Two N-terminal regions of the Sendai virus L RNA polymerase protein participate in oligomerization

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Abstract

The RNA dependent RNA polymerase of Sendai virus consists of a complex of the large (L) and phosphoprotein (P) subunits where L is thought to be responsible for all the catalytic activities necessary for viral RNA synthesis. We previously showed that the L protein forms an oligomer [Smallwood, S., Cevik, B., Moyer, S.A., 2002. Intragenic complementation and oligomerization of the L subunit of the Sendai virus RNA polymerase. Virology 304, 235–245] and mapped the L oligomerization domain between amino acids 1 and 174 of the protein [Çevik, B., Smallwood, S., Moyer, S.A., 2003. The oligomerization domain resides at the very N-terminus of the Sendai virus L RNA polymerase protein. Virology 313, 525–536]. An internal deletion encompassing amino acids 20 to 178 of the L protein lost polymerase activity but still formed an L–L oligomer. The first 25 amino acids of paramyxovirus L proteins are highly conserved and site-directed mutagenesis within this region eliminated the biological activity of the L protein but did not have any effect on P–L or L–L interactions. Moreover deletion of amino acids 2–18 in L abolished biological activity, but again the L–L binding was normal demonstrating that the oligomerization domain of L protein resides in two N-terminal regions of the protein. Therefore, sequences between both aa 2–19 and aa 20–178 can independently mediate Sendai L oligomerization, however, both are required for the activity of the protein.
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Introduction

Sendai virus (SeV) is a member of the genus *Respirovirus* of the Paramyxovirinae subfamily of the nonsegmented negative strand RNA viruses. It has a single-stranded genome of 15,384 nucleotides (nt) organized into six open reading frames encoding the NP, P/C/V, M, F, HN and L proteins. 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 RNA dependent RNA polymerase (RdRp) of SeV consists of the phosphoprotein (P, 528 amino acids [aa]) and the large protein (L, 2228 aa) subunits. P protein is a tetramer forming a coiled coil structure (Tarbouriech et al., 2000a, 2000b). Although the structure of the L protein has not been determined, we recently demonstrated that SeV L protein forms an oligomer (Smallwood et al., 2002), thus the minimal composition of the polymerase complex is L2–P4. Formation of functional SeV RdRp requires the coexpression of the P and L proteins. P binding stabilizes the L protein probably by facilitating its correct folding (Horikami et al., 1992; Horikami et al., 1997), but it is not required for oligomerization of L (Çevik et al., 2003). The P–L interaction has been extensively studied and the binding sites in both proteins have been identified. The L binding site was mapped to aa 412–479 on SeV P (Curran et al., 1994; Smallwood et al., 1994). The P binding site on the SeV L protein was first mapped to the first 400 aa by deletion analysis of the L gene (Chandrika et al., 1995; Horikami et al., 1994). Specific residues of SeV L protein involved in P binding were identified by site directed mutagenesis within aa 21–350 of L (Holmes and Moyer, 2002). The P proteins of SV5 and rinderpest viruses similarly bind to the N-terminus of their respective L proteins (Parks, 1994; Chattopadhyay and Shaila, 2004).

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The L subunit of the RdRp complex is thought to contain all the catalytic functions required for RNA synthesis. Sequence comparisons of the L proteins from a number of non-segmented negative strand RNA viruses revealed the presence of six conserved domains which were presumed to be involved in specific activities of L proteins (Poch et al., 1990; Sidhu et al., 1993). Characterization of SeV L mutants in each of the six domains by site directed mutagenesis revealed that mutation in different domains resulted in similar or the same defective phenotype suggesting that multiple domains contribute to the different steps in viral RNA synthesis (Chandrika et al., 1995; Cortese et al., 2000; Feller et al., 2000; Horikami and Moyer, 1995; Smallwood et al., 1999, 2002). Coexpression of two inactive L mutants with mutations in different domains showed intragenic complementation restoring viral RNA synthesis suggesting that the L protein may form an oligomer. We demonstrated oligomerization of L protein directly by the coimmunoprecipitation of differentially epitope tagged full length and truncated L proteins and showed by progressive deletion from the C and N terminus that the L–L interaction was mediated by aa 1–174 (Smallwood et al., 2002; Çevik et al., 2003). The oligomerization of L protein was also shown for human parainfluenza virus 3 (hPIV3) (Smallwood and Moyer, 2004) and measles virus (Çevik et al., 2004) and the binding domain was mapped to the N-terminal half of the respective L proteins. These data suggest that oligomerization of L protein is common feature of paramyxoviruses. In recent work, moreover, the L protein of lymphocytic choriomeningitis virus, an arenavirus, was also shown to be an oligomer (Sanchez and de la Torre, 2005). In this study we show that a bipartite region within the very N-terminus is required for the oligomerization of the L protein of SeV.

Results and discussion

FLAG-L BsmI internal deletion mutant of L separates L–L and L–P binding sites

We previously demonstrated that deletion of aa 1–174 of the SeV L protein abolished both L–L and L–P binding, however, site directed mutagenesis encompassing this region showed that different amino acids in L were required for each of these interactions (Çevik et al., 2003). In addition just aa 1–174 could induce oligomerization of a heterologous protein that did not normally form a complex (Çevik et al., 2003). To further study L complex formation, aa 20–178 of L in the FLAG-L protein were deleted by BsmI digestion of the gene as described in Materials and methods. To test for oligomerization of this L mutant, VVT7 infected cells were transfected with the HA-L BaK plasmid which contains the N-terminal 1146 aa of L alone or together with the FLAG-L WT or FLAG-L BsmI plasmids and labeled with Express-^{35}S. Gel analysis of total cytoplasmic cell extracts showed that all the L proteins were synthesized (Fig. 1A, Total). Immunoprecipitation (IP) with α-FLAG antibody gave only a small background amount of HA-L BaK protein when it was expressed alone (Fig. 1A, α-FLAG, lane 2), showing the specificity of the antibody. As a positive control
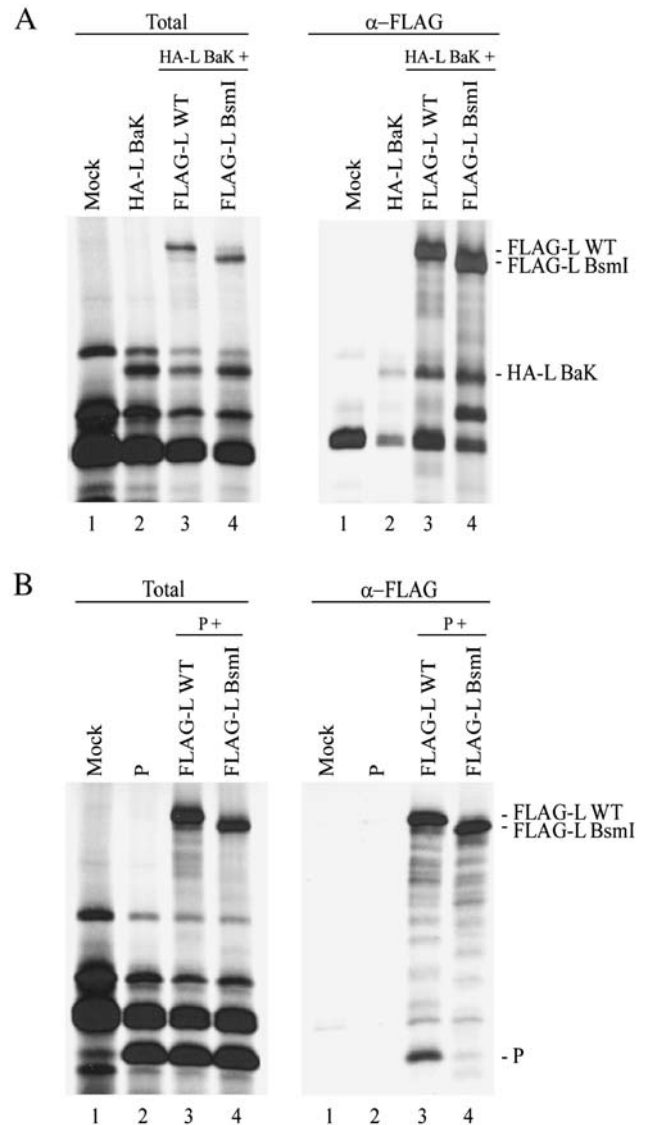

Fig. 1. L–L and P–L complex formation with an internal deletion of L. VVT7 infected cells were either not transfected (Mock) or transfected with the indicated HA-L BaK (A) or P (B) plasmids in the absence or presence of the FLAG-L WT or FLAG-L BsmI plasmids. The cells were radiolabeled with Express-^{35}S, cytoplasmic cell extracts prepared and either separated by SDS-PAGE directly (Total) or after IP with α-FLAG antibody (α-FLAG). The positions of the proteins are indicated.

HA-L BaK was coimmunoprecipitated by the α-FLAG antibody when coexpressed with FLAG-L WT, and also when expressed with FLAG-L BsmI (Fig. 1A, α-FLAG, lanes 3 and 4). The band in lane 4 just below HA-L BaK appears to be a mock band (see totals) that in just this sample was also immunoprecipitated. Only background amounts of this band were immunoprecipitated in other experiments, like in lanes 1–3 of Fig. 1B. These data suggest that the L oligomerization site resides in the N-terminal 19 aa and not in the deleted region of the L protein.

The internal deletion mutant was also tested for the ability to bind to SeV P. VVT7 infected cells were transfected with the P plasmid alone or together with FLAG-L WT or FLAG-L BsmI plasmids and pulse labeled with Express-^{35}S. Gel analysis of

the total samples showed that all the proteins were synthesized (Fig. 1B, total) and IP with α-FLAG antibody did not show any P protein when it was expressed alone (Fig. 1B, α-FLAG, lane 2). However, P protein was coimmunoprecipitated when it was coexpressed with FLAG-L WT, but not with FLAG-L *Bsm*I (Fig. 1B, α-FLAG, lanes 3 and 4) showing that the deleted region was required for P binding. These data are consistent with our previous finding that although both the P and L binding sites are located in N-terminus of L, different amino acids are involved in these interactions (Çevik et al., 2003).

Site directed mutagenesis in the N-terminus abolishes the activity of L protein

We previously demonstrated that the L proteins from SeV, hPIV3 and measles virus all form an oligomer (Smallwood et al., 2002; Çevik et al., 2003, 2004; Smallwood and Moyer, 2004), suggesting there may be conserved sequences in the L proteins involved in oligomerization. Analysis of the internal deletion at the N-terminus (Fig. 1) suggested that the first 19 aa of SeV L protein may be required for oligomerization, and sequence analysis (Poch et al., 1990; Sidhu et al., 1993) showed that aa 13–25 were highly conserved in the L protein of paramyxoviruses (Fig. 2A). To determine the importance of this region for the formation and activity of the polymerase complex, seven alanine scanning site-directed L mutations from aa 2–18 were constructed as described in Materials and methods. In a previous study mutagenesis of aa 20–25 inactivated the SeV polymerase by abolishing P but not L binding (Holmes and Moyer, 2002).

The mutant L proteins were tested for their biological activity. For transcription VVT7 infected cells were mock transfected or transfected with the P and WT or mutant FLAG-L plasmids. Cytoplasmic extracts were prepared and incubated with polymerase-free WT RNA-NP template and [α-^{32}P]CTP, and the total transcription products were analyzed by gel electrophoresis as described under Materials and methods. No transcription was observed in the absence of viral proteins indicating that the template was free of endogenous polymerase, but significant RNA synthesis occurred with FLAG-L WT protein as expected (Fig. 2B, lanes 1 and 2). FLAG-L 5–7 and 11–13 gave about one third the activity of WT L (Fig. 2B, lanes 4 and 6), however, the activity of all the other mutants including the single mutations at aa 14 and 15 was almost or completely abolished (lanes 3, 5, 7–9). Immunoblot analysis of a portion of the cell extracts showed that P protein and the FLAG-L mutants were all expressed, and although the amounts of two of the inactive L mutants (5–7 and 16–18) were somewhat reduced this did not account for their complete loss of activity. Thus conserved residues (aa 14, 16, 17) as well as non-conserved amino acids (aa 2–4, 8–10, 18) are required for polymerase function.

The L mutants were also assayed for biological activity in supporting DI-H genome replication *in vitro*. Cytoplasmic extracts of cells expressing each of the WT and mutant FLAG-L proteins together with the P and NP proteins were prepared and incubated with polymerase-free DI-H RNA-NP template in the
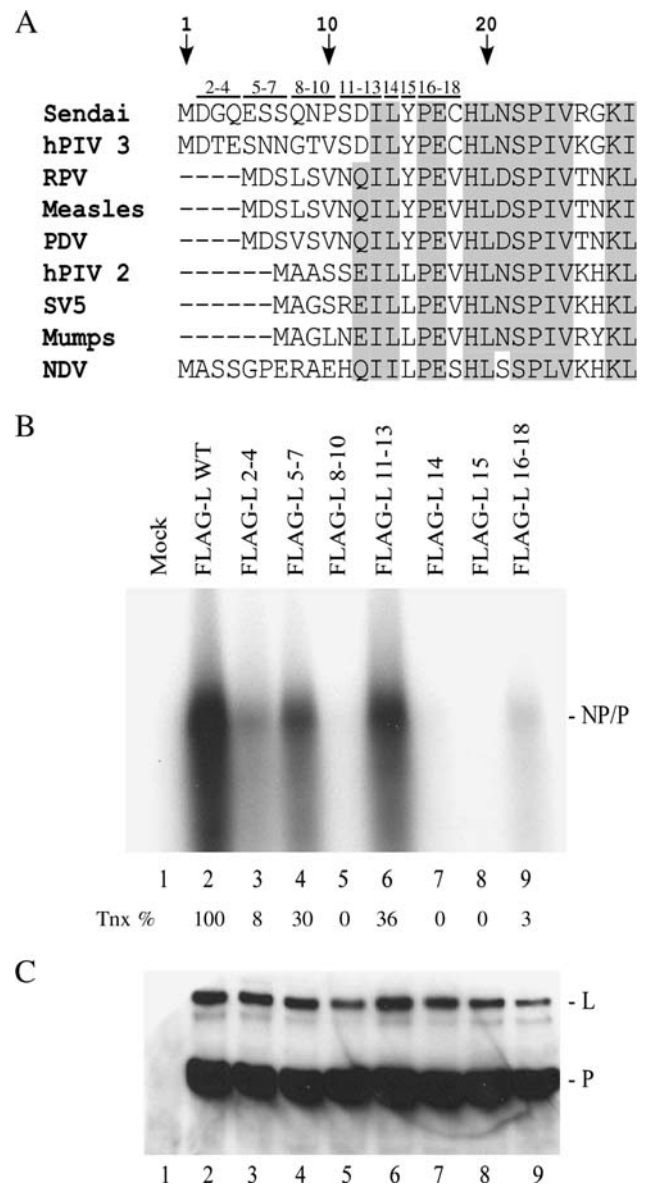

Fig. 2. In vitro transcription with site-directed SeV L mutants. (A) Comparison of sequences at the N-terminus of paramyxovirus L proteins. hPIV2 or 3, human parainfluenza virus 2 or 3; RPV, rinderpest virus; PDV, phocine distemper virus; SV5, simian virus 5; NDV, Newcastle disease virus. The positions indicated above: 2–4; 5–7; 8–10; 11–13; 14; 15; and 16–18 were changed to alanine in the mutant. (B) VVT7 infected cells were either Mock transfected or transfected with the indicated P and WT or mutant L plasmids. After 18 h cytoplasmic cell extracts were incubated with SeV 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 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%. (C) Immunoblot analysis of samples of the extracts with α-FLAG and α-P antibodies. The positions of the proteins are indicated.

presence of [α-^{32}P]CTP. The nuclease resistant replication products were isolated and analyzed as described under Materials and methods. *In vitro* DI-H RNA replication occurred with the WT L protein, but no replication occurred in the absence of viral proteins (Fig. 3A top, lanes 1 and 2) showing
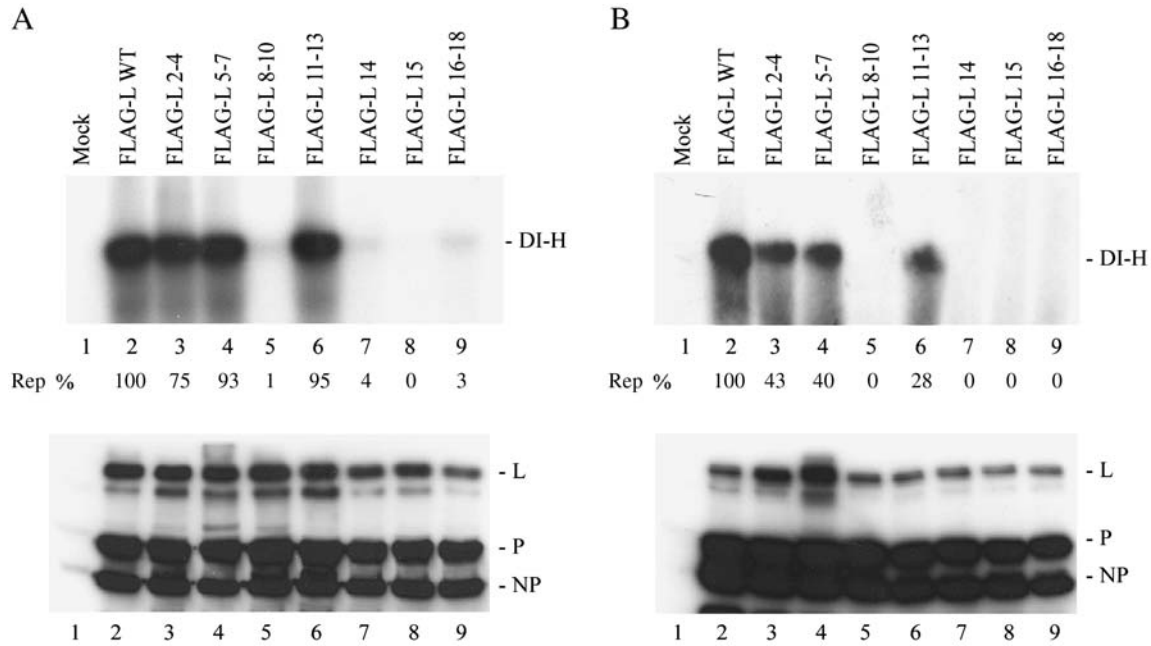
Fig. 3. *In vitro* and *in vivo* DI-H RNA replication with the site directed L mutants. (A) VVT7-infected A549 cells were transfected with no plasmids (Mock) or cotransfected with the NP and P plasmids together with the indicated WT or mutant L plasmids. Cytoplasmic cell extracts were incubated with polymerase-free DI-H RNA-NP template in the presence of [α-^{32}P]CTP. RNA was extracted from nuclease resistant nucleocapsid products and analyzed by gel electrophoresis (top) as described under Materials and methods. (B) VVT7-infected A549 cells were transfected with DI-H plasmid alone (Mock) or cotransfected with the DI-H, NP and P plasmids together with the indicated WT or mutant L plasmids. Cytoplasmic cell extracts were nuclease treated, the RNA extracted and detected by Northern blot analysis with a (−) strand specific DI-H riboprobe (top) as described under Materials and methods. The position of DI-H RNA is indicated. The amount of DI-H product (Rep) was quantitated on a phosphorimager and calculated relative to WT L as 100%, where in 2–3 experiments the values varied by <10%. (Bottom) Immunoblot analysis on samples of the extracts with α-FLAG and α-SeV antibodies, where the positions of the L, P and NP proteins are indicated.

that this template alone had no activity and replication was dependent on added viral proteins. The mutant proteins FLAG-L 2–4, 5–7 and 11–13 gave significant or close to WT replication activity (75%, 93% and 95% of FLAG-L WT protein, respectively) (Fig. 3A top, lanes 3, 4 and 6). A significant difference from the transcription results for these mutants was that FLAG-L 2–4 was active in DI-H replication but reduced by 92% in mRNA synthesis (Fig. 2). The replication activities of mutant proteins FLAG-L 8–10, 14, 15 and 16–18 were abolished (Fig. 3A, top, lanes 5, 7, 8 and 9) similar to the results with transcription (Fig. 2). Immunoblot analysis of a portion of the cell extracts showed that the L, NP and P proteins were all expressed (Fig. 3A, bottom).

The activities of the N-terminal site directed FLAG-L mutants were also tested for their activity in replication in vivo. This assay differs from its *in vitro* counterpart in that following VVT7 infection, cells were transfected with a plasmid containing a cDNA copy of the DI-H genome along with plasmids encoding P, NP and the indicated FLAG-L mutants. T7 transcription from the DI-H plasmid yields (+) sense DI-H genome RNA, which is then nonspecifically encapsidated by the NP protein encoded by pGEM-NP. The encapsidated (+) sense DI-H is then replicated *in vivo* by the viral polymerase with multiple rounds of amplification required to detect product. Extracts of the cells were prepared and digested with micrococcal nuclease to eliminate any non-encapsidated RNA. The nuclease-resistant (−) sense replication products were then isolated, separated by gel electrophoresis, and detected by

Northern blot analysis with a (+) sense DI-H genome riboprobe. In the absence of viral proteins no DI-H RNA was replicated, while full-length DI-H RNA was synthesized in the presence of the WT viral proteins (Fig. 3B top, lanes 1 and 2). The mutants FLAG-L 2–4, 5–7 and 11–13 which all showed WT levels of in vitro replication activity (Fig. 3A) gave somewhat reduced levels of in vivo replication ranging from 28% to 43% of WT L (Fig. 3B top, lanes 3, 4 and 6). This is due to the difference in the assays where in vivo replication is more sensitive to partial defects since multiple rounds of replication give an additive effect not seen in the single round of replication assayed in vitro. All the other N-terminal mutants, however, were inactive in replication in vivo (Fig. 3B top) as they were in vitro. Immunoblot analysis showed that all the proteins were expressed and the defects in the replication activity were not due to limited protein (Fig. 3B, bottom). Thus one or more of the amino acids in the blocks of 8–10 and 14–18 in the N-terminal region of L were essential for virus replication in vivo and in vitro.

The SeV L mutant changing aa 2–4 to alanine gave the interesting result that transcription was significantly impaired (only 8% of WT) while replication was still significant (75% of WT). In our previous studies of a significant number of SeV L mutants (Chandrika et al., 1995; Cortese et al., 2000; Feller et al., 2000; Horikami and Moyer, 1995; Smallwood et al., 1999, 2002) we have never identified any other mutant with this phenotype. There were a number of L mutants that were inactive in both transcription and replication as well as

some that were able to transcribe, but were defective in replication. Apparently by unknown mechanisms different mutations can differentially and selectively affect either transcription or replication. In a study of the hPIV3 L protein a deletion of aa 2–5 gave a mutant that could still both transcribe and replicate, although at 30% of the level of WT L (Malur et al., 2002), so different viruses may respond differently to mutation.

N-terminal site directed L mutants form P–L and L–L complexes

Some site directed mutagenesis in the very N-terminus of the SeV L protein eliminated or significantly reduced polymerase activity suggesting that these mutants might be unable to form functional polymerase complexes which requires both P–L and L–L interactions. The mutants were analyzed for both complexes by pull down experiments. VVT7 infected cells were transfected with P plasmid alone or together with the WT or mutant FLAG-L plasmids and incubated overnight. Immunoblot analysis of the total samples showed that all the proteins were synthesized except FLAG-L 16–18 which was quite low in this experiment (Fig. 4A). When the P protein was coexpressed with WT and mutant L proteins, IP with α-P antibody followed by immunoblotting with α-FLAG antibody showed that WT L and all the FLAG-L mutants coimmuno-precipitated with P in proportion to the amount of L protein synthesized (Fig. 4B), indicating that none of these mutations affected L binding to P protein. To confirm these results gstP was coexpressed with the L mutants and labeled with Express-^{35}S. The GST bead binding showed that gstP alone bound to the beads, while the FLAG-L WT protein alone did not (Fig. 4C, lanes 2 and 3) as expected. When coexpressed with gstP, FLAG-L WT as well as all the L mutants cobound to the beads (Fig. 4C, lanes 4–11), showing again that all the L mutants formed a complex with P protein.

To test for oligomerization of the L mutants VVT7 infected cells were transfected with the WT or mutant FLAG-L plasmids and the truncated HA-L Bak plasmid and pulse labeled with Express-^{35}S. Gel analysis of the total samples showed that all the proteins were synthesized (Fig. 5A). IP with α-FLAG antibody brought down WT FLAG-L, but not HA-L BaK, when they were expressed alone (Fig. 5B, lanes 2 and 3), however, when HA-L BaK was coexpressed with FLAG-L WT it was coimmunoprecipitated by α-FLAG antibody (Fig. 5B, lane 4). Similarly HA-L BaK was coimmunoprecipitated with α-FLAG antibody when it was coexpressed with each of the site directed L mutants (Fig. 5B, lanes 5–11). These data show that all the individual mutants in the very N-terminal region of L protein are still able to form both P–L and L–L interactions, yet for the L mutants that synthesize no RNA one or both of these interactions must somehow be defective. One possibility is that the mutants bind L or P incorrectly due to the mutations, and thus do not fold correctly giving inactive complexes. Alternatively the lack of activity could be due to an unknown defect(s) in other steps in RNA synthesis that go beyond these protein–protein interactions.
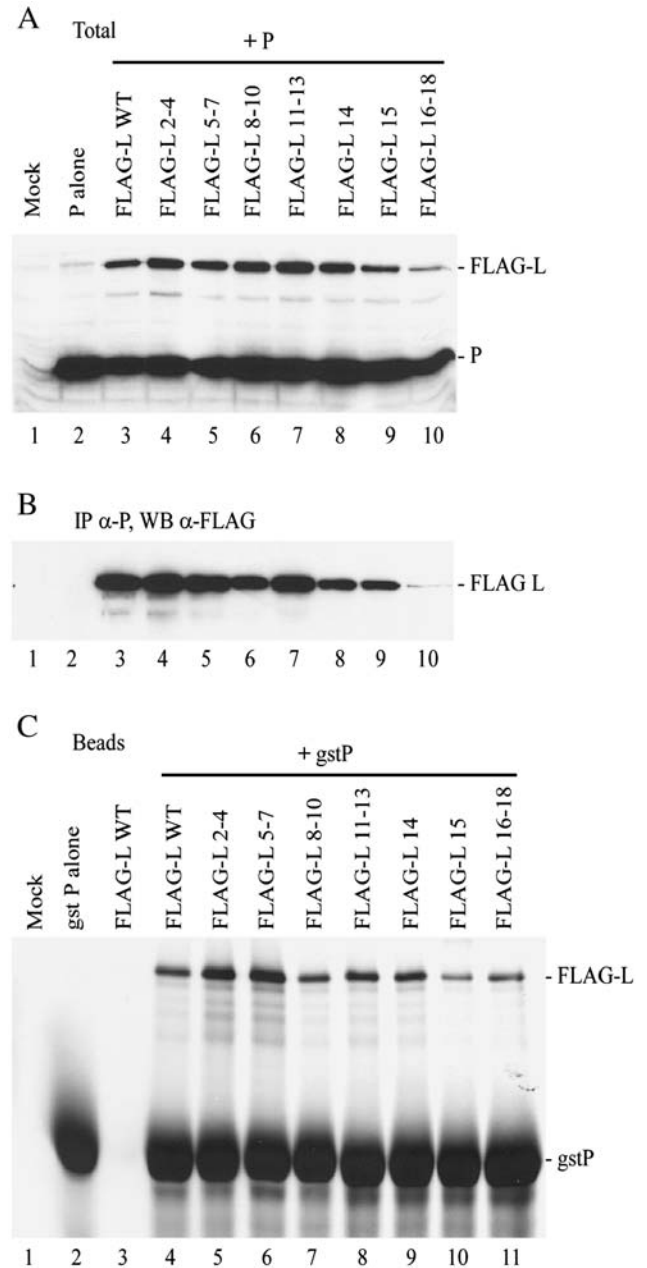

Fig. 4. P–L complex formation is not inhibited by the site-directed mutant L proteins. (A and B) VVT7 infected cells were either not transfected (Mock) or transfected with the indicated FLAG-L WT or mutant plasmids with P plasmid and incubated overnight. Cytoplasmic extracts were prepared and (A) total samples analyzed by immunoblot with α-P and α-FLAG antibodies or (B) samples were immunoprecipitated with α-P, separated by SDS-PAGE and immunoblotted with α-FLAG. (C) The cells were transfected with gstP plasmid and the WT or mutant L plasmids and radiolabeled with Express-^{35}S for 1 h at 16 h p.t. Cytoplasmic cell extracts were prepared and separated by SDS-PAGE after binding to glutathione-Sepharose beads. The positions of the proteins are indicated.

A small N-terminal deletion mutant also loses functional activity but retains protein–protein interactions

Since limited amino acid changes in the very N-terminus of L did not overtly affect the protein–protein interactions it was
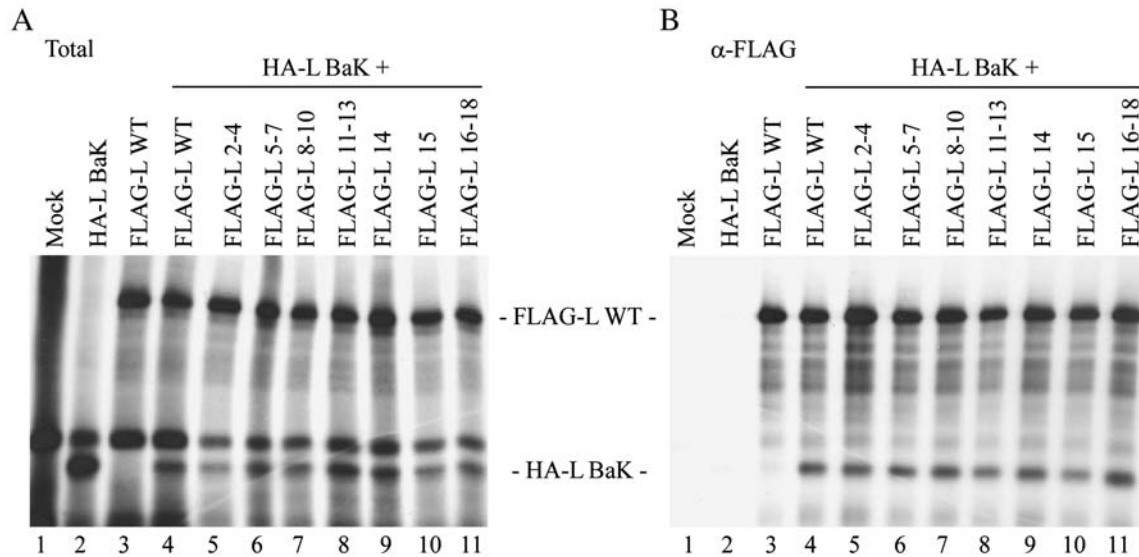
Fig. 5. L–L complex formation occurs with the site-directed L mutants. VVT7 infected cells were either not transfected (Mock) or transfected with HA-L BaK and the indicated WT or mutant L plasmids. The cells were radiolabeled with Express-^{35}S for 1 h at 16 h p.t. Cytoplasmic cell extracts were prepared and either separated by SDS-PAGE directly (A, Total) or after IP (B) with α-FLAG antibody. The positions of the proteins are indicated.

possible that deletion of the entire region might be required to actually inhibit binding of P and/or L. Accordingly we deleted aa 2–18 (Δ2–18) in FLAG-L and tested for function and protein binding. Extracts of cells expressing P with FLAG-L Δ2–18 or FLAG-L BsmI which deletes aa 20–178 gave no transcription compared to WT L (Fig. 6A). Similarly cell extracts expressing P and NP with these L deletions also were completely inactive in DI-H replication (Fig. 6B). This was expected since some of the individual changes in L Δ2–18 were inactive (Figs. 2 and 3) and the BsmI deletion could not bind P
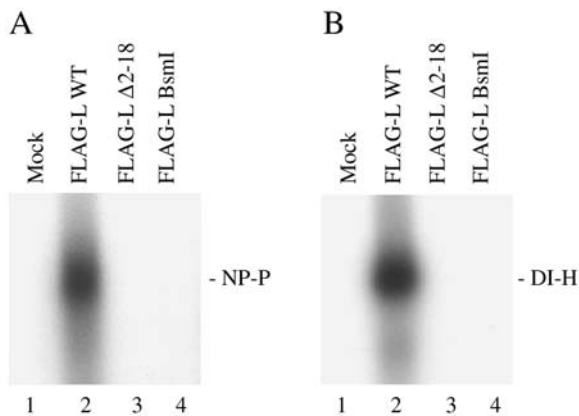

Fig. 6. Transcription and replication with SeV L deletion mutants. (A) VVT7 infected cells were either Mock transfected or transfected with the P and the indicated WT or deletion L plasmids. After 18 h cytoplasmic cell extracts were incubated with polymerase-free wt RNA-NP template and [α-^{32}P]CTP. 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. (B) VVT7 infected cells were transfected with no plasmids (Mock) or cotransfected with the NP and P plasmids together with the indicated WT or mutant L plasmids. Cytoplasmic cell extracts were incubated with polymerase-free DI-H RNA-NP template in the presence of [α-^{32}P]CTP. RNA was purified from nuclease resistant nucleocapsid products and analyzed by gel electrophoresis as described under Materials and methods. The position of DI-H RNA is indicated.

(Fig. 1). To test for P–L and L–L protein–protein interactions the Δ2–18 deletion was expressed with either gstP or HA-L BaK, respectively as described above. In each case the proteins were all expressed (Figs. 7A and B, Total). P protein bound to the deletion L Δ2–18 like WT L since each L protein cobound with gstP to glutathione beads (Fig. 7A, Beads). Interestingly L Δ2–18 also still retained the ability to oligomerize since compared to the controls, the α-HA antibody coimmunoprecipitated L Δ2–18 with HA-L BaK (Fig. 7B). Apparently sequences between both aa 2–19 and aa 20–178 can independently mediate L oligomerization, however, both are required for the activity of the protein as indicated in Fig. 8.

Similar site directed mutagenesis was performed on the conserved N-terminal region of the L protein of hPIV3 by Malur et al. (2002) to study the effect of the mutant polymerase complex on viral RNA synthesis and P protein binding. Mutation of amino acids 14 and 15 which are identical in both SeV and PIV3 gave similar results in the two L proteins where at least some P protein binding is retained, but the polymerase complex is inactive. However, in the case of PIV a deletion of aa 2–15 did abolished P binding, while for SeV L a deletion of aa 2–18 retained P binding, and as expected both proteins were inactive. Thus conserved residues in the N-terminus of L are essential for polymerase function although the details appear slightly different.

While the L proteins of a number of negative strand RNA viruses appear to form oligomers, the function and degree of L oligomerization in the polymerase P–L complex are unknown. One possibility is that oligomerization is required for the binding of Sendai L to the 4 subunits of the P protein. Moreover, L protein has multiple functions in viral RNA synthesis that may require oligomerization to create the enzymatic activities. The finding that L shows intragenic complementation (Smallwood et al., 2002) clearly demonstrates that each subunit of L in the polymerase complex is essential and can function
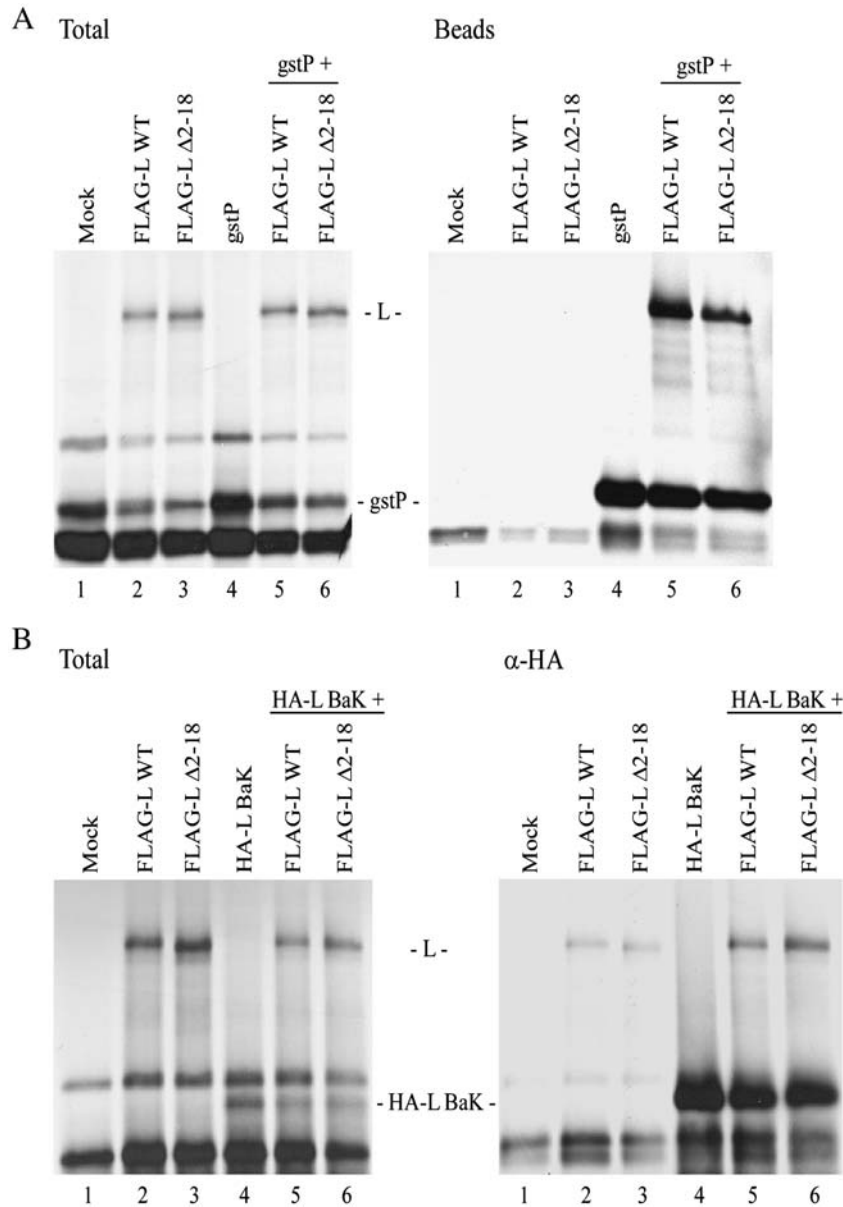
Fig. 7. P–L and L–L complex formation with the Δ2–18 L mutant. (A) VVT7 infected cells were either not transfected (Mock) or transfected with the indicated FLAG-L WT or Δ2–18 plasmids with gstP plasmid and radiolabeled with Express-^{35}S. Cytoplasmic cell extracts were prepared and either separated by SDS-PAGE directly (Total) or after binding to glutathione-Sepharose beads. The positions of the proteins are indicated. (B) VVT7 infected cells were either Mock transfected or transfected with the FLAG-L WT or FLAG-L Δ2–18 plasmids in the absence or presence of HA-L BaK plasmid and radiolabeled with Express-^{35}S. Cytoplasmic cell extracts were prepared and either separated by SDS-PAGE directly (Total) or after IP with α-HA antibody. The positions of the proteins are indicated.
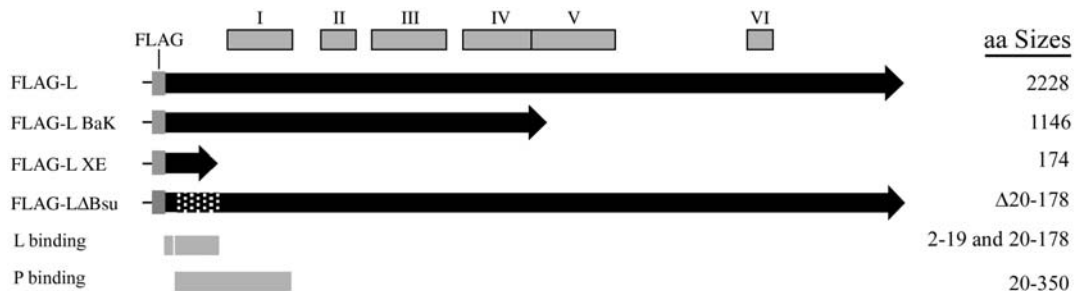

Fig. 8. Schematic diagram of L protein and the regions of protein–protein complex formation. The wild type FLAG-tagged L protein is indicated at the top with the conserved domains I–VI indicated by boxes above. The three truncated and deletion proteins are indicated below where the dotted box on FLAG-LΔBsu shows the deletion with their sizes indicated on the right. At the bottom are indicated by boxes the regions of L required for L binding and for P binding.

independently. Ultimately a crystal structure of the polymerase complex of a non-segmented negative strand RNA virus will be needed to understand its structure and function and the phenotypes of the various L mutants.

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) and the Sendai virus defective interfering particle (DI-H) were propagated on embryonated chicken eggs and wildtype (WT) polymerase-free Sendai and DI-H templates were prepared as described previously (Carlsen et al., 1985). Plasmids encoding the SeV 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 HA and FLAG-tagged SeV L plasmids, pGEM-HA-L, pGEM-HA-L BaK and pGEM-FLAG-L, were described previously (Çevik et al., 2003, see Fig. 8) and the pTM-gstP plasmid was described in Chandrika et al. (1995). The genes were all cloned downstream of the T7 promoter. Antibodies utilized for immunoblot and immunoprecipitation assays were anti-Sendai virus (α-SV) and anti-P peptide rabbit antibodies (Çevik et al., 2003), 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 L mutants

The FLAG-L *Bsm*I deletion was constructed by digestion of FLAG-L at the two *Bsm*I sites and religation to make an in frame deletion of aa 20–178 of L (see Fig. 8). The site directed mutants were constructed by changing the amino acids in FLAG-L at positions 2–4, 5–7, 8–10, 11–13, 14, 15 and 16–18 to alanine by oligonucleotide directed mutagenesis with the Quick Change Mutagenesis Kit (Stratagene). The changes were verified by sequencing. The FLAG-L Δ2–18 deletion was constructed by overlap PCR deleting aa 2–18.

Protein analysis

For the analysis of protein–protein interactions A549 cells in 35 mm dishes were infected with VVT7 at a m.o.i. of 2.5 pfu/cell for 1 h at 37 °C and then transfected with pGEM-P (2 µg) or pTM-gstP (0.1 µg) and wt or mutant pGEM-FLAG-L or pGEM-HA-L BaK (2 µg) with Lipofectamine (Life Technologies) in Opti-MEM according to the manufacturer's protocol as indicated in the figure legends. Fifteen hours post-transfection the cells were labeled with Express-[35S] (100 µCi/ml, Dupont NEN) in medium with 0.1× cysteine and methionine for 1 h. Cytoplasmic extracts were prepared in 200 µl NP-40 lysis buffer [0.15 M NaCl, 50 mM Tris–HCl (pH 8.0), 1% Nonidet P-40 (NP-40), and 1 µg/ml aprotinin]. The cell lysate was clarified by pelleting for

30 min at 15,000 rpm. For immunoprecipitation the extracts were incubated with α-HA (1 µg) or α-FLAG (2 µg) antibodies and selected with Protein A Sepharose CL 4B (Pharmacia) according to manufacturer's instructions. Samples containing gstP were selected with glutathione-Sepharose beads. Total and selected samples were analyzed by 7.5% SDS-PAGE and autoradiography. When unlabeled extracts were analyzed, samples were immunoprecipitated with one antibody and then immunoblotted with the second antibody as described below.

The samples of extracts used for RNA synthesis were separated by 7.5% SDS-PAGE and electroblotted onto PVDF membrane (Osmonics). The blots were incubated with the primary antibody, α-HA (0.4 µg/ml) or α-FLAG (4 µg/ml) antibodies or α-SeV antibody, as indicated in the figure legends and developed with HRP-conjugated rabbit anti-mouse secondary antibody or HRP-conjugated goat anti-rabbit as appropriate using the Enhanced Chemiluminescence Plus (ECL+) protein detection system (Amersham Life Science).

RNA synthesis

For *in vitro* mRNA synthesis 60 mm dishes of A549 cells were infected with VVT7 and transfected with the P (1.5 µg) and the WT or mutant L (0.5 µg) plasmids with Lipofectamine. At 18 h post transfection (p.t.) cytoplasmic cell extracts (100 µl) were prepared by lysolecithin permeabilization, micrococcal nuclease treated and assayed for transcription with 1 µg polymerase-free wt SeV nucleocapsid template (RNA-NP), and 20 µCi of [α-32P]CTP as described previously (Chandrika et al., 1995; Horikami et al., 1992). 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-citrate gel electrophoresis.

For *in vitro* replication 60 mm dishes of A549 cells were infected with VVT7 and transfected with the WT or mutant L (0.5 µg), P (5 µg) and the NP (2 µg) plasmids and cell extracts assayed for replication with 50 µCi [α-32P]CTP, and 2 µg polymerase free DI-H RNA-NP as described previously (Chandrika et al., 1995). The samples were then digested with micrococcal nuclease (MN) (20 µg/ml) with 1 mM CaCl$_2$ for 30 min at 30 °C followed by 2.2 mM EGTA to inactivate the MN. The nuclease-resistant nucleocapsid RNA was isolated using the Qiagen RNeasy Total RNA kit, and analyzed by electrophoresis on a 1.5% agarose/6 M urea-citrate gel.

For *in vivo* replication, infected cells were transfected as for *in vitro* replication with the L, NP and P plasmids with the addition of plasmid pSPDI-H (2.5 µg). Extracts were prepared, treated with micrococcal nuclease as above, and the nuclease-resistant nucleocapsid RNA isolated using the Qiagen RNeasy™ Total RNA kit. The RNA was separated on a 1.5% agarose/6 M urea gel and transferred to Hybond-N nylon membrane and the DI-H replication products (− sense) were detected by Northern Blot analysis with 32P-labeled T7 DI-H transcript (+ sense) generated from *Xba*I-linearized pSPDI-H plasmid as described previously (Chandrika et al., 1995). The RNA products were visualized by autoradiography and quantitated on the phosphorimager (Molecular Dynamics).

Acknowledgment

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