JOURNAL OF VIROLOGY, Feb. 1995, p. 849–855 Vol. 69, No. 2
0022-538X/95/$04.00+0

An N-Terminal Domain of the Sendai Paramyxovirus P Protein Acts as a Chaperone for the NP Protein during the Nascent Chain Assembly Step of Genome Replication

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Received 11 July 1994/Accepted 4 November 1994

Two domains involved in RNA synthesis have recently been found within the N-terminal 77 amino acids of the Sendai virus P protein. One domain is required for RNA synthesis per se and has properties in common with the transactivation domains of cellular transcription factors. The second domain is thought to be specifically required for the nascent chain assembly step in genome replication. We have further mapped this second domain by the construction of chimeric and deleted P proteins to amino acids 33 to 41 of P and by examining the abilities of these P proteins to support DI genome replication in vivo. Using glycerol gradient sedimentation, we have shown that this domain is required to form a stable complex with unassembled NP (P-NP^0) and to prevent NP from assembling illegitimately, i.e., independently of the concurrent assembly of a nascent viral genome. Since the P-NP^0 complex represents the functional form of unassembled NP which is delivered to the nascent chain during genome replication, and since amino acids 33 to 41 are not required for the stable interaction of P with the assembled NP of the nucleocapsid, this chaperone function of P is not required for mRNA synthesis or the RNA synthesis step of genome replication.

Paramyxoviruses contain nonsegmented negative-strand RNA genomes which are found as helical nucleocapsids that are assembled with the nucleocapsid protein NP. These core nucleocapsids (NP-RNA), rather than free RNA, are the templates for both mRNA synthesis and genome replication. Using NP-RNA templates and cell extracts in which the various viral proteins were expressed by plasmid transfection, only the P and L proteins, which form a polymerase complex (P-L), were found to be required for mRNA synthesis (4). Genome replication, in contrast, requires the additional participation of NP, which is consistent with the idea that genome synthesis (unlike that of mRNAs) is coupled to the concurrent assembly of the nascent chain (2, 11, 33). Unassembled NP (NP^0), however, is thought to function as a P-NP^0 complex, since only NP which has been coexpressed with P in vivo can function in genome replication in vitro, and this activity correlates with P-NP complex formation (3, 11).

Genome replication can be reconstituted in vitro either with extracts in which all three proteins have been coexpressed, or by combining extracts in which P/NP and P/L have been separately coexpressed; all other combinations were inactive (11). It has thus been possible to coexpress mutant and wild-type forms of P with either NP or L and thereby experimentally separate the P protein domains required for RNA synthesis per se (together with L) from those required for nascent chain assembly (together with NP) (5). For example, a region in the C-terminal half of P (amino acids [aa] 413 to 445 [Fig. 1]) which was defined by deletion analysis as being required for stable binding to L (5, 29) was found to be required for the RNA synthesis step of replication (together with L) but not for the nascent chain assembly step (together with NP). On the other hand, a region at the N-terminal end of P (aa 1 to 77), which was originally identified because it was required for genome replication as a whole but not for mRNA synthesis, was found to be required for the nascent chain assembly step of replication (together with NP) but not for the RNA synthesis step. In further contrast, P protein missing the C-terminal 29 residues appeared to be inactive in both steps of genome replication.

During these studies (5), another region near the N-terminal end of P (aa 78 to 144) which had unusual properties was uncovered. For example, aa 78 to 144 were required for mRNA synthesis in the absence of aa 1 to 77 but could be deleted as long as aa 1 to 77 were present. The segment from aa 78 to 144, furthermore, could not replace the segment from aa 1 to 77 for genome replication. Given this unusual situation, we postulated that aa 1 to 77 contained two (possibly separate) activities. One is involved in RNA synthesis per se (i.e., mRNA synthesis and the RNA synthesis step of genome replication) and is redundant in that it can be replaced by aa 78 to 144. The other, which is unique, is presumably involved in the nascent chain assembly step of replication. This paper further explores the nature of the latter domain by examining its requirement for genome replication in vivo and for P-NP^0 complex formation.

MATERIALS AND METHODS

Construction of subclones. Many of the subclones used in this study have been previously described (2, 5, 22). All Sendai virus (SeV) mutant P genes were constructed in a background containing the influenza virus HA1 epitope tag (8) fused at the N terminus of the protein (pGEM-P^{HA1}), and this tagged P protein was found to be fully active in all aspects of RNA synthesis (5). The series of nine aa deletions in the N terminus of P were introduced into pGEM-P^{HA1} by fusion PCR, essentially as described by Mikaelian and Sergeant (18).

The chimeras of the SeV and bovine parainfluenzavirus type 3 (bPIV3) P proteins (P3/Se and Se/P3 [see Fig. 6A]) were prepared by introducing an *Xba*I site at nucleotide 1069 in pGEM-P/C of bPIV3 (22, 28); a *Xba*I site was already present in an equivalent position (nucleotide 1028) within the SeV gene. Domain exchanges were then made using this common site. For the P3/Se chimera shown in Fig. 2B, the first 83 aa of the SeV P protein were substituted with the first 79 aa of the bPIV3 protein by replacing a *Sac*I-*Cla*I fragment of pGEM-P/C(SeV) with a *Sac*I-*Hpa*I fragment from pGEM-P/C(bPIV3).

RNA synthesis in vivo. DIH4^{uv} amplification in vivo was performed as de-

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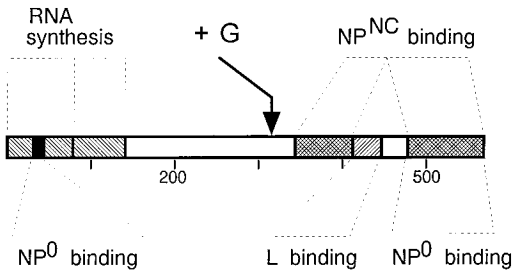

FIG. 1. Schematic representation of the SeV P protein. The P protein (568 residues) is represented by a rectangle. The various domains identified from this work and previous work (as described in the text) are indicated by boxes within the rectangle that are shaded. Two blocks of residues at the N terminus (aa 1 to 77 and 78 to 144) are involved in RNA synthesis; aa 33 to 41 within the first block is also involved in NP^0 binding. Two blocks with the C-terminal half (aa 345 to 411 and 479 to 568) are involved in binding to NP^{NC}; the latter block is also required for NP^0 binding, but the former block is not (unpublished data). Residues 412 to 445 represent the L protein binding site. The heavy arrow above indicates the site at which the alternate C-terminal open reading frames of the V and W proteins are fused in place by mRNA editing.

scribed previously (2). Briefly, CV1 monolayers in 9-cm dishes were infected with 2 to 5 PFU of a vaccinia virus recombinant expressing T7 RNA polymerase (vTF7-3 [9]) per cell. At 1 h postinfection, infecting medium was replaced with a transfection mix composed of 2 ml of modified Eagle medium and 15 μl of TransfectACE (24) combined with the following plasmid DNAs: 2.5 μg of pGEM-NP, 1.5 μg of tagged pGEM-P^{wt} or P^{Δ}, or 5 μg of untagged P plasmid and 1 μg of pGEM-L, except as indicated. After 3 h at 33°C, the monolayers were infected with $DIH4^{uv}$ and incubated at 33°C for 18 to 20 h. The cells were then solubilized in 150 mM NaCl–50 mM Tris (pH 7.4)–10 mM EDTA–0.6% Nonidet P-40 (lysis buffer). Nuclei were removed by pelleting at 12,000 × *g* for 5 min. Nucleocapsids were recovered by banding the extracts on linear 20 to 40% CsCl gradients. The RNA was phenol extracted and precipitated with ethanol. Nucleocapsid RNA from half a dish was resolved on a 1.5% agarose-HCHO gel, transferred to nitrocellulose, and probed with a ^{32}P-labeled (+) riboprobe transcribed from pEX5′ (19).

Glycerol gradient analysis of in vivo-expressed proteins. Cytoplasmic extracts were prepared from HeLa cells as described by Curran et al. (3, 4) and Horikami et al. (11). Briefly, 5-cm cell monolayers were infected with vTF7-3 and transfected with plasmid DNAs as described above (2 μg of pGEM-NP and 1.5 μg of tagged pGEM-P^{wt} or P^{Δ} mutant subclones). At 24 h postinfection, the monolayers were washed once in ice-cold phosphate-buffered saline before incubation on ice with 1 ml of buffer A (5% sucrose, 80 mM KCl, 35 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4], 5 mM K_2PO_4 [pH 7.4], 5 mM $MgCl_2$, 0.5 mM $CaCl_2$) containing 250 μg of lysolecithin (Sigma) per ml for 1 min. Buffer A was removed, the cells were scraped into 200 μl of RM salts (100 mM HEPES [pH 8.5], 150 mM NH_4Cl, 4.5 mM MgAc, 1 mM dithiothreitol), and the cell membranes were disrupted by pipetting up and down 20 times. Nuclei and cellular debris were removed by spinning at 12,000 × *g* for 5 min at 4°C. Extracts were layered onto linear 5 to 20% (vol/vol) glycerol gradients prepared in RM salts and containing a 100-μl cushion of 65% sucrose-D_2O. Gradients were centrifuged in an SW60 rotor at 40,000 for 18 to 24 h at 4°C. Gradient fractions (400 μl) were collected from the bottom of the tube, and 15-μl aliquots were analyzed by Western blotting (immunoblotting)–enzyme-linked immunosorbent assaying using the chemiluminescent substrate AMPPD (Boehringer).

Antibodies. A monoclonal antibody to an epitope of the influenza virus HA1 protein, which was designated 12CA5 (8), was obtained from the Berkeley Antibody Co. and is referred to in this work as anti-HA. The monoclonal antibody NP877 (21), which recognizes an epitope near the C terminus of NP, was kindly provided by Claes Orvell, Stockholm, Sweden. The monoclonal antibody to a C-terminal peptide of the L protein (7), which is referred to as anti-L, was kindly provided by this laboratory.

RESULTS

Chimeric P proteins. SeV, which is also referred to as murine PIV1 (mPIV1), and human PIV1 (hPIV1) are closely related viruses; along with the human and bovine strains of PIV type 3 (PIV3), these viruses form the *Parainfluenzavirus* genus of the *Paramyxoviridae* family. Although the N-terminal halves of these PIV P proteins are poorly conserved (17), their functions are presumably better conserved. If so, it should be possible to construct P proteins which are chimeras between those of SeV and the other viruses, and whether or not these chimeric proteins are active should yield additional information about these domains. We began by determining that within the SeV background (i.e., with the SeV NP and L genes), the entire SeV P protein could be replaced with that of hPIV1 for both transcription and replication, with only a modest loss of activity, therefore, SeV chimeras containing hPIV1 sequences would not be informative. However, replacement with the bPIV3 protein ablated all activity (not shown); hence, the bPIV3 P protein was a suitable candidate for this approach.

We began by exchanging the entire N-terminal halves of the SeV and bPIV3 P proteins, using a unique *Xba*I site at aa 308 in the SeV gene (within the hypervariable central region) and an engineered *Xba*I site at aa 331 of the PIV3 gene. The activities of these chimeric P proteins were examined by cotransfecting the corresponding genes along with either the SeV or bPIV3 L genes (and the SeV NP gene) into cells infected with $DIH4^{uv}$ (a viral stock in which the nondefective helper virus was selectively inactivated by UV irradiation), and their abilities to amplify the DI genome were determined (2). This is the most stringent of our tests for P, since P must participate in repeated rounds of genome replication. We found that within a completely SeV background (i.e., with the SeV NP and L genes), aa 1 to 308 of the SeV protein could be replaced with aa 1 to 331 of bPIV3 P with only a twofold reduction in activity (Fig. 2A). The activity of this bPIV3/SeV (P3/Se) protein chimera was abolished in the presence of the bPIV3 L gene, and all other combinations of chimeric P genes and homologous L genes (plus the SeV NP gene) were equally inactive. Examination of the various P protein levels by Western blotting indicated that all of the P proteins had accumulated to approximately the same extent (to within a factor of 2 [data not shown]).

The P gene functions required for RNA synthesis and nascent chain assembly in the N-terminal region of the protein have thus been conserved between these two viruses, even though there is little homology present in these amino acid sequences. This may not be surprising for the RNA synthesis function, since the SeV gene apparently carries a redundant sequence (aa 78 to 144) which is at best only partially related. However, the nascent chain assembly function is unique to aa 1 to 77 of SeV; therefore, we subsequently determined whether this function was located at a similar position in the bPIV3 protein. This was examined by substituting aa 1 to 83 with aa 1 to 79 of bPIV3, and this chimeric P protein was also found to be relatively active in supporting DIH4 amplification (Fig. 2B). The nascent chain assembly function of P is thus located in similar regions of both proteins.

When the P proteins of SeV and bPIV3 are aligned, the N-terminal halves are found to be 25% identical, and the first 77 residues have even less conservation (only 11% identity). However, a closer inspection of the alignment in this region shows an 11-aa region which is better conserved, in which only 2 of the 11 aa are strictly conserved but 6 more are conservative replacements (Fig. 3C). We were interested in whether the nascent chain assembly function could be mapped to a specific sequence such as this or whether, like the acidic activation domain of Gal4 (16) and possibly the redundant SeV RNA synthesis function, it could not be assigned to a specific region because there were redundant elements. Therefore, we scanned aa 1 to 77 of SeV by introducing a series of short amino acid deletions (Δ5-11, Δ11-18, Δ19-25, Δ29-32, Δ33-41, Δ42-50, Δ51-59, and Δ60-69) and again tested the abilities of these P proteins to participate in the amplification of DIH4. As shown in Fig. 3, all of the deletion mutants except one (Δ33-41) showed nearly wild-type activity, whereas Δ33-41 showed no

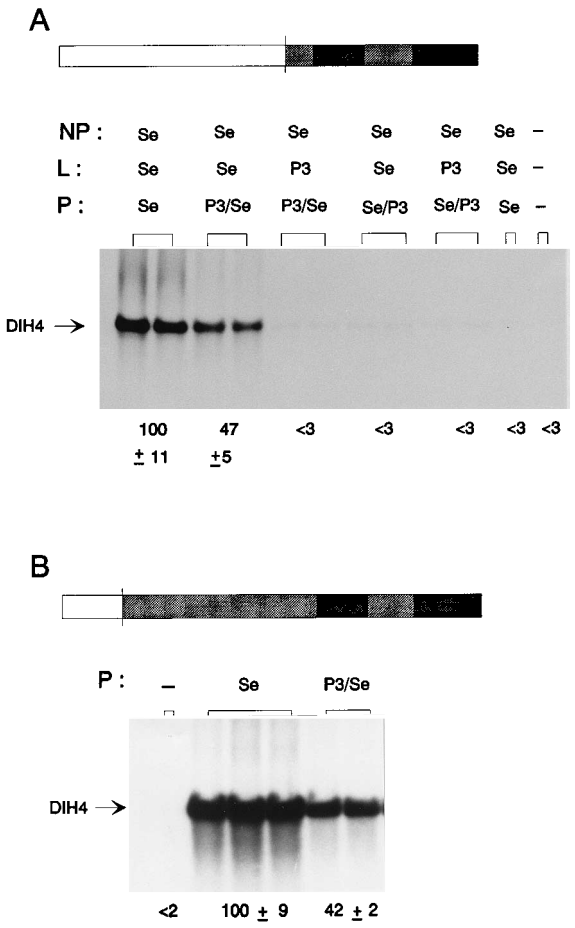

FIG. 2. Activities of chimeric SeV/bPIV3 P proteins in genome replication. Chimeric P proteins (Materials and Methods) containing sequences of both bPIV3 (white boxes) and SeV (shaded boxes) were coexpressed along with the SeV (Se) and bPIV3 (P3) L genes and the SeV NP gene (as indicated) in cells which were also infected with DIH4^{uv}. Genomic RNAs were then isolated on CsCl gradients, and their amounts were estimated by Northern (RNA) blotting. (A) The double lanes represent duplicate transfections. The active chimeras are listed above the lanes, and the two single lanes on the right represent controls lacking DIH4^{uv} infection or the NP, P, and L genes, respectively. (B) A different chimera, in which residues 1 to 83 of SeV were replaced by residues 1 to 79 of bPIV3, was coexpressed along with the SeV NP and L genes. In the control left-hand single lane, the P gene was left out. The darker shaded regions represent the two NP^{NC} binding sites. The numbers below each set of lanes show the relative intensities of the bands and the ranges of the duplicate (and triplicate) transfections, as determined in a PhosphorImager.

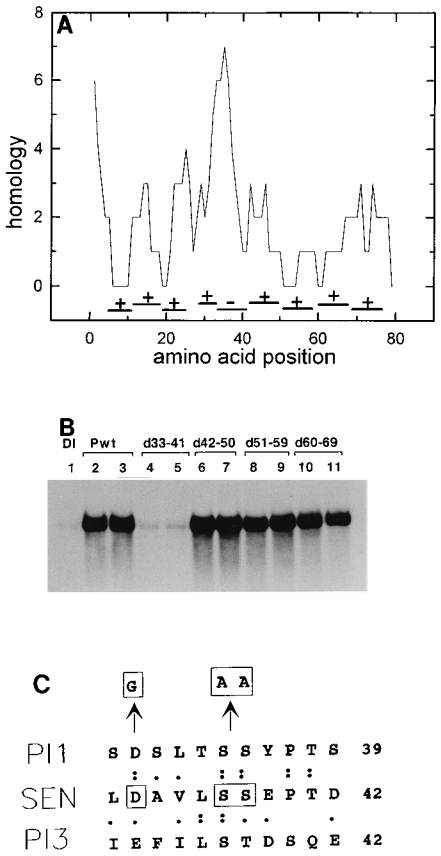

FIG. 3. Mapping of the nascent chain assembly function. A series of short amino acid deletions (Δ5-11, Δ11-18, Δ19-25, Δ29-32, Δ33-41, Δ42-50, Δ51-59, and Δ60-69) were made within the N-terminal 77 aa of the SeV P protein; their positions are indicated by the boldface horizontal lines at the bottom of panel A. (A) Homology between the SeV and bPIV3 P proteins over the N-terminal 80 aa. The homology was determined by moving a window of 5 aa through the alignment of the two sequences; two points were given for an exact match and one point was given for a conservative replacement (see panel C). The deleted P proteins were tested for their abilities to support in vivo amplification of DIH4 as outlined in Materials and Methods. ±, results from all of the mutants tested. (B) Northern blot analysis from a selection of these P mutants. Lane 1 (DI) is a control from which the NP, P, and L genes were omitted. d, Δ. (C) depicts the primary sequence of the 11-aa domain in SeV found by the homology search, as discussed in the text. Single point mutations introduced into the SeV sequence, as discussed in the text, are indicated by closed boxes. PI1 and PI3, sequences from the PIV1 and PIV3 P gene, respectively.

activity at all. (The weak bands visible in lanes 4 and 5 of Fig. 3 are also found at the same level in control lane 1 and represents the nonamplified input genomes [2]). It is unlikely that the ablation of genome replication by deletion of aa 33 to 41 is due to a longer-range conformational effect, since most of the remainder of this region was scanned by deletional analysis (including Δ29-32 and Δ42-50, which flank Δ33-41) and this did not uncover other sequences that appear to be important. aa 33 to 41 are, therefore, more likely to be directly involved in nascent chain assembly. We then tried to identify the critical residues within this sequence. Inspection of the aligned PIV1, SeV, and PIV3 sequences (Fig. 3C) shows that all three proteins contain an invariant Ser followed by either a Ser or Thr in the middle of the critical region. Given the limited conservation here, we examined whether these two residues were important as sites for phosphorylation. However, we found no loss of activity of the protein when both Ser residues were simultaneously changed to Ala (not shown). The Asp-33 in the SeV protein is also conserved in the other related proteins (as either Asp or Glu); therefore, we also examined the effect of changing this residue to Gly, a nonconservative replacement. However, this protein also did not display an altered activity in our standard test. Although only three of the nine residues have so far been tested, it is possible that our inability to identify critical residues within this limited sequence is a result of the fact that it is the peptide backbone (rather than the conserved side chains themselves) that is interacting directly with the transcription machinery. The substitutions that we have made would apparently not perturb the structure in such a way as to disrupt such a backbone-mediated interaction. However, further mutations in this region will be required to examine this possibility.

The nascent chain assembly function of P correlates with its ability to bind to NP^0. The P protein is known to bind tightly

to nucleocapsids, and Ryan and Portner (25) have mapped the sequences required for this interaction to the C-terminal half of P. Two noncontiguous regions (aa 345 to 411 and aa 479 to 568) were identified for this function (Fig. 1), and, remarkably, a recombinant protein containing only these two regions fused together could bind to nucleocapsids (26). Cytoplasmic extracts of infected cells were used as the source of the nucleocapsids (25, 26), and since these holonucleocapsids contained approximately 300 P proteins and 50 L proteins in addition to the NP-RNA core (15), the nucleocapsid protein which bound to the C terminus of P was not determined in these studies. We have recently repeated some of these binding studies using core nucleocapsids devoid of the P and L proteins, and since our results are similar to those reported previously, it appears that these regions of P are in fact required for binding to the NP protein of the nucleocapsids (NP^{NC}) (14). Thus, the only regions of P known to bind NP are located in the C-terminal half of the chain.

The fact that deletions at the N terminus of P ($P^{\Delta 1-77}$ and $P^{\Delta 33-41}$) specifically ablate the nascent chain assembly step of genome replication suggests that this region might be interacting with NP^0. Moreover, since NP^0 is thought to function as a P-NP^0 complex, an attractive explanation of these results is that a binding site for NP^0 is located at the N terminus of P. Therefore, we examined this interaction (in the absence of nonionic detergents [12]) using glycerol gradient sedimentation of transfected cell extracts (11). As shown in Fig. 4B and C, when P is expressed alone under these conditions, it sediments as a relatively homogeneous band (fractions 5 and 6) and none of the protein is found at the bottom of the tube. When P is coexpressed with NP, its sedimentation increases (it is found predominantly in fractions 3, 4, and 5) and P cosediments with a band of the coexpressed NP protein. Fifteen percent of P is also found at the bottom of the tube.

When NP is expressed by itself, the majority of NP (65%) is found at the bottom of the tube (see Fig. 6B), and this presumably represents the self-assembled form of NP. We have not examined this material in the electron microscope; however, others have shown that NP expressed by itself can form helical nucleocapsid-like structures (30) and that there is a close correlation between the regions of NP required for the nascent chain assembly step of genome replication and this illegitimate self-assembly of NP when it is expressed by itself (1). There is also a small peak of NP near the top of the gradient (fractions 7 and 8 [see Fig. 6B]), possibly representing a monomeric form of the protein. When NP and P are coexpressed (Fig. 4), <30% of NP is found at the bottom of the tube, the small peak at fraction 8 (see Fig. 6) is much reduced, and >60% of NP is found near the middle of the gradient, cosedimenting with P (Fig. 4). These results indicate that P forms a complex with NP, and since this complex sediments only slightly faster than P protein expressed by itself, this represents a complex with the unassembled form of NP. P-NP^0 complex formation also prevents NP from aggregating (12, 23). The fact that some P (ca. 15%) is found at the bottom of the tube when it is coexpressed with NP (Fig. 4) is consistent with the finding that P can also bind to the self-assembled form of NP. All of these results agree well with those reported previously (11).

When $P^{\Delta 1-77}$ is expressed by itself, it sediments like wild-type P, i.e., mostly in fractions 5 and 6 (Fig. 5B). However, when $P^{\Delta 1-77}$ is coexpressed with NP, its sedimentation rate is unaffected by the presence of the coexpressed NP (Fig. 5A and C). Moreover, the presence of the coexpressed P does not reduce the amount of NP found at the bottom of the tube, which remains at >60% (Fig. 5 and 6). $P^{\Delta 33-41}$ behaves similarly to

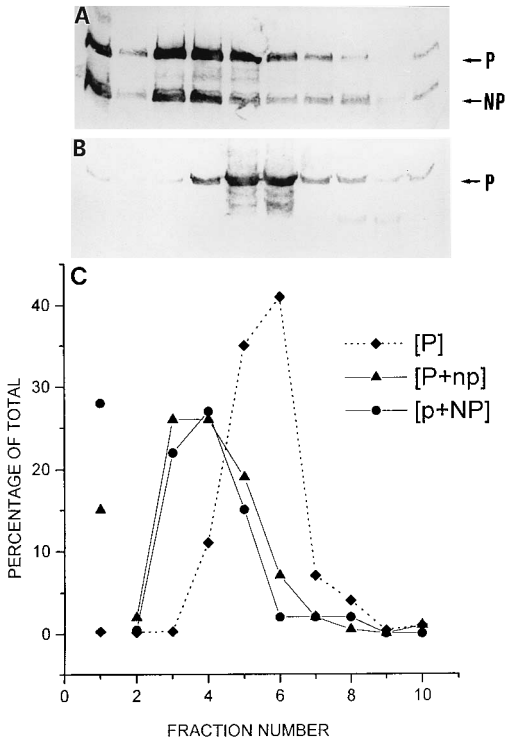

FIG. 4. Glycerol gradient analysis of the P-NP^0 complex. HeLa cells were infected with vTF7-3 and transfected with either P^{wt} or P^{wt} and NP. Cytoplasmic extracts were prepared and fractionated on linear 5 to 20% glycerol gradients, and aliquots from each fraction were analyzed by immunoblotting (Materials and Methods). (A) Western blot analysis of the P plus NP gradient using monoclonal antibodies against the epitope-tagged P protein (the more slowly migrating band) and the NP protein (bottom band). (B) Western blot analysis of the P gradient using the anti-tag monoclonal antibody. (C) The bands in panels A and B were quantitated on a densitometer, and the amount in each fraction was then plotted as a percentage of the total. Fraction 1 contains the material which pelleted onto the 65% sucrose-D_2O cushion which was placed at the bottom of each gradient. P+np, profile of the P protein when P and NP were coexpressed; p+NP, profile of the NP protein.

$P^{\Delta 1-77}$ in these respects (Fig. 7); however, we also note that less NP is found in the pellet when it is coexpressed with $P^{\Delta 1-77}$ and $P^{\Delta 33-41}$ than when it is expressed alone (Fig. 6 and 7) and that there is a small amount of NP (12%) which cosediments with $P^{\Delta 1-77}$ (Fig. 5, fraction 5). These results suggest that the N-terminally deleted P proteins can no longer interact stably with NP^0 but that these deletions may not have eliminated all P-NP interactions. We also note that 15 to 20% of the deleted P proteins are still found at the bottom of the tube, and this suggests that although they can no longer form a stable complex with NP^0, they may still be able to bind to the self-assembled form of NP. In other experiments (data not shown), $P^{\Delta 1-77}$ was found to bind to core nucleocapsids as tightly as the wild-type protein.

The C-terminal sequences of P are also required for P-NP^0 complex formation. We previously reported that aa 539 to 568 of P were required for a functional P-L complex, presumably because these residues are required for binding to the nucleocapsid template (2). However, these residues were also found to be required for the nascent chain assembly step of replication (5), indicating that they may play a role in interacting with NP^0. This putative interaction was also examined by glycerol gradient sedimentation. As shown in Fig. 8, when $P^{\Delta 539-568}$ (listed as PNde) is expressed by itself, it sediments similarly to P^{wt} and $P^{\Delta 1-77}$ (Fig. 8B, fractions 5 and 6), but there is also a

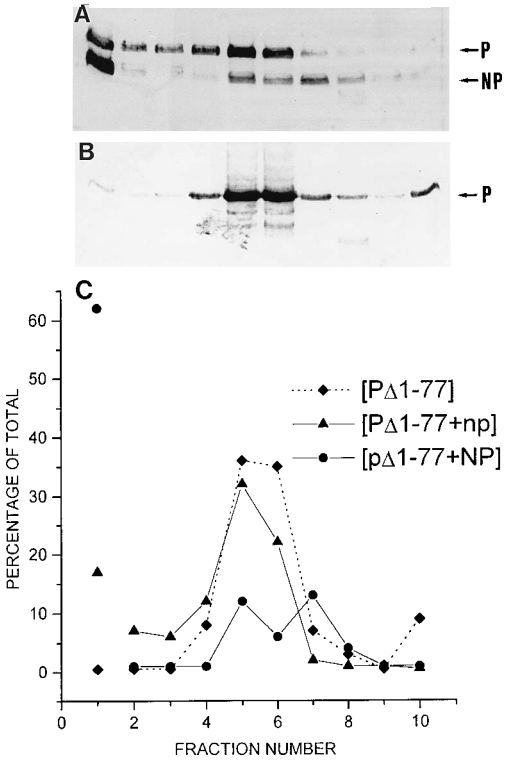

FIG. 5. Glycerol gradient analysis of the $P^{\Delta 1\text{-}77}$-NP^0 complex. Panels are as those described in the legend to Fig. 4, except that the interaction of $P^{\Delta 1\text{-}77}$ was examined.

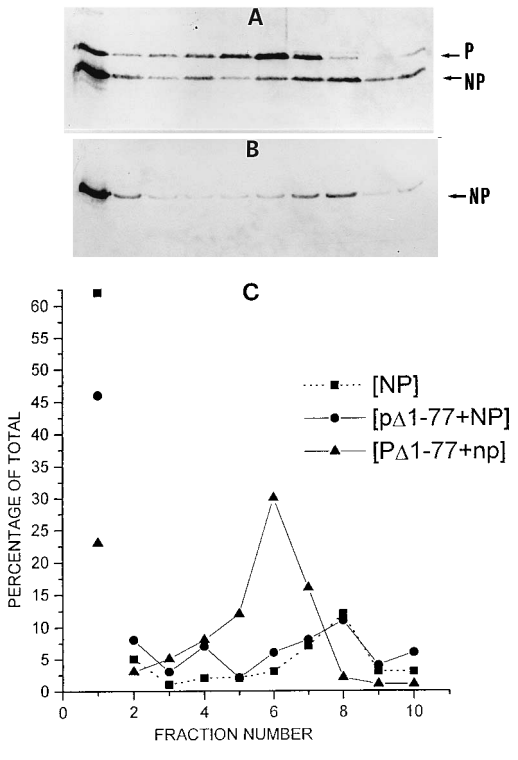

FIG. 6. Glycerol gradient analysis of the $P^{\Delta 1\text{-}77}$-NP^0 complex. Panels are as those described in the legend to Fig. 4, except that NP expressed by itself (rather than P expressed by itself) was examined (shown in panel B).

small amount of $P^{\Delta 539\text{-}568}$ (ca. 8%) at the bottom of the tube. When $P^{\Delta 539\text{-}568}$ and NP are coexpressed (Fig. 8A), the presence of the coexpressed NP shifts a small fraction of the P protein to a slightly faster rate, but less than that which occurs on coexpression of P^{wt} (Fig. 4). This indicates that these C-terminal residues are also required for the formation of a stable P-NP^0 complex. We also note that the amount of $P^{\Delta 539\text{-}568}$ at the bottom of the tube has been reduced to <1% of the total, which is consistent with the requirement of these residues for the binding of P to NP^{NC}. It is also clear that the amount of NP at the bottom of the tube when it is coexpressed with $P^{\Delta 539\text{-}568}$ has been reduced to levels similar to those seen in the presence of P^{wt} (<30%) and that a large fraction of NP is found to sediment within the gradient (Fig. 8, peaks at fractions 3 and 5). These peaks of NP could result from an interaction between NP and the deleted P proteins, which, although they cannot form stable complexes, may nevertheless affect the way in which NP interacts with itself.

Interaction between N-terminal half of P and the L protein. During our studies with the chimeric P proteins, we noticed that the ability of the bPIV3/SeV chimera (Fig. 2A) to function in genome replication was more sensitive to the levels of L expression than P^{wt}. As shown in Fig. 9, similar levels of genome replication occurred in this in vivo test when between 0.1 and 1 μg of pGEM-L per dish was used with P^{wt}; however, replication increased fivefold between these amounts of pGEM-L when the bPIV3/SeV chimera was used. The L protein levels in cytoplasmic extracts of each transfection were determined by immunoblotting (Materials and Methods), and L protein was found to be expressed equally in the presence of either P protein (not shown). That this chimera requires higher levels of L to carry out the same amount of genome replication as P^{wt} could mean that the nature of the N-terminal half of P can indirectly alter P-L interaction via the stable L binding site (aa 412 to 445). However, these results could also mean that the N-terminal half of P is directly interacting with L. This

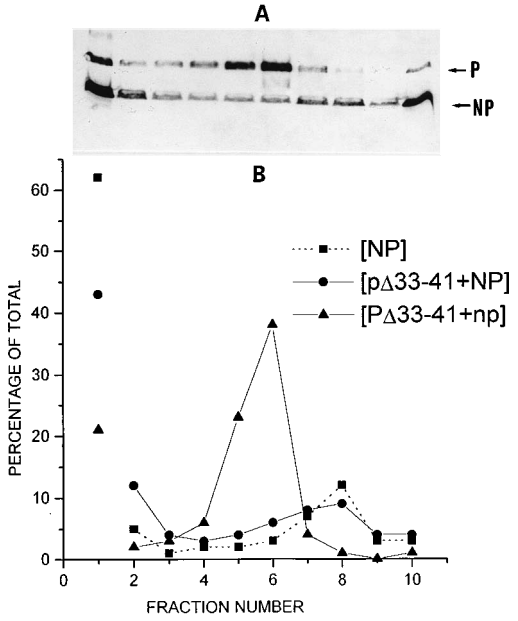

FIG. 7. Glycerol gradient analysis of the $P^{\Delta 33\text{-}41}$-NP^0 complex. Panels are as described in the legend to Fig. 4, except that the $P^{\Delta 33\text{-}41}$-NP^0 interaction was examined; only the immunoblot of the coexpressed proteins is shown. The profile of NP expressed by itself is reproduced from Fig. 6.

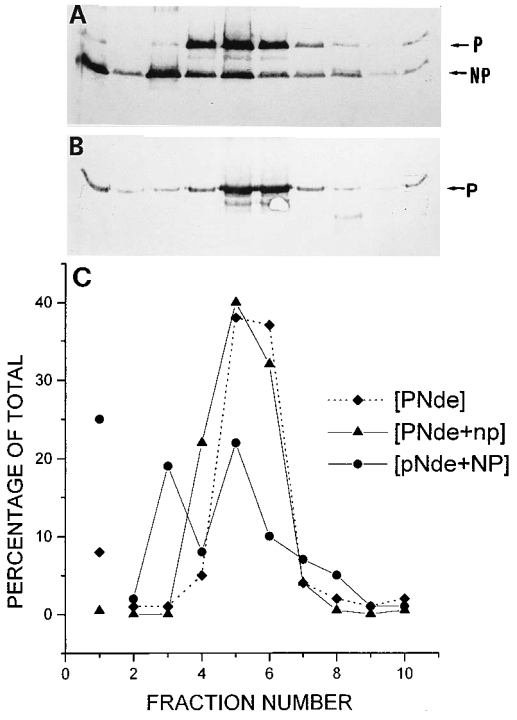

FIG. 8. Glycerol gradient analysis of the $P^{\Delta 539\text{-}568}$-$NP^0$ complex. Panels are as those described in the legend to Fig. 4, except that the interaction of $P^{\Delta 539\text{-}568}$ (PNde) with NP^0 was examined.

point is of some interest, since the N-terminal half of P is also found in two other viral proteins which are translated from edited P gene mRNAs (Fig. 1), namely, W, which contains the N-terminal half of P by itself, and V, which contains this half fused to a Cys-rich domain (13, 31). Moreover, V and W specifically inhibit genome replication (5), and this inhibition

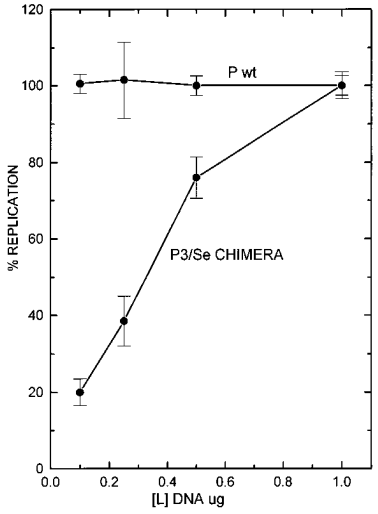

FIG. 9. The L gene dependence of the chimeric P protein. Duplicate transfections were carried out with the standard amounts of plasmids expressing the NP and either the P^{wt} or the $P^{bPIV3/SeV}$ (P3/Se) gene and variable amounts of pGEM-L (as indicated) in cells infected with $DIH4^{uv}$. The extent of genome replication was determined and quantitated as described in the legends to Fig. 2 and 3 and was shown relative to 1 μg of pGEM-L set at 100 for each P gene. The vertical bars show the ranges of the duplicate determinations.

can be overcome by overexpression of L (2), which also suggests that the N-terminal half of P and L may be interacting. However, L forms a complex sufficiently stable to withstand immunoselection only with P and not with V or W (5, 29), and the region in P that is responsible for this binding (residues 413 to 445) is absent in V and W. This region(s) at the N terminus of P (which has not been further refined) could represent an L interaction domain, as opposed to a stable binding site.

DISCUSSION

Since replication is thought to be RNA synthesis coupled with the concurrent assembly of the nascent chain, the finding that the SeV V and W proteins inhibit genome replication but not mRNA synthesis suggested that the N-terminal half of the P protein would contain a domain(s) specifically involved in nascent chain assembly. Little was known about this half of P, except that it contains the sites at which the protein is phosphorylated (6, 32). We have since found two quite different, and probably overlapping, functions within aa 1 to 77 at the N terminus of the SeV P protein. The first is required for RNA synthesis per se and is distinguished by its lack of strict sequence requirements; it can be functionally replaced with aa 78 to 144 of P. The second is required for genome replication but not for transcription and is presumably required for nascent chain assembly. This domain appears to have strict sequence requirements in that it can be mapped by deletion analysis to a 9-aa segment (aa 33 to 41) and can be replaced only with the same region from a closely related virus (PIV3) which contains a conserved sequence, and not by the N-terminal regions of more distantly related viruses (such as mumps virus or vesicular stomatitis virus [not shown]). This domain has now been shown to be required for binding to NP^0, which is consistent with the idea that a P-NP^0 complex is required to deliver NP^0 to the nascent chain during genome replication.

Helical assemblies such as paramyxovirus nucleocapsids form when the binding site of a protein is complementary to a region of its surface that does not include the binding site itself. Three regions of P, aa 33 to 41, aa 345 to 411, and aa 479 to 568 (Fig. 1), are now known to be important for binding to NP. Ryan et al. have previously shown that aa 345 to 411 and aa 479 to 568 were necessary and sufficient for binding to nucleocapsids. Since this latter C-terminal region is also required for binding to NP^0 (Fig. 8), aa 479 to 568 would presumably be binding to a region or surface that is present on both NP^0 and NP^{NC}. aa 33 to 41, on the other hand, are required only for binding to NP^0, and these residues might then be binding to a surface that is absent from NP^{NC} because of a conformational change which occurs on assembly or because it interacts with the subunit binding site or its complement, both of which are unavailable in nucleocapsids. This latter idea is attractive, since it also provides a mechanism by which P-NP^0 complex formation prevents NP^0 from aggregating nonspecifically. In any event, it is this selective requirement of aa 33 to 41 for binding to NP^0 which allows P to act as a chaperone. It will be of interest to determine where aa 33 to 41 of P interact on the NP^0 chain. The NP protein is thought to contain a globular body that is formed from the N-terminal 80% of the chain which is well conserved among related viruses, with a hypervariable C-terminal tail. In a recent structure-function study of the SeV protein (1, 3), tailless NP^0 was found to retain part of its function in genome replication, in that it could encapsidate the nascent chain and allow the polymerase to continue to the end of the template to form a full-length complementary genome nucleocapsid. However, nucleocapsid templates which were themselves composed of tailless NP were unable to func-

tion as templates. The tailless NP, like the wild-type protein, was active in the nascent chain assembly step of replication only when it was coexpressed with P, indicating that aa 33 to 41 of P are not interacting (at least in a simple fashion) with this tail for assembly. On the other hand, Ryan et al. (27) have recently shown that when holonucleocapsids were reacted with monoclonal antibodies whose epitopes were in this C-terminal tail of NP, the normally tightly bound P protein was released. It will, therefore, also be of interest to determine whether this release of P was due to a direct competition of P and the monoclonal antibody for the same region of the NP tail (which is known to be highly accessible on nucleocapsids [10, 20]) or whether this effect was indirect.

The impetus for these studies was the finding that V and W specifically inhibited the nascent chain assembly step of genome replication, and these studies have begun to offer clues as to how this specific inhibition might occur. V and W do not contain the C-terminal half of P and, as expected, do not form a stable complex with NP^0 (not shown). However, like $P^{\Delta 539\text{-}568}$, V and W can partially prevent NP^0 from nonspecifically self-assembling (not shown), and it is possible that although this V- and W-NP^0 interaction does not lead to stable complex formation, it can nevertheless affect the ability of NP^0 to function. In this speculative vein, it is also possible that V and W specifically inhibit replication not only because they can interact with NP^0, but because they can also interact with L. L overexpression can relieve the inhibition due to V and W (2), and increased amounts of L are required to carry out genome replication with a chimeric P protein containing the N-terminal half of bPIV3 (Fig. 9). This can be construed as evidence that a site(s) in the N-terminal half of P interacts with L, again without forming a stable complex. In this latter case, this unstable interaction between P (of the P-NP^0 complex) and L would somehow be required for the nascent chain assembly step of genome replication, thereby coupling this step to that of RNA synthesis in genome replication.

ACKNOWLEDGMENTS

We thank Laurent Roux (Geneva), for numerous discussions and careful reading of the manuscript.

This work was supported by the Swiss National Science Fund.

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