated in the converse experiment, where samples immunoprecipitated with α-HA and then immunoblotted with α-FLAG showed the pull down of each of the truncated L proteins with wt HA-L (data not shown). These data show that even the smallest of the truncations, FLAG-L XE, with aa 1-174 forms a complex with L, identifying the binding site within this N-terminal fragment.

A relevant question was then whether the short C-terminally truncated L proteins could also bind P protein. We previously showed that the untagged protein equivalent to BaK did bind P; however, the BsK protein did not (Chandrika et al., 1995). VVT7-infected cells were transfected with the P and C-terminal L truncation plasmids and immunoblot analysis of the extract with α-FLAG antibody showed that the truncations were all expressed (Fig. 2C, top). Immunoblot analysis with α-P antibody showed that P protein was uniformly expressed in each sample except mock (data not shown). When the samples were first immunoprecipitated with α-FLAG and then immunoblotted with α-P antibody, wt FLAG-L and the FLAG-L BaK, but none of the other truncations, bound P (Fig. 2C, bottom). This is consistent with the data that P-L binding is not a requirement for L oligomerization (Fig. 1).

*Short C-terminal truncations of the L protein can complement transcription of a defective L mutant*

We next asked if these short C-terminal truncations of L could complement transcription as could the longer truncated proteins (Smallwood et al., 2002a). The defective L mutant, L15, which contains a three amino acid insertion at aa 379 in domain I (Chandrika et al., 1995) or the plasmids with the various truncations of L were expressed by transfection of VVT7-infected cells with P plasmid or L15 was coexpressed with each truncation. Cell extracts were prepared and incubated with wt polymerase-free RNA-NP in the presence of a radiolabeled nucleotide to assay for

mRNA synthesis. Total RNA was isolated and analyzed by agarose gel electrophoresis as described under Materials and methods. In mock extracts without viral proteins there was no mRNA synthesis showing that the template was free of endogenous polymerase activity (Figs. 3A and B, Lane 1). Extracts expressing the wt P and L proteins gave significant synthesis of the NP/P mRNAs (Figs. 3A and B, Lane 2); however, neither L15 or nor any of the truncated L proteins were able to support transcription when expressed alone (Lanes 3–6). In contrast, L15 coexpressed with FLAG-L BaK, BsK, and EE (Fig. 3A, Lanes 7–9) or with FLAG-L PE (Fig. 3B, Lane 7) were all able to restore some level of viral mRNA synthesis. The shorter truncations, FLAG-L NE and XE, however, did not complement transcription (Fig. 3B, Lanes 8 and 9). These latter truncations were expected to be negative since the 174 aa FLAG-L XE protein did not contain the L15 mutation site at aa 379 and FLAG-L NE with 381 aa was truncated at the mutation site. Immunoblot analysis of the samples showed that all the truncated L proteins were expressed, although at somewhat variable levels (Figs. 3A and B, bottom). The data show that some of the short C-terminally truncated proteins can complement transcription of a defective L mutant.

One characteristic of proteins in a complex is that they can often act as dominant negative mutants and inhibit the activity of the wt protein. We, therefore, tested if FLAG-L BsK, which complemented transcription but which did not bind P (Fig. 2C), could inhibit transcription by wt L protein. VVT7-infected cells were mock transfected or transfected with the P plasmid, a constant amount of wt L plasmid, and the indicated increasing amounts of the FLAG-L BsK plasmid. Cytoplasmic extracts were incubated with wt polymerase-free RNA-NP template and a radiolabeled nucleotide and total product RNA was isolated and analyzed. The wt L and P proteins gave good synthesis of the viral mRNAs, while there was no synthesis in the absence of viral proteins (Fig. 4, Lanes 1 and 2). Increasing amounts of FLAG-L BsK progressively decreased the level of viral transcription (Fig. 4, Lanes 3–5). As the control for this experiment, we have previously shown that the addition of increasing amounts of wt L plasmid does not change the level of transcription (Horikami et al., 1992), which is maintained at the same level from 0.5 to 2.0 μg of L. Therefore, increasing the amount of the truncation does not reflect a loss of the wt L, but rather a dominant negative effect of the truncated protein. Immunoblot analysis showed that increasing FLAG-L BsK protein was synthesized with increasing plasmid transfected (data not shown). These data show that this L truncation mutant did act as a dominant negative mutant and is consistent with wt L-L BsK complex formation.

*N-terminal deletions of the L protein lose binding to L and P but still bind C*

We wanted to further delineate the L–L binding site with the use of N-terminal deletions of the protein. Two deletions were constructed as described under Materials and methods:
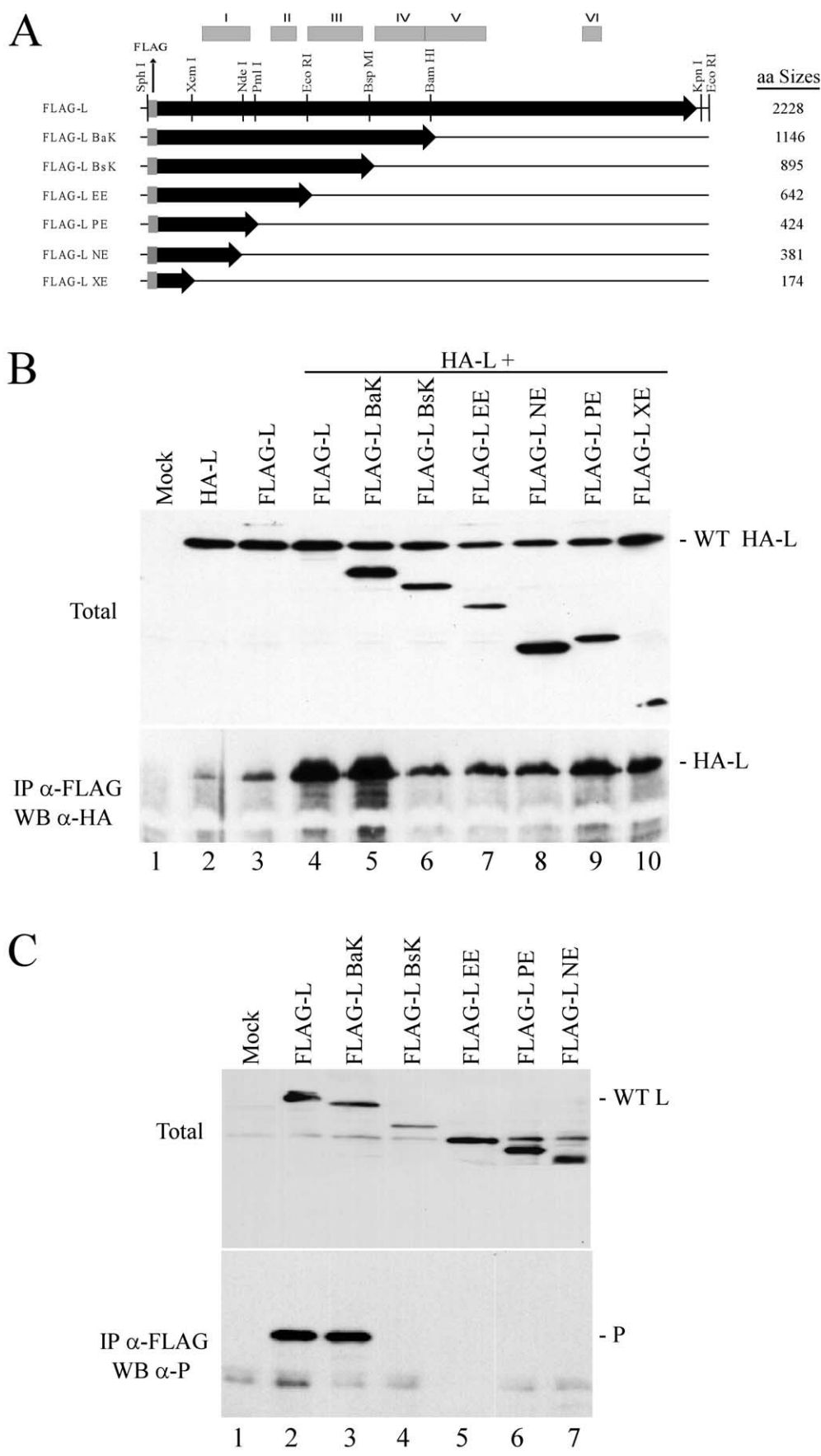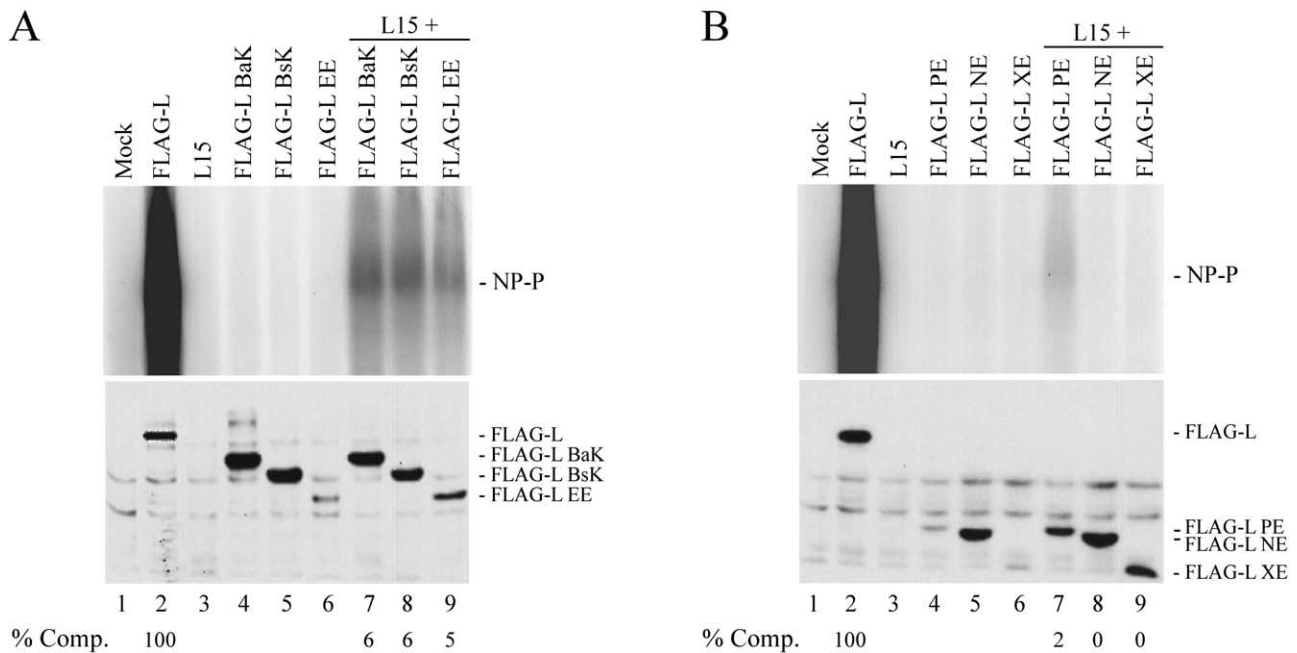
Fig. 3. Truncated L proteins complement defective L15 transcription. (A and B) VVT7-infected cells were either mock-transfected or transfected with the P and the indicated tagged wt or mutant L plasmids alone or in pairs with L15 and each tagged truncation. After overnight incubation cytoplasmic extracts were incubated with wt polymerase-free RNA-NP template and [$\alpha$-$^{32}$P]CTP. Total RNA was isolated and analyzed on an agarose urea gel as described under Materials and methods (top). The position of the comigrating NP and P mRNAs is indicated. The data from three experiments were quantitated and expressed for the pairs as the % complementation (Comp.) after subtracting the activity of the mutants alone compared to wt L as 100%. The values of the multiple experiments varied by less than 10%. (Bottom) Samples of the extracts in (A) and (B) were analyzed by immunoblotting with $\alpha$-FLAG antibody and the positions of the proteins are indicated.

LX deleted the entire region (aa 1-189) encompassed in FLAG-L XE, which was the smallest binding fragment; and LXA deleted the region to the AvrII site removing most of the segment (aa 1-734) encompassed in FLAG-L EE (Fig. 5A). These proteins were unstable in transfected cells, but could be detected by pulse labeling. To test for binding to L, VVT7-infected cells were transfected with the P, wt FLAG-L, HA-L BaK, or LXA and LX plasmids alone or together and labeled for 1 h with Express-$^{35}$S. Gel analysis of the total cytoplasmic cell extracts showed that all the L proteins were synthesized (Fig. 5B, top). Immunoprecipitation with $\alpha$-FLAG antibody brought down wt FLAG-L, but neither HA-L BaK nor the truncated L proteins when they were expressed alone (Fig. 5A, bottom, Lanes 2–5), showing the specificity of the antibody. When the wt and truncated L proteins were coexpressed, as a positive control HA-L BaK was coimmunoprecipitated by $\alpha$-FLAG antibody with FLAG-L (Fig. 5, bottom, Lane 6); however, virtually no LXA and only a small amount of LX were

pulled down (Lanes 7 and 8). Quantitation of the proteins showed that the LX and LXA binding to wt L was reduced by 80 and 94%, respectively, compared to the positive control. These data suggest that the L oligomerization site resides in the N-terminal 189 aa of L protein, consistent with the smallest protein, FLAG-L XE (aa 1-174), that still bound L.

The N-terminal deletions were also tested for their ability to bind to Sendai P and C proteins utilizing glutathione-S-transferase (GST) fusion proteins and pull-down experiments with glutathione-Sepharose beads. VVT7-infected cells were transfected with the GSTP (Fig. 5C) or GSTC (Fig. 5D) plasmids and wt and N-terminal deletion plasmids alone or together and pulse labeled with Express-$^{35}$S. Gel analysis of the total samples showed that all the proteins were synthesized (Figs. 5C and D, top). For P binding as controls GSTP alone bound to the beads, while the wt or mutant L proteins alone did not (Fig. 5C, bottom, Lanes 2–5). When coexpressed with GSTP, wt L, but not LXA or

Fig. 2. L protein C-terminal truncations form oligomers with wt L. (A) Schematics of the wt L protein with domains I–VI and the C-terminal truncations of L with the sizes of the proteins on the right. L15 is a 3 aa insertion at aa 379 in domain I. (B) VVT7-infected cells were either mock-transfected or transfected with P and the indicated FLAG and/or HA-tagged full-length L plasmids or C-terminal truncated FLAG-L truncations. After overnight incubation, cytoplasmic extracts were prepared and separated by SDS–PAGE directly and immunoblotted with both $\alpha$-HA and $\alpha$-FLAG antibodies (Total, top). Samples were also isolated by IP with $\alpha$-FLAG and then immunoblotted (WB) with $\alpha$-HA antibody (bottom). (C) VVT7-infected cells were either mock-transfected or transfected with P plasmid and the indicated FLAG-tagged wt L or C-terminal L truncation plasmids. After overnight incubation cytoplasmic cell extracts were prepared and samples of the total separated by SDS–PAGE and immunoblotted with $\alpha$-FLAG antibody (top). Samples were also isolated by IP with $\alpha$-FLAG antibody and then immunoblotted (WB) with $\alpha$-P antibody (bottom). The positions of the proteins are indicated.
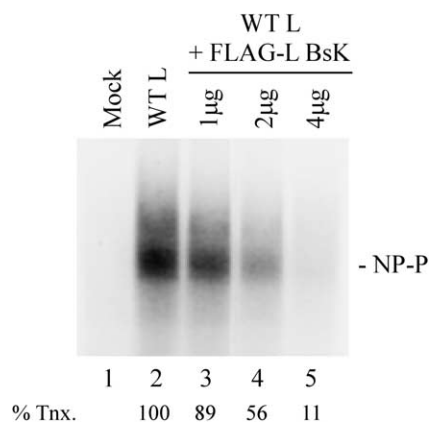
Fig. 4. Inhibition of WT Sendai transcription by a C-terminal deletion of L. VVT7-infected cells were either mock-transfected or transfected with the P (0.75 μg) and a constant amount of wt L (0.25 μg) plasmids and the indicated increasing amounts of BsK plasmid. pGEM plasmid was added to maintain constant DNA for transfection. After overnight incubation, cytoplasmic cell extracts were incubated with wt polymerase-free RNA-NP template and [α-³²P]CTP. Total RNA was isolated and analyzed on an agarose urea gel as described under Materials and methods. The position of the NP and P mRNAs is indicated. The data from two experiments were quantitated and expressed as the % transcription (Tnx.) after subtracting the activity of the mock compared to wt L as 100%. The values of two experiments varied by less than 10%.

LX, bound to the beads (Fig. 5C, bottom, Lanes 6-8), showing that these deletions abolished P binding. As controls for C binding, GSTC alone, but not wt L protein alone, bound to the beads (Fig. 5D, bottom, Lanes 2 and 3). In contrast, when coexpressed with GSTC, wt L and both LXA and LX bound to the beads (Fig. 5D, bottom, Lanes 4–6), showing that these deletions did not abolish C binding. Thus the N-terminal deletions inhibited both L–L and L–P complex formation, but did not apparently disrupt the overall structure of the protein, since C binding was retained.

*Site-directed L mutants deficient in P binding still bind wt L*

We previously identified specific amino acids in the N-terminal 350 aa of Sendai L that are required for binding to P (Holmes and Moyer, 2002), a region which overlaps the sequences necessary for L–L complex formation (Fig. 2). To determine whether any of the same amino acids might be involved in both interactions, we constructed FLAG-tagged mutant L proteins to test binding to HA-L BaK. VVT7-infected cells were transfected with the P, wt, and mutant FLAG-L plasmids and HA-L Bak plasmid and pulse labeled with Express-³⁵S, since the mutant L proteins were unstable for long-term labeling. Gel analysis of the total samples showed that all the proteins were synthesized (Fig. 6, top). Immunoprecipitation with α-FLAG antibody brought down wt FLAG-L and P, but not HA-L BaK or P, when they were expressed (Fig. 6, bottom, Lanes 2 and 3), as expected. As a positive control when HA-L BaK was coexpressed with

wt FLAG-L and P, it was coimmunoprecipitated by α-FLAG antibody (Fig. 6, bottom, Lane 4). In addition the α-FLAG antibody also pulled down HA-L BaK and P with all of the mutant FLAG-L proteins when they were coexpressed (Fig. 6, bottom). Similarly, in the reverse experiment α-HA antibody coimmunoprecipitated each of the FLAG-L mutants (data not shown). These data show that the site-directed L mutations which altered or abolished P binding had no effect on L–L complex formation.

*L protein C-terminal truncations can complement transcription with the L516 mutant*

In the earlier results, the shortest C-terminal L truncations, FLAG-L NE and FLAG-L XE, did form an L–L complex, although they did not complement a defective L mutant at aa 379 because they did not contain the site (Figs. 2 and 3). We showed previously that the site-directed L mutant, L516, with amino acid changes at aa 77-81, gave some residual P binding and minimal transcription (Holmes and Moyer, 2002), but could form a complex with wt L (Fig. 6). We asked if the short C-terminal truncations could complement L516 as a measure of whether the L–L interaction was functional with a suitable mutant. The defective mutant, L516, was coexpressed by transfection of VVT7-infected cells with P plasmid and the indicated FLAG-tagged C-terminal truncations of L and assayed for mRNA synthesis (Fig. 7). Cell extracts were prepared and incubated with wt polymerase-free RNA-NP in the presence of a radiolabeled nucleotide. Analysis of total RNA showed extracts expressing the wt P and L proteins gave significant synthesis of the NP/P mRNAs compared to the mock-transfected extract (Fig. 7, Lanes 1 and 2), while L516 gave a small amount of transcription (Lane 3) as observed previously (Holmes and Moyer, 2002). None of the truncated L proteins were able to support transcription when expressed alone (Lanes 4–6). In contrast, L516 coexpressed with FLAG-L PE, NE, and XE all gave reproducible levels of viral mRNA synthesis (Fig. 7, Lanes 7–9). Immunoblot analysis of the samples with α-FLAG antibody showed that all the truncated L proteins were expressed (data not shown). Thus these data show that even the smallest of the C-terminal L truncations can rescue transcription of an appropriate defective mutant, consistent with their ability to form the L–L complex.

*The N-terminus of L confers complex formation on a heterologous protein*

Since deletion analyses showed the very N-terminus of L is required for L–L complex formation, we asked if this region could direct the oligomerization of a heterologous protein with L. As described under Materials and methods, we constructed a fusion protein with the N-terminal fragment of L encompassing aa 1-381 (NE) fused to the N-terminus of maltose binding protein (MBP). The L–NE–MBP fusion as well as the nonfusion MBP were expressed
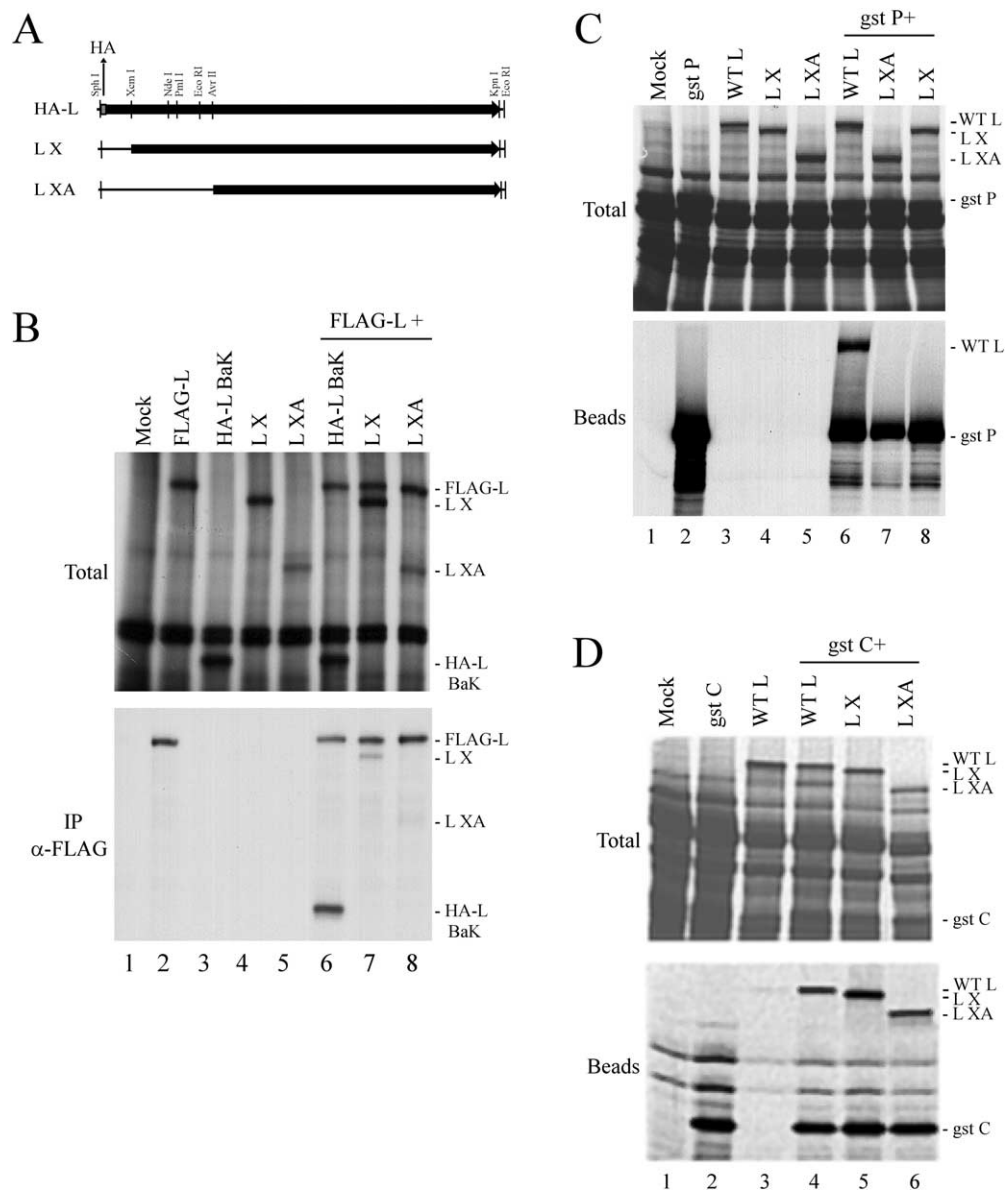
Fig. 5. N-terminal deletions of the L protein bind GSTC, but not GSTP or wt L. (A, top) Schematic of the N-terminal L deletions. (B) VVT7-infected cells were either mock-transfected or transfected with the P plasmid and the indicated wt FLAG-L plasmid alone or together with the L N-terminal truncation LX or LXA plasmids. The cells were radiolabeled with Express-³⁵S for 1 h at 14 h pt; cytoplasmic extracts were prepared and either separated by SDS–PAGE directly (Total, top) or after immunoprecipitation with α-FLAG (B) antibody (IP, bottom). VVT7-infected cells were also transfected with the GSTP plasmid (B) or GSTC plasmid (C) and the indicated wt L or L N-terminal truncation plasmids. The cells were radiolabeled with Express-³⁵S for 1 h at 14 h pt; cytoplasmic extracts were prepared and either separated by SDS–PAGE directly (Total, top) or samples were selected by binding to glutathione-Sepharose beads (Beads, bottom) and analyzed by SDS–PAGE. The positions of the tagged proteins are indicated.

in the absence or presence of full-length HA-L in VVT7-infected, transfected cells (Fig. 8, top). Immunoprecipitation of cell extracts with both α-HA and α-MBP antibodies showed that all the proteins were synthesized (Fig. 8, left). Immunoprecipitation with α-MBP alone of samples with the proteins expressed individually showed the specificity of the antibody for MBP, since no cross-reactivity with HA-L was observed (Fig. 8, right, Lanes 1–4). However, when the proteins were coexpressed with HA-L, IP with α-MBP showed complex formation of HA-L with L-NE-MBP, but not with MBP (Fig. 8 right, Lanes 5 and 6). In the converse

experiment IP with α-HA also brought down the L-NE-MBP with the HA-L protein (data not shown), confirming complex formation. These data show that just the N-terminal 381 aa of L are sufficient to confer complex formation of a heterologous protein with wt L.

## Discussion

We previously showed that pairs of inactive L mutants give intragenic complementation to restore viral RNA syn-
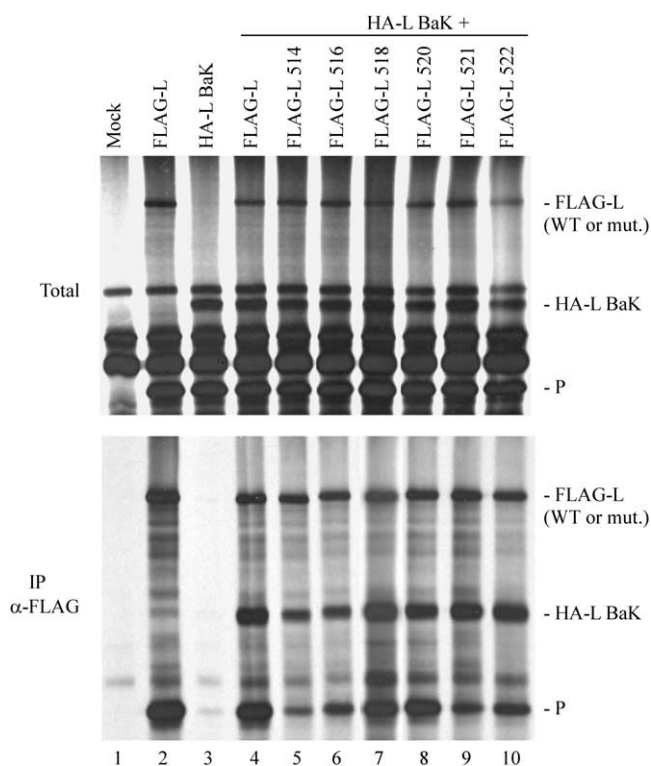
Fig. 6. L oligomerization occurs with L mutants that abolish P binding. VVT7-infected cells were either mock-transfected or transfected with the P plasmid and the indicated FLAG-tagged WT or mutant L plasmids together with HA-L BaK plasmid. The cells were radiolabeled with Express-$^{35}$S for 1 h at 14 h pt; cytoplasmic extracts were prepared and either separated by SDS–PAGE directly (Total, top) or after immunoprecipitation with α-FLAG antibody (IP, bottom). The positions of the proteins are indicated.

a ratio of L:P of 1:2 or 1:3, similar to the data we obtained using a different selection technique (Horikami et al., 1997). These types of experiments, however, have been shown to be inaccurate for the determination of the ratio of subunits, since this method suggested that the Sendai P protein is a trimer, whereas biophysical studies showed that P is a tetramer (Tarbouriech et al., 2000a,b). Based on the ratios we obtained, the minimal polymerase composition could be $L_2P_4$ or $L_2P_8$. Further studies will be required to resolve the higher order structure of the Sendai RNA polymerase.

We next asked if these short C-terminal truncations of L could complement transcription of a defective mutant L, as could the longer truncated proteins (Smallwood et al., 2002a), as a measure of the activities of the polypeptides. The inactive L mutant, L15, which contains a three amino acid insertion at aa 379 in domain I (Chandrika et al., 1995), coexpressed with C-terminal truncations up to 895, 642, and 424 aa, FLAG-L BsK, FLAG-L EE, and FLAG-L PE, respectively, gave low, but reproducible viral transcription. For this complementation these L truncation mutants did not bind P, but L15 does bind P. The portion of L comprising aa 1-895, furthermore, acts as a dominant negative mutant to inhibit the transcription of wild-type L in vitro (Fig. 4). Thus the FLAG-L BsK, EE, and PE truncations are functional. Since they do not bind P protein, they are part of the polymerase complex by virtue of the L–L interaction and not through a P–L interaction.

To further define the L–L binding site, L proteins with N-terminal deletions of aa 1-189 and aa 1-734 were demonstrated to have lost the ability to form the L–L complex as

thesis and further that L oligomerizes where the L–L interaction site was initially mapped to the N-terminal half of the protein (Smallwood et al., 2002a). In these studies of the L–L interaction we now show that L oligomerization does not depend on P protein, since the complex forms in the absence of P (Fig. 1). However, the stability of wt L does require P protein since L oligomers are degraded without the coexpression of P (Horikami et al., 1997). We propose that L oligomerizes and then binds the P tetramer which promotes the proper folding of L. To further delineate the L–L binding site, progressively smaller N-terminal regions of L containing 1146, 895, 642, 424, 381, and 174 aa were all shown to bind wt L; however, only polypeptides with aa 1-1146 and 1-895 bound the P protein (Fig. 2). Similar binding experiments with differentially tagged L proteins of different sizes showed that L–L complex formation did not depend on the presence of full-length L, but occurred with sets of smaller fragments (data not shown). At least as indicated by the immunoblots, some of the L oligomers formed by the truncated proteins in the absence of P binding were in fact stable (Fig. 2). Using the tagged L proteins of different sizes and immunoprecipitation experiments with tag antibodies, we attempted to determine the ratio of L to P in the polymerase complex. We got variable results with
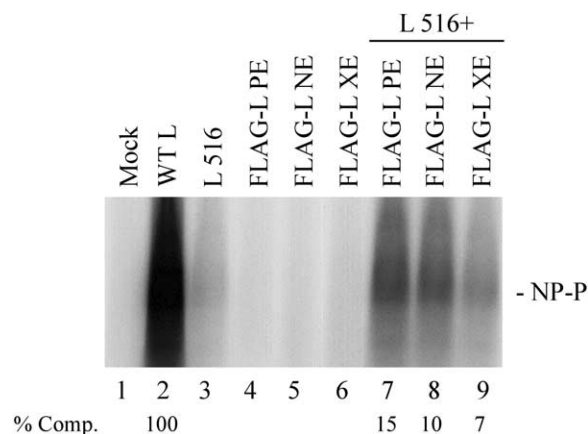


Fig. 7. Truncated L proteins complement defective L516 transcription. VVT7-infected cells were mock-transfected (−) or transfected with the wt P and L plasmids or the P plasmid with the indicated C-terminal truncated FLAG-L plasmids (shown in Fig. 2) alone or in pairs with L516 plasmid. After overnight incubation, cytoplasmic cell extracts were incubated with wt polymerase-free RNA-NP template and [α-$^{32}$P]CTP. Total RNA was isolated and analyzed on an agarose urea gel as described under Materials and methods. The position of the NP and P mRNAs is indicated. The data from two experiments were quantitated and expressed for the pairs as the % complementation (Comp.) after subtracting the activity of the mutants alone compared to wt L as 100%. The values of the two experiments varied by less than 10%.
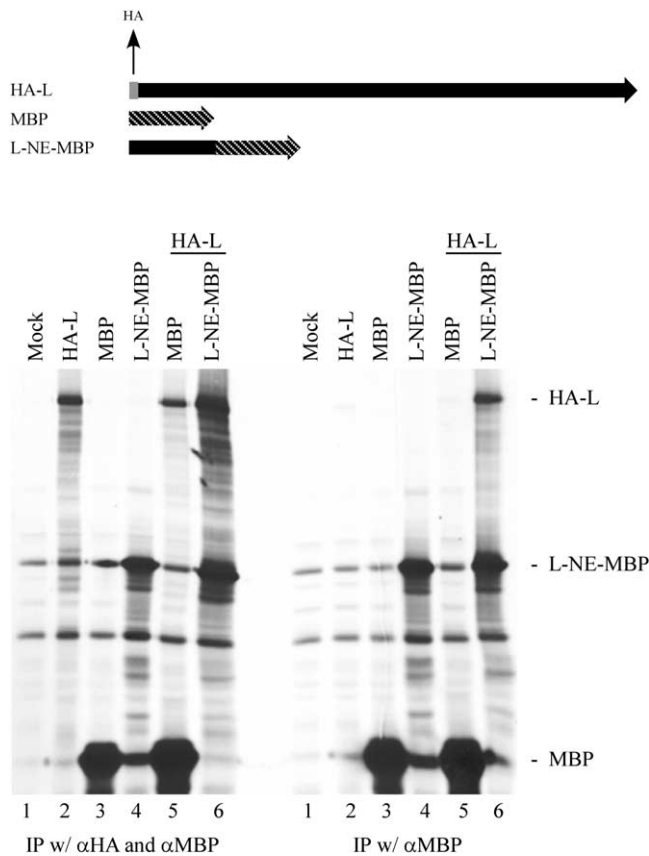
Fig. 8. Complex formation between L and an L-NE-MBP fusion protein. The top shows schematics of HA-L, maltose binding protein (MBP), and a fusion of the L-NE fragment to the N-terminus of MBP (L-NE-MBP). VVT7-infected cells were either mock-transfected or transfected with the indicated HA-WT L, MBP, and L-NE-MBP plasmids. The cells were radiolabeled with Express-$^{35}$S for 1 h at 14 h pt; cytoplasmic extracts were prepared and separated by SDS–PAGE after immunoprecipitation (IP) with α-FLAG and α-MBP antibodies (right) or α-MBP antibody alone (right). The positions of the proteins are indicated.

well as the L–P complex (Fig. 5). However, these N-terminal truncations still bind C protein, suggesting that the overall integrity of the proteins was maintained. These data are consistent with the L–L interaction site residing in aa 1-189, which is consistent with the smallest polypeptide, aa 1-174, which still forms the L–L complex. In fact we show that fusion of just the N-terminal aa 1-381 of L to MBP confers the ability of the heterologous protein to bind L (Fig. 8). In addition, site-directed mutations in the N-terminal 347 aa of L which either abolished or greatly reduced P binding (Holmes and Moyer, 2002) do not affect L–L complex formation (Fig. 6), so while the L and P binding sites on L are overlapping, they are mediated by different amino acids. Most of these site-directed L mutants at aa 20-25, 77-81, 209-213, 262-266, 287-291, and 345-347 changed clustered hydrophobic amino acids to ala, with the exception of L516 (aa 77-81), which changed two charged residues. This would suggest that the L–L interaction may be mediated by charged residues, or perhaps other hydrophobic amino acids in regions that were not altered. Interestingly,

the N-terminal portions of L, contained in FLAG-L PE, FLAG-L NE, and to a lesser extent FLAG-L XE, with aa 1-424, aa 1-381, and aa 1-174, respectively, can complement the transcription defect in an L mutant (L516) altered at aa 77-81, showing their L–L interaction is functional. Again none of these mutants bind P so their activity in the polymerase is due to the L–L interaction. Together these data support the idea that independent domains reside in the L protein.

In a recent study of the L protein of human parainfluenza virus 3 (hPIV3), a member of the same family as Sendai virus, the N-terminus, is also necessary for polymerase function. Deletion of the nonconserved aa 2-15 abolished both P binding and viral transcription (Malur et al., 2002). In addition changing individual aa in a region highly conserved with Sendai L from aa 13-25 also significantly reduced or eliminated P–L complex formation and RNA synthesis. The Sendai L mutant L514, changing aa 20-25, had a similar deleterious effect on Sendai P binding and RNA synthesis (Holmes and Moyer, 2002), but no effect on L–L complex formation (Fig. 6). In the case of hPIV3 L, as was also the case for Sendai L, additional regions of L were required for P binding. Based on the homology of the proteins, we predict that the PIV3 L and perhaps all paramyxovirus L proteins will also oligomerize, which is currently under investigation. It is interesting that recent data have shown that the RNA polymerases of the positive-strand RNA viruses, poliovirus, and hepatitis C virus also require oligomerization for function (Pata et al., 1995; Hobson et al., 2001; Qin et al., 2002; Wang et al., 2002).

## Materials and methods

### Cells, viruses, plasmids, and antibodies

Human lung carcinoma cells (A549 cells, ATCC) were used for all experiments. Recombinant vaccinia virus expressing the phage T7 RNA polymerase (VVT7) (Fuerst et al., 1986) was grown on A549 cells. Sendai virus (Harris) was propagated on embryonated chicken eggs and wt polymerase-free Sendai template was prepared as described previously (Carlsen et al., 1985). Plasmids encoding the Sendai virus genes, pGEM-L, pGEM-NP, pGEM-Pstop (expressing only the P protein and not the C proteins, designated here as pGEM-P), were described previously (Curran et al., 1991). The Sendai L mutant L15 constructed in domain I at aa 379 was described and characterized previously (Chandrika et al., 1995). The clustered hydrophobic-to-alanine or charged-to-alanine scanning mutants, L514–L522, constructed between aa 20 and 347 in the L protein were described and characterized previously (Holmes and Moyer, 2002). The viral genes were all cloned downstream of the T7 promoter. Antibodies utilized for immunoblot and immunoprecipitation assays were anti-FLAG M-2 monoclonal

antibody (α-FLAG, Sigma) and anti-HA probe F-7 monoclonal antibody (α-HA, Santa Cruz Biotechnology, Inc.).

### Construction of epitope-tagged and truncated L genes

The HA and FLAG epitopes were integrated in-frame to the 5′ end of the L gene of Sendai virus by PCR using the (+)-sense HA primer SM530 (5′ CTCTGCATGC ATG-TACCCAT ACGATGTTCC AGATTACGCT AGCTT-GGGTG GTCCAATGGA TGGGCAGGAG TCCTCC) or the (+)-sense FLAG primer SM531 (5′ CTCTGCATGC ATGATCGATT ACAAGGATGA CGATGACAAG CTT-GCAATGG ATGGGCAGGA GTCCTCC), respectively. These primers, which contain an *Sph*I site at the 5′ end in front of the coding sequence, the epitope tag, and 17 nt from the ATG start site for L, were used in PCR with Vent polymerase and the (−)-sense primer SM410 (5′ CCCT-GCTCGA GTCAACTTGT TCAGTATC) downstream from the *Xcm*I site. The products were digested with *Sph*I and *Xcm*I and cloned into those sites in pGEM-L*Sph*I/*Bst*EII, where *Sph*I and *Bst*EII sites were inserted upstream of the 5′ end of the L gene in pGEM-L (J. Feller and S.A. Moyer, unpublished data). The resulting clones were designated as pGEM-HA-L and pGEM-FLAG-L, respectively, and were confirmed by sequencing. To construct the C-terminal truncations, pGEM-FLAG-L was digested with either: *Bam*HI and *Kpn*I; *Bsp*MI and *Kpn*I; *Eco*RI; *Pml*I and *Eco*RI; *Nde*I and *Eco*RI; or *Xcm*I and *Eco*RI to drop out increasing amounts of the 3′ end of L. The remaining region of vector and L for each was blunted with T4 DNA polymerase and ligated with T4 DNA ligase. The resultant clones were designated FLAG-L BaK, FLAG-L BsK, FLAG-L EE, FLAG-L PE, FLAG-L NE, and FLAG-L XE, respectively.

To construct the first N-terminal truncation mutant, the region between the *Xcm*I and *Nco*I sites of the L gene was amplified by PCR using Vent polymerase with primer SM533 (5′CTCTGCATGC ATGACGCGTC CCGGGG-GACC CCTC 3′) and SM170 (5′ ATCAGGGAA GTCA-CAGG 3′). The PCR products were digested with *Sph*I and *Nco*I and cloned into those sites in pGEM-L*Sph*I/*Bst*EII. The resultant L clone contained a deletion to the *Xcm*I site as well as mutations for the generation of silent *Sma*I, *Xma*I, and *Mlu*I sites and it was designated pGEM-LX. This clone was digested with *Xma*I and *Avr*II, blunted with T4 DNA polymerase, and ligated with T4 DNA ligase to construct the pGEM-LXA deletion mutant.

Six of the clustered hydrophobic-to-alanine or charged-to-alanine scanning mutants (L514, L516, L518, L520, L521, and L522) previously constructed between aa 20 and 347 of Sendai L (Holmes and Moyer, 2002) were tagged with the FLAG epitope by PCR or subcloning. The 5′ end of the mutants L514 and L516 was amplified by PCR using Vent polymerase and FLAG primer SM531 and SM410. The PCR products were digested with *Sph*I and *Xcm*I and cloned into those sites in pGEM-FLAG-L to construct

FLAG-L 514 and FLAG-L 516, respectively. The mutants L518, L520, L521 were digested with *Xcm*I and *Nco*I and the mutant L522 was digested with *Xcm*I and *Pml*I. These fragments containing all the desired mutations were cloned in to these sites in pGEM-FLAG-L to construct FLAG-L 518, FLAG-L 520, FLAG-L 521, and FLAG-L 522.

To fuse the maltose binding protein to the N-terminal fragment of the Sendai L, MBP was amplified from pMAL-2c plasmid (NEB) by PCR using Vent polymerase and SM569 5′ GTGCACGTGGCCATATGAAAATC-GAAGAAGGTAAACTGG3′ and SM570 5′CGGGG-TACCT CATCCGCCAA AACAGCCAAGC3′, a primer pair specific for MBP. Restriction sites for *Nde*I and *Kpn*I were incorporated into the 5′ and 3′ ends of the MBP during PCR amplification by primers SM569 and SM570, respectively. The PCR-amplified MBP was cloned into the *Nde*I and *Kpn*I sites of the pGEM-L plasmid from which the *Nde*I-*Kpn*I fragment was removed. The resulting construct was designated as pGEM-L-NE-MBP, where L-NE was joined to the N-terminus of MBP.

### RNA synthesis

For in vitro mRNA synthesis, 60-mm dishes of A549 cells were infected with VVT7 at a m.o.i. of 2.5 PFU/cell for 1 h at 37°C and transfected with the P (1.5 $\mu$g) and the wt or mutant L (0.5 $\mu$g) plasmids with Lipofectin (Life Technologies) according to the manufacturer's protocol in Opti-MEM (GIBCO). In complementation 0.25 $\mu$g of each of two mutant L plasmids and 1.5 $\mu$g of P were used for transfection. At 18 h posttransfection (pt) cell extracts (100 $\mu$l) were prepared by lysolecithin permeabilization in incomplete reaction mix (RM) [0.1 M HEPES (pH 8.5), 50 mM NH$_4$Cl, 7 mM KCl, 1 mM DTT, 1 mM spermidine, 1 mM each ATP, GTP, and UTP, 10 $\mu$M CTP, and 10% glycerol] as described previously (Chandrika et al., 1995; Horikami et al., 1992). The nuclei were removed and the extracts were incubated with 20 $\mu$g/ml micrococcal nuclease (MN) plus 1 mM CaCl$_2$ at 30°C for 30 min, followed by 2.2 mM EGTA for inactivation of the MN. The nuclease-treated extracts were then supplemented with 0.1 vol of 10× supplemental mix [45 mM MgOAc, 5 U/$\mu$l RNasin, 200 $\mu$g/ml actinomycin D, 400 U/ml creatine phosphokinase (CPK), and 33 mg/ml creatine phosphate], 1 $\mu$g polymerase-free wt Sendai nucleocapsid template (RNA-NP), and 20 $\mu$Ci of [$\alpha$-$^{32}$P]CTP, and the samples were incubated for 2 h at 30°C. RNA was isolated using the Qiagen RNeasy Total RNA kit according to the manufacturer's protocol and analyzed by 1.5% agarose/6 M urea gel electrophoresis and visualized by autoradiography. All RNA products were quantitated on the phosphorImager (Molecular Dynamics).

### Protein analysis

For the analysis of the L–L interaction of the stable C-terminally truncated L mutants, A549 cells in 60-mm

dishes were infected with VVT7 and transfected with pGEM-P (6 $\mu$g) and pGEM-HA-L or pGEM-FLAG-L (3 $\mu$g) or both (1.5 $\mu$g each). Cytoplasmic extracts were prepared at 18 h pt in 200 $\mu$l NP-40 lysis buffer [0.15 M NaCl, 50 mM Tris–HCl (pH 8.0), 1% Nonidet P-40 (NP-40), and 1 $\mu$g/ml aprotinin]. For immunoprecipitation, the unlabeled cell extract was incubated with $\alpha$-HA (1 $\mu$g) or $\alpha$-FLAG (2 $\mu$g) antibodies and selected with *Staphylococcus aureus* (Cowen strain) as described previously (Carlsen et al., 1985) or Protein A-Sepharose CL 4B (Pharmacia) according to manufacturer's instructions. The total and immunoprecipitated samples were separated by 7.5% SDS–PAGE and electroblotted onto PVDF membrane (Osmonics). The blots were incubated with the primary antibody, $\alpha$-HA (0.4 $\mu$g/ml), or $\alpha$-FLAG (4 $\mu$g/ml) antibodies, as indicated in the figure legends and developed with HRP-conjugated rabbit anti-mouse secondary antibody using the Enhanced Chemiluminescence Plus (ECL+) protein detection system (Amersham Life Science).

For the analysis of the L–L interaction of the unstable N-terminal truncation or site-directed L mutants, A549 cells in 35-mm dishes were infected with VVT7 and then transfected with pGEM-P (2 $\mu$g) with wt or mutant pGEM FLAG-L, pGEM- HA-L BaK, pGEM-LX, or pGEM-LXA (2 $\mu$g) or pGEM-P (4 $\mu$g) and both appropriate wt or mutant FLAG and HA-tagged or untagged L (2 $\mu$g each). Fourteen hours posttransfection cells were labeled with Express-[$^{35}$S] (100 $\mu$Ci/ml, DuPont NEN) in medium with no cysteine and methionine for 1 h. Cytoplasmic extracts were prepared by scraping into 100 $\mu$l NP-40 lysis buffer. The cell lysate was clarified by pelleting for 30 min at 15,000 rpm. For immunoprecipitation, the labeled cell extracts were incubated with $\alpha$-HA or $\alpha$-FLAG antibodies as above and selected with Protein A-Sepharose CL 4B. Total and IP samples were analyzed by 7.5% SDS–PAGE and quantitated on the phosphorImager.

## Acknowledgment

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