Mapping of a Region of the Paramyxovirus L Protein Required for the Formation of a Stable Complex with the Viral Phosphoprotein P

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The paramyxovirus large protein (L) and phosphoprotein (P) are both required for viral RNA-dependent RNA polymerase activity. Previous biochemical experiments have shown that L and P can form a complex when expressed from cDNA plasmids *in vivo*. In this report, L and P proteins of the paramyxovirus simian virus 5 (SV5) were coexpressed in HeLa T4 cells from cDNA plasmids, and L-P complexes were examined. To identify regions of the SV5 L protein that are required for L-P complex formation, 16 deletion mutants were constructed by mutagenesis of an SV5 L cDNA. Following coexpression of these L mutants with cDNA-derived P and radiolabeling with ^{35}S -amino acids, cell lysates were analyzed for stable L-P complexes by a coimmunoprecipitation assay and by sedimentation on 5 to 20% glycerol gradients. Mutant forms of L containing deletions that removed as much as 1,008 residues from the C-terminal half of the full-length 2,255-residue L protein were detected in complexes with P by these two assays. In contrast, large deletions in the N-terminal half of L resulted in proteins that were defective in the formation of stable L-P complexes. Likewise, L mutants containing smaller deletions that individually removed N-terminal regions which are conserved among paramyxovirus and rhabdovirus L proteins (domain I, II, or III) were also defective in stable interactions with P. These results suggest that the N-terminal half of the L protein contains sequences important for stable L-P complex formation and that the C-terminal half of L is not directly involved in these interactions. SV5-infected HeLa T4 cells were pulse-labeled with ^{35}S -amino acids, and cell extracts were examined by gradient sedimentation. Solubilized L protein was detected as an ~8 to 10S species, while the P protein was found as both a ~4S form (~85%) and a species that cosedimented with L (~15%). These data provide the first biochemical evidence in support of a simple domain structure for an L protein of the nonsegmented negative-sense RNA viruses. The results are discussed in terms of a structural model for the L protein and the interactions of L with the second viral polymerase subunit P.

The parainfluenza virus simian virus 5 (SV5) is a prototype of the paramyxovirus family of nonsegmented negative-sense RNA viruses. Six major viral proteins are found within the paramyxovirus particle (reviewed in reference 13). Three of these proteins, the fusion (F), hemagglutinin-neuraminidase (HN), and membrane (M) proteins, are found within or closely associated with the viral lipid envelope. The nucleocapsid protein (NP) is the most abundant of the paramyxovirus structural proteins (~2,600 copies per Sendai virion [24]). NP is tightly bound to the ~15-kb viral genomic RNA to form an RNase-resistant nucleocapsid (NC) structure. Two other NC-associated polypeptides, the phosphoprotein (P) and the large protein (L), together constitute the viral RNA-dependent RNA polymerase. Both the L and P polypeptides are required for transcription and replication of the NP-encapsidated genomic RNA to produce viral mRNAs and progeny virus genomes (4, 14, 15, 26). Although the exact role of L and P in the synthesis of viral RNA is unknown, the relative abundance of the L and P polypeptides in virions (~40 and ~300 molecules per Sendai virion, respectively [24]) and their distribution on the NC template (36) are consistent with the proposal that L acts as the catalytic subunit of the viral

polymerase while P functions both in conjunction with L and in a noncatalytic role.

By contrast to the understanding of the structure and characteristics of the P protein ($M_r \approx 46,000$), there is less known about the structure of the paramyxovirus L polypeptide (13). The majority of this limited information concerning the paramyxovirus L is based on either comparisons of protein sequences deduced from cDNA clones (34, 39) or analogies with the L protein of vesicular stomatitis virus (VSV), a rhabdovirus which employs a similar replication strategy (reviewed in references 1 and 2). Nearly half of the paramyxovirus and rhabdovirus genomes (>6,000 bases) is devoted to encoding the L protein. The large size of the L polypeptide chain ($M_r \approx 250,000$) may reflect the presence of multiple distinct catalytic domains responsible for performing the individual steps in RNA synthesis (8, 13). In support of the proposal that L is multifunctional, both genetic and *in vitro* reconstitution experiments using VSV proteins have implicated the L polypeptide in RNA capping (1), methylation (17), chain initiation, elongation (16, 40), termination, and polyadenylation (20). A comparison of the deduced amino acid sequences of those viral L protein genes which have been cloned has shown that the predicted N- and C-terminal regions of these L proteins are diverse, whereas the middle region contains at least six domains of high amino acid sequence identity and spacing (32, 34, 39). Several of these conserved regions contain limited homology with amino acid motifs associated with functions in other cellular and viral proteins (34, 35). Although

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the importance of two of these conserved regions in the synthesis of viral RNA has been confirmed experimentally for the VSV L protein (5, 40), no specific function has been assigned to any region of a viral L polypeptide.

In the case of the paramyxovirus Sendai virus, recent biochemical experiments have shown that cDNA-derived P and L proteins expressed *in vivo* can be detected as a complex by coimmunoprecipitation and velocity sedimentation assays (18). Furthermore, the formation of an L-P complex correlates with the ability of the cDNA-derived L and P proteins to synthesize viral RNA (18). Thus, the well-established observation that both L and P polypeptides are required for viral RNA synthesis (14, 15, 28) can be explained, at least in part, by a need to form an L-P complex for polymerase activity (18). As it appears that interaction with the P protein is essential for L function, locating polypeptide domains that direct these L-P interactions is critical for our understanding of both the structure-function relationships of individual regions of the multifunctional L protein as well as the steps involved in viral replication. Mapping of an L-binding domain to a region of the VSV P protein has been reported previously (9). However, the complementary regions on an L polypeptide which are involved in interactions with P have not yet been identified. The recent finding that cDNA-derived L and P can form a complex when expressed *in vivo* (18) provides an opportunity to map the regions of the L protein involved in L-P complex formation. On the basis of the assumption that it is likely that a polypeptide the size of L contains distinct domains which could fold independently (29), I have carried out a deletion analysis of the SV5 L protein with the primary goal of mapping a P-binding site to a region within the L polypeptide. This report presents biochemical evidence that the SV5 L and P proteins form a complex when expressed from cDNA plasmids *in vivo* or during a virus infection. cDNA-derived deletion mutants of L were tested for the ability to form a complex with P protein *in vivo*. The results of this analysis indicate that an N-terminal region of L is important for the formation of stable L-P complexes. These data provide the first experimental evidence for a simple domain structure for a viral L protein and are discussed as part of a model that will form a basis for future mutagenesis.

MATERIALS AND METHODS

Cells and viruses. Monolayer cultures of CV1 and HeLa T4 (25) cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Vaccinia virus vTF7.3 (12) was grown and titers were determined in CV1 cells. Infections with the W3A strain of SV5 (6) were as described previously (30).

Plasmid construction and deletion mutagenesis. A cDNA plasmid (pGem2-P) that encodes the SV5 P protein under control of the T7 RNA polymerase promoter (33, 41) was kindly provided by Reay Paterson, Northwestern University. The cloning and sequencing of the SV5 L protein gene have been described previously (32), and this cDNA was used as starting material for the deletion mutagenesis. To facilitate expression of the L protein from this cDNA, ATG codons which were located 5' to that initiating the L open reading frame were removed by using 5' mRNA-sense (bases 12 to 43) and 3' vRNA-sense (bases 705 to 734) synthetic DNA primers in a PCR with Amplitaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) as described previously (7, 32). Briefly, the 5' portion of the L gene (L-5', bases 7 to 474 [32]) was modified to include both a unique 5' *Xba*I site in the noncoding region and an *Nco*I site (CCATGG) in the sequences which

encode the L protein-initiating AUG. This modified DNA fragment was then linked to the remaining L protein gene sequences (bases 475 to 6822) through the *Pml*I site at base 475. The resulting complete SV5 L protein gene was inserted into the *Xba*I and *Sal*I sites of pGem3 (Promega, Madison, Wis.) to produce pGem3-L. The orientation of the L gene was such that mRNA-sense transcripts could be produced by using the T7 RNA polymerase promoter.

Plasmids encoding L protein mutants with in-frame deletions were constructed by using standard techniques. The restriction sites illustrated schematically in Fig. 1A that were used to construct the L deletion mutants and the amino acids removed from the full-length 2,255-residue protein were as follows: mutant 1 (*Nco*I to *Dra*I), residues 2 to 101; mutant 2 (*Nco*I to *Msc*I), residues 2 to 419; mutant 3 (*Dra*I to *Msc*I), residues 102 to 419; mutant 4 (*Pml*I to *Bst*EII), 151 to 815; mutant 5 (*Bgl*II to *Bgl*II), 354 to 962; mutant 6 (*Xho*I to *Xho*I), residues 623 to 1235; mutant 7 (*Bsp*EI to *Bsp*EI), residues 1146 to 2153; mutant 8 (*Bsp*EI to *Sca*I), residues 1146 to 1957; mutant 9 (*Sca*I to *Eco*RV), residues 1958 to 2074; mutant 10 (*Hpa*I to *Bsp*EI), residues 879 to 2153; mutant 11 (*Bst*EII to *Eco*RV), residues 817 to 2074; mutant 12 (*Xho*I to *Bsp*EI), residues 621 to 2153; and mutant 13 (*Hinc*II to *Bsp*EI), residues 566 to 2153. In brief, mutants 3 and 5 to 7 were constructed by digestion of pGem3-L with either *Dra*I and *Msc*I together (to create mutant 3) or with *Bgl*II (mutant 5), *Xho*I (mutant 6), or *Bsp*EI (mutant 7) only, and the plasmid DNA was subsequently ligated. Likewise, mutants 1 and 2 were constructed by digestion of pGem3-L with *Nco*I followed by treatment with the Klenow fragment of DNA polymerase I to create a blunt-end DNA segment, which was in turn ligated to a 3' *Dra*I (mutant 1) or *Msc*I (mutant 2) site. Mutant 4 was constructed by digestion of pGem3-L with *Bst*EII, treatment with Klenow fragment, and digestion with *Pml*I before religation. Mutants 8 to 13 were constructed by ligating the appropriate fragment containing the 5' unique *Xba*I site and a 3' site shown in Fig. 1A to pGem3-L digested with *Xba*I and either *Sca*I (mutant 8), *Eco*RV (mutants 9 and 11), or the *Bsp*EI site at bases 6481 to 6486 that had been blunt-ended by prior treatment with Klenow DNA polymerase (mutants 10, 12, and 13).

Mutants 14 to 16 were constructed by PCR as described previously (7) to encode deletions that precisely removed conserved domain I (amino acid residues 228 to 418), domain II (residues 510 to 621), or domain III (residues 656 to 878). To construct mutant 14, a DNA fragment containing pGem3 sequences 5' to the L gene and L gene bases 1 to 1280 was used as template in a PCR along with 5' and 3' synthetic primers complementary to the T7 polymerase promoter and L protein gene bases 687 to 707 (5'-GCGGTCGACAATATCTCAGT GTTAAATAATGC-3'), respectively. The 3' primer was designed to introduce changes which converted bases 705 to 710 of the L cDNA clone to an *Ssp*I site (underlined). After digestion with *Ssp*I and *Xba*I, the resulting 750-bp PCR product was cloned into pGem2 for sequencing. The *Pml*I-*Ssp*I fragment (bases 475 to 707) was then excised and linked by blunt-end ligation to the *Msc*I site of the wild-type L protein gene at base 1281. To construct mutant 15, a DNA fragment containing bases 487 to 2474 was used as a template in a PCR along with 5' and 3' synthetic primers corresponding to bases 705 to 734 (5'-AATCATACACTAACATACAT GACCTTTGAA-3') and the complement of bases 1533 to 1552 (5'-GAGGCCTCTCGAGGATTGTAGGGGACCT G-3'), respectively. The 3' primer was used to convert bases 1552 to 1557 to an *Xho*I site (underlined). After digestion with *Xho*I and *Bgl*II, the resulting DNA fragment was ligated into a

FIG. 1. Schematic diagram of the SV5 L protein gene, deletion mutants, and L protein. (A) L protein gene. The cDNA encoding the SV5 L protein is depicted as an open box, with relevant endonuclease restriction sites shown above their positions in the gene. Numbered black lines indicate portions of the L gene that were deleted to create mutants 1 to 16. (+) and (-) refer to the ability of the mutants to form a stable complex with the P protein in the two assays described in the text. Abbreviations: N, *Nco*I; D, *Dra*I; Pm, *Pml*I; Bg, *Bgl*II; M, *Msc*I; H, *Hinc*II; X, *Xho*I; Bs, *Bst*EII; Hp, *Hpa*I; Be, *Bsp*EI; Sc, *Sca*I; RV, *Eco*RV. (B) L protein. The L polypeptide is depicted as a hatched box, with brackets indicating the location of regions of high amino acid homology among L protein sequences (I to VI). ABCD denotes the location of the polymerase core domain described by Poch et al. (34, 35). The position of the synthetic peptide used to generate an antiserum to the L protein (L peptide) and amino acid numbers within the L polypeptide are indicated. N, amino terminus; C, carboxy terminus.

pGem3 vector for sequencing. A DNA fragment corresponding to bases 1153 to 1557 was excised by digestion with *Pst*I and *Xho*I and linked to the corresponding sites at bases 1153 and 1888 of the wild-type L gene. To construct mutant 16, a DNA fragment (bases 1153 to 3538) was used as a template in a PCR along with 5' and 3' synthetic primers corresponding to L gene bases 1303 to 1321 (5'-ATGCTTCAAAGGGTCTCAC-3') and the complement of bases 1971 to 1991 (5'-CGCGCCCGGGAATTCAAAGTCATCACTTGG-3'), respectively. The 3' primer was designed to introduce changes which converted bases 1986 to 1991 of the L cDNA clone to an *Eco*RI site (underlined). The resulting 680-bp DNA was digested with *Eco*RI and *Xho*I and cloned into the corresponding sites of pGem7 vector for sequencing. Plasmid DNA was digested with *Eco*RI, blunt ended with Klenow fragment, and digested with *Xho*I. The resulting 100-bp fragment was ligated into the *Xho*I and *Hpa*I sites of the wild-type L gene at bases 1888 and 2660, respectively. The nucleotide sequence of all of the above PCR-derived DNA fragments was determined by dideoxynucleotide sequencing (37).

Isotopic labeling of polypeptides, immunoprecipitation, and PAGE. Proteins were expressed in HeLa T4 cells from cDNA plasmids as described previously (31), using a modified version of the vaccinia virus/T7 RNA polymerase system of Fuerst et al. (12). Briefly, 35-mm-diameter dishes of subconfluent HeLa T4 cells were infected with vaccinia virus vTF7.3 (multiplicity of infection of ≈ 10) for 1 h and then transfected with pGem2-P ($\sim 2 \mu\text{g}$) and/or pGem3-L (~ 2 to $6 \mu\text{g}$) mutant plasmid, using Lipofectin reagent ($12 \mu\text{g}$ in $800 \mu\text{l}$ of Opti-MEM [Gibco-BRL, Gaithersburg, Md.]) according to the manufacturer's instructions. Differing amounts of the various mutant L plasmids were used in transfections to obtain approximately the

same level of L expression. Cells were radiolabeled from 11 to 12 h postinfection (hpi) with 50 to $100 \mu\text{Ci}$ of Tran[^{35}S]label (ICN Radiochemicals Inc., Irvine, Calif.) per ml in Dulbecco's modified Eagle's medium lacking cysteine and methionine, washed in cold phosphate-buffered saline (PBS), and lysed in buffer containing the appropriate detergent. Proteins were radiolabeled in SV5-infected HeLa T4 cells as described previously (30). For immunoprecipitation of sodium dodecyl sulfate (SDS)-denatured proteins, radiolabeled cells were lysed in $250 \mu\text{l}$ of 1% SDS and boiled for 5 min. Cell extracts were clarified by centrifugation and diluted with 4 to 6 volumes of buffer (60 mM Tris-HCl [pH 7.4], 190 mM NaCl, 6 mM EDTA, 1.25% Triton X-100 [11]). Proteins were immunoprecipitated as described previously (23), using *Staphylococcus aureus* protein A-agarose (Gibco-BRL). The rabbit polyclonal antiserum to detergent-disrupted SV5 virus (anti-SV5 serum) has been described elsewhere (32); this serum recognizes all of the known SV5 proteins except the L polypeptide. The rabbit polyclonal L-specific (anti-L protein) serum was the kind gift of Robert Lamb, Howard Hughes Medical Institute. This serum was raised to a synthetic peptide that had been chemically linked to keyhole limpet hemocyanin. The sequence of the peptide corresponded to C-terminal amino acids 2241 to 2254 of the SV5 L protein (one-letter amino acid code, $\text{NH}_2\text{-N-I-N-E-E-I-D-R-G-I-D-G-E-E-COOH}$ [32]). Samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 10% polyacrylamide gels followed by fluorography (22). Autoradiograms and figure labels were electronically scanned with a Microtek Scanmaker 600ZS, using Adobe Photoshop software.

Glycerol gradient centrifugation. L-P protein complexes were analyzed by glycerol gradient centrifugation, using a

FIG. 2. Coimmunoprecipitation of cDNA-derived L and P proteins. Vaccinia virus vTF7.3-infected HeLa T4 cells were cotransfected with a plasmid encoding P protein (2 μ g) along with plasmids encoding either wild-type L protein (3 μ g) or L protein deletion mutants 4 to 7 (2 to 4 μ g). Cells were radiolabeled from 11 to 12 hpi with Tran^[35S]label. Equal aliquots of detergent cell lysates were immunoprecipitated under nondenaturing conditions with either anti-L protein (A) or anti-SV5 or preimmune (B) serum before analysis by SDS-PAGE. Lanes 1 to 5 represent samples from cells cotransfected with plasmids encoding P with either wild-type L (lane 1) or L mutants 4 to 7 (lanes 2 to 5, respectively). Lanes 6 to 10 of panel B represent samples from the same lysates as lanes 1 to 5, respectively. P lanes in panel B are samples of proteins from cells transfected with P plasmid alone and immunoprecipitated with anti-SV5 or preimmune serum. M is a marker lane of SV5-infected cell polypeptides, with the positions of the wild-type L and P proteins indicated.

variation of the method described by Horikami et al. (18). Vaccinia virus vTF7.3-infected HeLa T4 cells (35-mm-diameter dishes) were transfected with plasmids encoding P and mutant L proteins as described above. Alternatively, cells were infected with SV5 (30). Cells were radiolabeled with 50 μ Ci of Tran^[35S]label per ml from 11 to 12 hpi for the vaccinia virus infection or for 30 min at 15 to 19 hpi for the SV5 infection. The cell monolayers were washed in ice-cold PBS before lysis in 0.5 ml of buffer G (25 mM Tris-HCl [pH 7.5], 0.5% Nonidet P-40 [NP-40], 250 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 14 mM β -mercaptoethanol). Cell lysates were clarified by centrifugation (10 min, 4°C, 160,000 $\times$ g, TLA100 rotor [Beckman Instruments, Palo Alto, Calif.]), and the resulting supernatant was operationally defined as the soluble fraction. An aliquot (0.4 ml) of the supernatant was layered onto a 12-ml preformed linear 5 to 20% (vol/vol) glycerol gradient in buffer G. Following centrifugation (25 h, 39,000 rpm, 5°C, SW41 rotor), 12 1-ml fractions were collected from the bottom of the tubes. Each fraction was adjusted to 0.5% SDS, boiled for 5 min, and diluted with buffer as described above. Samples were immunoprecipitated by using 8 μ l (cDNA-derived proteins) or 12 μ l (SV5-derived proteins) of a 1:1 mixture of anti-L protein and anti-SV5 sera before analysis of the immune complexes by SDS-PAGE. Protein standards (rabbit hemoglobin, ~4S; mouse immunoglobulin G1, 7S; potato β -amylase, 9S) were analyzed in parallel gradients, and gradient fractions were analyzed by SDS-PAGE followed by Coomassie blue staining.

Coimmunoprecipitation assays. Coimmunoprecipitation of cDNA-derived P and L proteins was carried out by a variation of the method described by Shaw et al. (38). Briefly, vaccinia virus vTF7.3-infected, cDNA-transfected cells were radiolabeled for 1 h with Tran^[35S]label as described above and then washed with ice-cold PBS before lysis in 1 ml of cold buffer R (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 5 mM EDTA, 0.4% deoxycholate, 1% NP-40, 0.5% bovine serum albumin, 25 mM iodoacetamide). Following centrifugation in the TLA100 rotor as described above, the supernatant was adjusted to 0.1% SDS and pretreated (1 h, 4°C) with 5 to 8 μ l of preimmune serum and 50 μ l of a 1:1 (vol/vol) slurry of protein A-agarose. The

agarose beads were pelleted, and the resulting precleared supernatant was then incubated for 2 h at 4°C with 10 μ l of anti-SV5 serum. After the addition of protein A-agarose beads, the immune complexes were washed three times at 4°C with 1 ml of ice-cold buffer R containing 0.1% SDS before analysis by SDS-PAGE. In parallel, aliquots representing one half of the cell lysate used in the coprecipitation assay were adjusted to 1% SDS, boiled, and immunoprecipitated with the anti-L protein serum.

RESULTS

Effect of L mutations on the formation of an L-P complex. It has been previously shown by using a coimmunoprecipitation assay that cDNA-derived Sendai virus L and P proteins form a complex in vivo (18). To determine if SV5 L and P proteins could also form a complex when expressed in vivo from cDNA plasmids, a similar coimmunoprecipitation assay was used as described previously (38). Monolayers of HeLa T4 cells were first infected with vTF7.3, a recombinant vaccinia virus which expresses the bacteriophage T7 RNA polymerase (12). These infected cells were then cotransfected with a cDNA plasmid encoding the SV5 L protein (pGem3-L; Fig. 1A) and a plasmid encoding the SV5 P protein (pGem2-P [41]), both of which were under control of the T7 RNA polymerase promoter. Following radiolabeling with Tran^[35S]label, detergent cell lysates were prepared under nondenaturing conditions described in Materials and Methods. Lysates were immunoprecipitated either with an antiserum raised to a synthetic peptide whose sequence corresponds to a C-terminal region of the SV5 L protein (anti-L protein serum; Fig. 1B) or with an antiserum raised to disrupted SV5 virus (anti-SV5 [32]). This anti-SV5 serum does not contain antibodies that recognize the L protein (data not shown).

As shown in Fig. 2, the SV5 L protein was immunoprecipitated by the anti-L protein serum from extracts of vTF7.3-infected cells that had been cotransfected with pGem3-L and pGem2-P (Fig. 2A, lane 1). Immunoprecipitation of an aliquot of the same lysate with the anti-SV5 serum showed precipitation of the P protein, along with a polypeptide that comigrated

with L (Fig. 2B, lane 1). This additional polypeptide was not found in immune complexes isolated from cells that had been transfected with the P plasmid alone (Fig. 2B, anti-SV5 panel, lane P). Neither P or L polypeptides were precipitated from these lysates with preimmune serum (preimmune lanes). For unknown reasons, the amount of P in immune complexes isolated by using the anti-L protein serum (Fig. 2A, lane 1) was always less than that of the converse precipitation using anti-SV5 serum (Fig. 2B, lane 1). As described above, the presence of L in the immune complexes isolated with the anti-SV5 serum was not due to contaminating antibodies specific for the L polypeptide. These data are consistent with coimmunoprecipitation studies reported for the Sendai virus P and L proteins (18) and indicate that SV5 cDNA-derived L and P can form a complex in the absence of other viral components.

To locate the regions of L important for interactions with the P protein, a series of L protein deletion mutants was constructed (Fig. 1). There are presently no data concerning the structure of a viral L protein from which to target these mutations. Therefore, the rationale for the location of these deletions was based on the goal of creating a panel of mutants that would span nearly the entire coding region of L. Initially, four mutant L protein genes encoding predicted internal deletions of 665, 609, 613, and 1,008 amino acids were constructed by using restriction enzyme sites within the L cDNA (Fig. 1, mutants 4 to 7). In all cases, the sequences of these altered genes retained the L protein translational reading frame, so that the mutant polypeptides could be immunoprecipitated with the anti-L protein serum. When coexpressed with P in the vaccinia virus/T7 system outlined above and immunoprecipitated from lysates with anti-L protein serum, L protein mutants 4 to 7 were detected as distinct polypeptide species (Fig. 2A, lanes 2 to 5, respectively) that migrated slightly faster than the full-length L protein (lanes 1 and M). Analysis of aliquots from these same lysates by using the anti-SV5 serum resulted in the detection of P (Fig. 2B, lanes 2 to 5), along with additional nonspecific polypeptides that were also seen when preimmune serum was used (lanes 6 to 10). Of the four altered L polypeptides tested, mutant 7 was the only L-specific polypeptide detected in immune complexes with P under these conditions at levels comparable to that obtained with the full-length L (Fig. 2B, lane 5). Immunoprecipitation of lysates from cells coexpressing L mutants 4 to 6 with P (Fig. 2B, lanes 2 to 4, respectively) resulted in a pattern similar to that found when P was expressed alone (anti-SV5 panel, lane P). Only very small amounts of mutants 4 to 6 were found in the immune complexes, and these mutants were never detected in the immune complexes at levels comparable to that found for wild-type L or mutant 7. Taken together, these data indicate that an L mutant lacking 1,008 C-terminal residues from the 2,255-residue full-length protein (mutant 7) is still capable of forming a stable L-P complex by this coimmunoprecipitation assay, but large deletions in the N-terminal half of L (mutants 4 to 6) result in proteins that are defective in these interactions.

The L and P proteins do not coimmunoprecipitate from mixed lysates of cells separately transfected with P and L plasmids. A series of mixing experiments was carried out to determine if the formation of the SV5 L-P complex described above required the coexpression of these polypeptides in the same cell. Separate dishes of vTF7.3-infected HeLa T4 cells were individually transfected with plasmid DNA encoding P, HN, L, or mutant L protein. After radiolabeling with Tran³⁵S]label, cell lysates were prepared under nondenaturing conditions and mixed prior to immunoprecipitation with the anti-SV5 serum. As shown in Fig. 3, when lysates of cells

FIG. 3. The L and P proteins do not coimmunoprecipitate from mixed lysates of cells separately transfected with P and L plasmids. Separate dishes of vTF7.3-infected HeLa T4 cells were mock transfected or individually transfected with plasmids encoding either the P protein (2 μ g), HN protein (5 μ g), L protein (5 μ g), or L protein mutant 5 or 7 (5 μ g). Following a 1-h radiolabeling period with Tran³⁵S]label, cell lysates were prepared under nondenaturing conditions described in Materials and Methods. Equal aliquots of clarified cell lysate expressing radiolabeled P protein (lanes 1 to 4) or HN protein (lanes 5 to 8) were mixed and held at 4°C for 3 h with mock-transfected cell lysates (lanes 1 and 5) or with lysate from cells expressing radiolabeled L protein (lanes 2 and 6), L protein mutant 5 (lanes 3 and 7), or L protein mutant 7 (lanes 4 and 8). After immunoprecipitation with the anti-SV5 serum, proteins were analyzed by SDS-PAGE. Lanes 9 to 11 depict proteins immunoprecipitated with the anti-L protein serum from SDS-denatured extracts of cells expressing wild-type L protein (lane 9) or L protein mutant 5 (lane 10) or 7 (lane 11). M is a marker lane of SV5-infected cell polypeptides.

that had separately expressed P protein and L protein were mixed and immunoprecipitated, the proteins isolated (lane 2) were very similar to those seen from mixed lysates of cells expressing P and mock-transfected cells (lane 1). Thus, L protein was not detected in immune complexes from these mixed lysates, even though the L protein had been synthesized at significant levels (lane 9). The L protein was not precipitated by the anti-SV5 serum when expressed alone and cell lysates were mixed with lysates from cells separately expressing cDNA-derived HN protein (compare lanes 5 and 6) or M protein (data not shown). Likewise, the proteins immunoprecipitated from mixed lysates of cells separately expressing either L mutant 5 or 7 (Fig. 3, lanes 10 and 11) with lysates from cells expressing P (lanes 3 and 4) or HN (lanes 7 and 8) were the same as that found for mock-transfected cells. Together, these data supporting the contention that the presence of L in the immune complexes described above is due to interactions with P, and this conclusion is further supported by the results of glycerol gradient sedimentation analysis described below. Furthermore, these data indicate that the formation of the L-P complex with cDNA-derived proteins appears to require or be enhanced by coexpression of the two proteins in the same cell. This is a characteristic previously described for the Sendai virus P and L proteins (18) but different from that of the VSV proteins (e.g., references 5 and 9).

Effect of N- and C-terminal L mutations on formation of L-P complexes. Five additional L protein deletion mutants were constructed to confirm the conclusions presented above and to

FIG. 4. Effect of L protein N- and C-terminal deletions on the coimmunoprecipitation of L with P protein. (A) Effects of N- and C-terminal deletions. HeLa T4 cells infected with vTF7.3 were cotransfected with 2 μ g of a plasmid encoding P protein along with 6 μ g of either wild-type L (lane 1), N-terminal L mutants 1 to 3 (lanes 2 to 4), or C-terminal L mutants 8 and 9 (lanes 5 and 6). After radiolabeling of the cells from 11 to 12 hpi with Tran^[35S]label, proteins were immunoprecipitated from cell lysates with the anti-SV5 serum under non-denaturing conditions (lanes 1 to 6, anti-SV5 panel) and analyzed by SDS-PAGE. Likewise, one half of the material processed for the samples depicted in the anti-SV5 panel was denatured with SDS, diluted with buffer, and immunoprecipitated with the anti-L protein serum (anti-L panel). The positions of L and P polypeptides are indicated. (B) Effect of progressive C- to N-terminal L protein deletions. vTF7.3-infected HeLa T4 cells were cotransfected with plasmids encoding P and wild-type L (lane 1) or L mutants 10 to 13 (lanes 2 to 5, respectively). Radiolabeled cell lysates were prepared and immunoprecipitated with the anti-SV5 serum (left panel) or with the anti-L protein serum (right panel) as described above. M is a marker lane of SV5-infected cell polypeptides.

further determine the effect of smaller L deletions on the formation of stable L-P complexes. N-terminal residues 2 to 101, 2 to 419, and 102 to 419 were deleted to create mutants 1 to 3, respectively (Fig. 1). Likewise, C-terminal residues 1146 to 1957 and 1958 to 2074 were deleted to create mutants 8 and 9 (total of 2,255 L residues). Plasmids encoding these altered L proteins were cotransfected into vTF7.3-infected HeLa T4 cells along with a plasmid encoding P. After radiolabeling of the cells with Tran^[35S]label, cell lysates were immunoprecipitated with the anti-SV5 serum under nondenaturing conditions; alternatively, an aliquot of the lysate was denatured and immunoprecipitated with the anti-L protein serum. As shown in Fig. 4A, L mutants 1, 2, 3, 8, and 9 were synthesized as major

polypeptide species (anti-L panel, lanes 2 to 6, respectively) that migrated faster than wild-type L (anti-L panel, lane 1). Immunoprecipitation of aliquots of these same lysates with anti-SV5 serum under coimmunoprecipitation conditions showed wild-type L protein in immune complexes with P (anti-SV5 panel, lane 1) as described above. Likewise, C-terminal deletion mutants 8 and 9 were detected in immune complexes at levels that closely matched that found for the full length L (anti-SV5 panel; compare lane 1 with lanes 5 and 6). In contrast to these C-terminal mutants, only very small or undetectable amounts of the N-terminal deletion mutants 1 to 3 were reproducibly detected in these immune complexes with P (anti-SV5 panel, lanes 2 to 4).

To further define the C-terminal boundary of L required for stable interactions with P, the smallest L protein which bound to P in these assays (mutant 7) was used as starting material from which four additional mutants with progressive deletions in a C- to N-terminal direction were constructed (mutants 10 to 13; Fig. 1). Mutants were designed to contain deletions that would flank the C-terminal side (mutant 10 and 12) or delete into (mutant 11 and 13) two regions of L that are homologous among all viral L protein sequences (domains II and III, respectively [39]). When coexpressed with P, these mutants were readily detected in cell lysates by using the anti-L protein serum (Fig. 4B, anti-L panel, lanes 2 to 5) at levels that were consistently higher than that of wild-type L (anti-L panel, lane 1). However, the proportion of radiolabeled mutant proteins found in immune complexes isolated with the anti-SV5 serum (anti-SV5 panel, lanes 2 to 5) relative to the level of expression of the mutants (anti-L panel, lanes 2 to 5) was reproducibly much less than that found for wild-type L (compare lanes 1 of the two panels). Each of these L deletion mutants still contains 102 C-terminal residues which include the segment recognized by the antipeptide antibodies. Thus, the data from these coprecipitation assays suggest that L sequences located between C-terminal residues 1146 and 2153 (those deleted to create mutant 7) are not required for the formation of stable L-P complexes. In contrast, alterations in the N-terminal half of the SV5 L results in polypeptides which are defective in L-P interactions.

Glycerol gradient sedimentation analysis of L-P complexes. The formation of a complex between cDNA-derived P and L proteins demonstrated above by coimmunoprecipitation was confirmed by glycerol gradient sedimentation analysis. HeLa T4 cells infected with vTF7.3 were transfected with plasmids encoding L and P either alone or in combination, and cells were radiolabeled with Tran^[35S]label. Soluble cell lysates were sedimented on linear 5 to 20% glycerol gradients, and fractions from the gradient were denatured with SDS, diluted, and immunoprecipitated with a mixture of anti-L protein and anti-SV5 serum. When expressed in the absence of P, the cDNA-derived L protein was detected in fractions 1 to 5 near the bottom of the gradient, with a peak centered around fraction 4, and this sedimentation was found to correspond to ~8 to 10S (Fig. 5A). A second protein of unknown origin ($M_r \approx 110,000$) was seen in fractions 6 and 7 which may represent a vaccinia virus-specific polypeptide. When expressed in the absence of L, the P protein sedimented as a major species centered around fraction 8 (~4S; Fig. 5B). In contrast to the results obtained when P was expressed alone, coexpression of P with L gave a unique sedimentation profile (Fig. 5C). The L protein was again detected as a ~8 to 10S species (Fig. 5C, lanes 3 and 4), but the P protein was now present as two forms: one which sedimented like that seen when P was expressed alone (~4S), and a second form which cosedimented to the same portion of the gradient as the L protein (~8 to 10S;

FIG. 5. Glycerol gradient sedimentation analysis of cDNA-derived L-P complexes. vTF7.3-infected HeLa T4 cells were transfected alone or in combination with plasmids encoding the P protein (4 μ g), wild-type L, or L protein mutant 5 or 7 (6 μ g of each). Cells were radiolabeled from 11 to 12 hpi with Tran^[35S]label, and soluble cell lysates were analyzed by centrifugation through linear 5 to 20% glycerol gradients as described in Materials and Methods. Fractions collected from the bottom of the gradient were denatured with SDS and immunoprecipitated with a mixture of anti-L protein and anti-SV5 sera before analysis by SDS-PAGE. Shown are gradient profiles of polypeptides from cells transfected with plasmids encoding L protein only (A), P protein only (B), L and P proteins (C), L mutant 5 and P protein (D), or L mutant 7 and P protein (E). The position of a 110-kDa protein of unknown origin is marked (110 K). Centrifugation was from right (Top) to left (Bottom), and gradient fractions are numbered. Arrows indicate the positions of 9S, 7S, and 4S sedimentation markers that were analyzed in a parallel gradient.

fractions 3 and 4). Thus, these sedimentation data demonstrate that cDNA-derived L and P proteins can form an ~8 to 10S complex *in vivo* in the absence of other viral components, a result which is consistent with data obtained by Horikami et al. (18) for Sendai virus L and P proteins.

As a second assay to determine if the L protein deletion mutants described above interact with P, each of the 13 L protein mutants was coexpressed in HeLa cells along with P, and cell lysates were analyzed by glycerol gradient sedimentation. Two representative examples of the results obtained from this analysis are shown in Fig. 5D and E. When coexpressed with L mutant 5, the P protein was found as a ~4S peak (Fig. 5D), with no faster-sedimenting P protein detected. This

profile matched that seen when P was expressed by itself (Fig. 5B). The gel depicted in Fig. 5D was deliberately overexposed but did not reveal any P protein sedimenting in the ~8 to 10S region of the gradient. In contrast, analysis of lysates from cells coexpressing P with L deletion mutant 7 (Fig. 5E) revealed two distinct forms of P, one of which sedimented as ~4S (fractions 7 and 8), and a faster-sedimenting form was found in the same peak fraction as L mutant 7 (fraction 5). The slightly slower sedimentation of the mutant 7-P complex (Fig. 5E, lane 5) relative to that of the wild-type L-P complex (Fig. 5C, lane 4) may reflect the fact that this altered L protein is approximately half the size of full-length L. When expressed with P and analyzed by gradient centrifugation, each the L deletion mu-

tants produced a sedimentation profile that was similar to one of those shown in Fig. 5C, D, or E.

Alignments of L protein sequences have identified regions of high amino acid homology (34, 39). Three additional L protein mutants were constructed to examine the effects of small deletions in three of these conserved N-terminal domains on the formation of L-P complexes. On the basis of the locations of these conserved regions in the SV5 L protein, DNA segments encoding conserved domains I, II, and III were individually deleted from the SV5 cDNA to create mutants 14 to 16, respectively (see Materials and Methods). After coexpression of mutants 14 to 16 in HeLa T4 cells with P, lysates were assayed by gradient sedimentation for the presence of L-P complexes as described above. These three L mutants produced an L-P sedimentation profile matching that shown in Fig. 5D (data not shown) and thus were defective in the formation of stable ~8 to 10S complexes with P. Collectively, the ability of each altered L polypeptide to reproducibly interact with P in both the coimmunoprecipitation and cosedimentation assays was determined. From these data, the L mutants were scored as positive or negative for stable binding to P protein, and the summarized results are shown in Fig. 1. These results support the conclusion that regions of L important for the formation of stable SV5 L-P complexes reside in the N-terminal half of the L polypeptide.

Sedimentation analysis of L-P complexes from SV5-infected cells. Although cDNA-derived L and P can form an ~8 to 10S complex *in vivo* (18) (Fig. 5), it was unclear whether a similar complex is formed in SV5-infected cells. The analysis of paramyxovirus-infected cell lysates is complicated by the fact that it is likely that newly synthesized L protein rapidly associates with the viral NC structure (21, 36). Both L and P can be removed from NCs by high-salt conditions, but it is not known if the SV5 L-P complex is stable in high salt. As a first step in addressing this question, a titration experiment was carried out to determine the salt concentration needed to solubilize L from virus-infected cells. HeLa T4 cells infected with SV5 were radiolabeled for 30 min at 19 hpi with Tran[³⁵S]label, and cell lysates were prepared by using non-ionic detergent and increasing salt concentrations. Samples were centrifuged at speeds sufficient to pellet viral nucleocapsids (10). Aliquots representing equal fractions of the resulting pellet and supernatant were solubilized by boiling in SDS, and samples were immunoprecipitated with the anti-L protein or anti-SV5 serum. As shown in Fig. 6A, the majority of the L protein detected with anti-L protein serum was found in the pellet fraction when cell lysates were prepared with no additional salt present (0 lanes). However, cell lysis with detergent buffer containing progressively higher concentrations of salt resulted in a larger fraction of L recovered in the supernatant. Surprisingly, detergent buffer containing as little as 125 mM NaCl resulted in the solubilizing of >50% of the pulse-radiolabeled L protein from the cell lysates (Fig. 6A; compare lanes P and S, 125 mM NaCl). Nearly all of the pulse-radiolabeled L could be recovered in the supernatant fraction by using buffer with 500 mM NaCl, but a significant amount of this protein was detected as an aggregate near the top of the gel (500 mM, S lane). The salt-dependent fractionation profile of the pulse-radiolabeled P protein in these same lysates was similar to that of L (Fig. 6B) except that 250 mM NaCl was required to solubilize >50% of the newly synthesized P protein.

To determine if the solubilized P and L proteins could be found together in a distinct complex, radiolabeled SV5-infected cell lysates prepared by using 250 mM NaCl were analyzed by sedimentation through 5 to 20% glycerol gradi-

FIG. 6. Salt-dependent fractionation of L and P proteins from SV5-infected cells. HeLa T4 cells infected with SV5 (multiplicity of infection of ~5) were radiolabeled at 19 hpi with Tran[³⁵S]label for 30 min. Cell extracts were prepared in 0.5 ml of NP-40-containing buffer that had been supplemented with NaCl to the indicated final salt concentrations. Following centrifugation (160,000 × g, 10 min), aliquots corresponding to equal amounts of the resulting pellet (P) and supernatant (S) fractions were boiled in SDS, diluted with buffer, and immunoprecipitated with 12 µl of either the anti-L protein serum (A) or the antiserum to SV5 viral proteins (B) before analysis by SDS-PAGE.

ents. Aliquots from fractions of the gradient were immunoprecipitated with a mixture of anti-SV5 and anti-L protein sera before analysis by SDS-PAGE. The majority of the radiolabeled viral polypeptides, including the NP, M, and P proteins, sedimented slightly slower than the ~4S marker (Fig. 7, lanes 8 to 10). In contrast, the L protein was detected in the bottom one-third of the gradient as a major protein species that sedimented as an ~8 to 10S particle (lanes 4 and 5). Only trace amounts of L were recovered in the pellet at the bottom of the centrifuge tube (data not shown), suggesting that under these conditions the L protein had not formed insoluble aggregates. While the majority of P was found in the ~4S portion of the gradient with the NP protein (lanes 8 to 10), a smaller amount was detected in the same fractions as the L protein (lanes 4 and 5). Quantitation of the distribution of P in the gel depicted in Fig. 7 indicated that ~15% of the total radiolabeled P protein was found in the region of the gradient corresponding to ~8 to 10S (not shown). These data indicate that the SV5 P and L proteins can be isolated as a stable ~8 to 10S complex from virus-infected cell extracts, similar to the results obtained for cells cotransfected with P and L cDNAs.

DISCUSSION

The members of the family of nonsegmented negative-sense RNA viruses, including the paramyxoviruses, rhabdoviruses, and filoviruses, are similar in genome structure and replication strategy (1, 13, 27). A common theme in the replication of

FIG. 7. Glycerol gradient sedimentation analysis of SV5-infected cell polypeptides. SV5-infected HeLa T4 cells were radiolabeled for 30 min at 15 hpi with Tran[^{35}S]label, and lysates were prepared in 0.5 ml of NP-40-containing buffer supplemented with 250 mM NaCl. Clarified lysate was analyzed by sedimentation through a 12-ml linear 5 to 20% glycerol gradient as described in Materials and Methods. Fractions (1 ml) were collected from the bottom of the gradient, and an aliquot of each fraction was adjusted to 0.5% SDS, boiled, diluted with buffer, and immunoprecipitated with a mixture of anti-L protein and anti-SV5 serum before analysis by SDS-PAGE. Arrows indicate the positions of 9S, 7S, and 4S sedimentation markers that were analyzed in a parallel gradient. M is a marker lane of proteins immunoprecipitated from the cell lysate prior to loading onto the gradient.

these viruses is the use of a large multifunctional L polypeptide to catalyze many of the steps in RNA synthesis and processing. However, the L protein does not carry out these functions alone, as viral RNA synthesis requires a second polymerase-associated viral protein P (or VP35 for the filoviruses [27]). Indirect evidence from *in vitro* reconstitution experiments (10, 15), as well as results obtained by using biochemical and immunoelectron microscopic techniques, have indicated that the L and P polypeptides physically interact to form an active polymerase complex (18, 28, 36).

The goal of the experiments reported here was to use cDNA-derived L and P to identify regions of the paramyxovirus L protein that are important for interaction with P. The interaction of L with P was not disrupted by large deletions within the C-terminal portion of the L molecule, as determined by two independent assays. In contrast, all of the deletion mutants that were altered in the N-terminal half of L were defective in the formation of stable complexes with P. Surprisingly, L mutants that lacked only the N-terminal 100 amino acids (~5% of the full L protein; mutant 1) or contained small N-terminal deletions that individually removed conserved domains I to III (mutants 14 to 16) were not detected as ~8 to 10S L-P complexes on glycerol gradients. The defect in the ability of these N-terminal deletion mutants to interact with P could reflect the fact that these alterations have induced a global misfolding of L or that the P-binding site has been masked rather than deleted. A more interesting possibility is that the N-terminal site for P binding is composed of widely dispersed discontinuous regions in L that fold into a secondary structure easily disrupted by deletions.

It has been proposed on a theoretical basis alone that the viral L protein contains distinct domains that may be responsible for the individual steps in RNA synthesis (34, 39). The data presented here provide the first experimental evidence in support of a domain structure for L. These results are consis-

tent with a proposal that the structure of the L polypeptide consists of two independently folding halves; the N-terminal half of L contains the sites for P binding and the region which is thought to be the active site for RNA polymerization (34, 40), whereas the C-terminal half is not required for P binding but may be involved in interactions with the nucleocapsid. Alternatively, the C-terminal half of L could contain auxiliary functions not directly involved in RNA polymerization, such as the sites which catalyze the methylation or guanylation of mRNA (17). The deletions that created L protein mutants 7 and 8 removed large segments from the C-terminal half of L, and in both cases this included a precise deletion of conserved domain V (Fig. 1). All mutations that extended into L sequences located N-terminal to domain V resulted in polypeptides defective in P binding (e.g., mutants 10 to 13), suggesting that conserved region V may represent a border between two independently folding halves of L. Domain V is rich in cysteine (Cys) and histidine (His) residues that are conserved both in sequence and in spacing among viral L proteins (34, 39). A comparison of L protein sequences has led to the proposal that this Cys- and His-rich region may represent either a site for metal binding or a region that stabilizes the L structure (27, 34). In support of a possible role of this domain in L protein stability, it has been reported recently that amino acid changes associated with a transcription thermosensitivity phenotype for the VSV mutant *tsG16(I)* map to a Cys residue in conserved domain V of the L protein (19). Thus, the results from the deletion mutagenesis described here can be interpreted in the context of previous data to suggest a simple model for the structure of the L protein in which the Cys- and His-rich conserved domain V divides the molecule into two independently folding domains.

Although untested, it is very unlikely that the SV5 L protein deletion mutants described here would be active in RNA synthesis. Nevertheless, these results have important implications for future mutagenesis of other L proteins which might be targeted to disrupt an enzymatic activity without destroying the prerequisite interactions of L with the P protein. As a recent example, Canter et al. (5) have described a transcription-defective VSV L protein mutant that was constructed by deletion of a small C-terminal conserved region (domain VI). However, because of lack of prior knowledge of the location of the P-binding site on the VSV L protein and the ability of this altered L to bind to P, no conclusion concerning the exact role of this domain of L in RNA synthesis could be reached. By extension of the conclusions reached here to the structure of the VSV L protein, one would predict that the mutant VSV L protein containing this C-terminal deletion (5) would still bind P and that this C-terminal mutation has altered L activity by a different means.

The stoichiometry of the L and P polypeptides in the ~8 to 10S SV5 L-P complex is not presently known. The analysis of the ratio of these proteins is complicated by the fact that both free L and L-P sediment to the same region of the glycerol gradients, presumably as a result of the small contribution of P ($M_r \approx 46,000$) to the size of the L-P complex. Likewise, the ratio of L to P in the active polymerase complex that is bound to the NC template is unknown. However, after purification from virions by ion-exchange column chromatography, the VSV polymerase complex is reported to be an L_1P_1 heterodimer that sediments on glycerol gradients as a ~11S particle (28). The similarity in the SV5 ~8 to 10S L-P sedimentation value reported here to that of the ~11S VSV L-P particle (28) suggests that the SV5 L-P complex may also exist as a heterodimer.

At the present time, it is unclear why only a small portion of

the cDNA-derived soluble P protein is found as the ~8 to 10S species. Titration experiments (not shown) indicated that the relative amount of P in the ~8 to 10S fraction did not change dramatically with large increases in the amount of either P or L plasmid that was transfected, suggesting that L-P assembly may be governed by additional factors other than concentration. In lysates from SV5-infected cells, ~15% of the pulse-radiolabeled P protein that had been solubilized was found in the ~8 to 10S complex, while the remainder was found in the same gradient fractions as the NP protein. These two forms of P may correspond to the P-L and P-NP complexes, respectively, which have been previously described by using biochemical approaches (18) and by immunoelectron microscopy (36). Alternatively, the sedimentation of cDNA- or SV5-derived P as two separate populations may be the result of either an instability of the complex, an equilibrium between the L-bound and unbound forms of P, or two distinct pools of P protein which differ from each other in some manner. In support of this latter possibility, distinct subpopulations of the VSV P protein which differ in phosphorylation and transcription activity have been described (3, 8). This modification to the P polypeptide may control or be the result of interactions with the L polypeptide. Experiments are in progress to determine if the two forms of the SV5 P reported here differ by their phosphorylation patterns and to determine the role of phosphorylation in the assembly of the paramyxovirus L-P complex.

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