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Identification of Protein Regions Involved in the Interaction of Human Respiratory Syncytial Virus Phosphoprotein and Nucleoprotein: Significance for Nucleocapsid Assembly and Formation of Cytoplasmic Inclusions

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We have reported previously that the nucleoprotein (N), the phosphoprotein (P), and the 22-kDa protein of human respiratory syncytial virus (HRSV) are components of the cytoplasmic inclusion bodies observed in HEp-2-infected cells. In addition, coexpression of N and P was sufficient to induce the formation of N-P complexes detectable by either coimmunoprecipitation with anti-P antibodies or generation of cytoplasmic inclusions. We now report the identification of protein regions required for these interactions. Deletion mutant analysis of the P protein gene indicated that its C-terminal end was essential for interacting with N. This conclusion was strengthened by the finding that an anti-P monoclonal antibody (021/12P), reacting with a 21-residue P protein C-terminal peptide, apparently displaced N from N-P complexes. The same effect was observed with high concentrations of the C-terminal peptide. However, sequence requirements for the P protein C-terminal end were not absolute, and mutants with the substitution Ser-237→Ala or Ser-237→Thr were as efficient as the wild type in interacting with N. In addition, P and N proteins from strains of different HRSV antigenic groups, with sequence differences in the P protein C-terminal end, were able to coimmunoprecipitate and formed cytoplasmic inclusions. Deletion mutant analysis of the N gene indicated that large segments of this polypeptide were required for interacting with P. The relevance of these interactions for HRSV is discussed in comparison with those of analogous proteins from related viruses.

Human respiratory syncytial virus (HRSV), a member of the *Pneumovirus* genus of the *Paramyxoviridae* family, possesses a single negative-stranded genome RNA of 15,222 nucleotides for the A2 strain (21, 31). Similarities in gene order and certain gene products are observed between HRSV and other paramyxoviruses and related viruses, such as rhabdovirus (37). However, the HRSV genome encodes some unique proteins. For instance, two nonstructural polypeptides (NS1 and NS2) are encoded by genes located at the 3′ end of the genomic RNA, preceding the nucleoprotein (N) gene, which is nearest the 3′ end in the paramyxovirus and rhabdovirus genomes. In addition, HRSV encodes a 22-kDa (22K) protein with no homolog in other related viruses (20). The transcriptional map of the 10 separate gene products encoded by the HRSV genome is 3′-(leader)-NS1-NS2-N-P-M-SH-G-F-22K protein-L-(trailer)-5′ (for a recent review, see reference 6).

The actual view of HRSV genome replication and transcription is to a great extent inferred from data obtained with paramyxoviruses and rhabdoviruses. It is thought that the template for RNA synthesis is the ribonucleoprotein complex (RNP) of viral RNA and N and that the L protein, with the P protein cofactor, is the actual polymerase (for a recent review of paramyxovirus replication and transcription, see reference 33). Recent data obtained with in vitro transcription systems, based on viral RNP isolated from infected cells, support that view (22, 30). However, the fine details of HRSV RNA synthesis may differ from those for other viruses since extra viral and/or cellular proteins could be involved in such processes.

We have recently described the protein composition of inclusion bodies, first noted by electron microscopy in the cytoplasm of HRSV-infected cells (34). These inclusions could be immunolabelled with monoclonal antibodies (MAbs) directed against either the N, P, or 22K protein (14). Recent data indicate that L protein and viral RNA are also localized in the inclusion bodies (unpublished data), suggesting that these structures represent RNP aggregates. Transient coexpression in eukaryotic cells of N and P, independent of other HRSV gene products, resulted in the formation of cytoplasmic inclusions that resembled the inclusion bodies found in HRSV-infected cells (14). Although coexpression of the 22K protein with either N or P did not lead to microscopic inclusions, the former protein was incorporated into these structures if coexpressed simultaneously with N and P. Immunobinding assays demonstrated the existence of N-P and N-22K protein complexes in extracts of both HRSV-infected cells and transfected cells that coexpressed the corresponding proteins (14).

Although the N-22K protein complex is unique to HRSV, N-P complexes have been described for vesicular stomatitis virus (9, 10, 28), rabies virus (11), Sendai virus (8, 18, 19), and measles virus (23), among others. The picture emerging from those studies is that P protein interacts with recently made N (N^0) to prevent N assembling illegitimately and to deliver it to the nascent chain during genome replication (8). Then, a certain number of P molecules remain bound to RNPs.

As a first step toward elucidating the relevance of N-P complexes for the replicative cycle of HRSV, we have studied the sequence requirements of N-P interactions. The results obtained indicate certain parallelism with those derived from similar studies carried out with related viruses.

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MATERIALS AND METHODS

Cells and viruses. HEp-2 cells were grown in tissue culture petri dishes, using Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. HEp-2 cells were infected with HRSV as described previously (15).

The isolation and characterization of anti-N (except 021/2N)- and anti-P (except 021/12P)-producing hybridomas have been described elsewhere (13, 16).

Nucleic acid manipulations. Standard methods were followed as described by Sambrook et al. (42). pGEM-4-derived plasmids with inserts of the Long strain N (LN5) and P (LP5) protein genes under the T7 promoter have been described elsewhere (14).

Plasmids CHN and CHP, with inserts of the N and P protein genes of the CH18537 strain, respectively, were generated by reverse transcription-coupled PCR. Total RNA was isolated from HEp-2-infected cells by the isothiocyanate-CsCl method (5), and 2 μg was used for cDNA synthesis, using avian myeloblastosis virus reverse transcriptase and the primer LG3$^-$ (3′-T_{15}CGAAGGGCCC CG-5′; underlined is an *Ava*I site added for cloning purposes). PCR amplifications were carried out with Ampli-*Taq* polymerase and two pairs of primers that included LG3$^-$ and primers with the first 18 nucleotides of either N or P mRNA and a *Pst*I site at the 5′ end.

PCR-mediated mutagenesis was carried out by the procedure of Ho et al. (17) as described by Arbiza et al. (2). The basic protocol involved two separate amplifications with two pairs of primers. In each pair, one of the primers included the substituted nucleotide and was complementary to the mutagenic primer of the other pair. The amplified DNAs were mixed, denatured, and after dilution used for a third amplification with a pair of primers flanking the mutated site. This DNA was digested with appropriate restriction enzymes and used to replace a similar segment in plasmid LN5 or LP5 digested with the same enzymes. The presence of the mutant nucleotide in the new plasmids was confirmed by direct sequencing of the replaced segment. Details of the mutagenesis procedure and sequences of primers used to generate the different mutants can be obtained from the authors upon request.

Transient expression of HRSV P and NP proteins. The expression system used was the one described by Fuerst et al. (12), based on the expression of bacteriophage T7 RNA polymerase after infection of HEp-2 cells with the vaccinia virus recombinant vTF7-3. Subsequently, the cells were transfected with the different plasmids (2 to 6 μg/35-mm-diameter petri dish or proportionally adapted to other culture plates) by using Lipofectin. Transfected cells were then used for one of the following assays.

(i) Immunofluorescence staining. Twenty-four hours after transfection, cells growing in microchamber culture slides were fixed with cold methanol for 5 min followed by cold acetone for 30 s. After air drying, unspecific binding sites were saturated with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), incubated with the hybridoma supernatants indicated in the figure legends, and stained with a fluorescein isothiocyanate-labelled goat anti-mouse immunoglobulin antiserum.

(ii) Immunoprecipitation. Eighteen hours after transfection, the culture medium was replaced with methionine-free Dulbecco's modified Eagle's medium, and cells were incubated for 2 h at 37°C. Then 200 μCi of Tran^{35}S-Label was added to each 35-mm-diameter petri dish, and incubation continued for 3 to 4 h. At the end of this period, the cells were scraped off with a rubber policeman, pelleted by low-speed centrifugation, and washed with cold PBS. Extracts were prepared in lysis buffer (10 mM Tris-HCl [pH 7.6], 140 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]).

Immunoprecipitations were carried out with protein A-agarose beads (50 μl per sample) that were sedimented at 10,000 × *g* for 30 s and resuspended in 200 μl of antibody-containing hybridoma supernatant. After washing with PNS (PBS containing 0.5% Nonidet P-40 and 0.25 mg of BSA per ml), 250 μl of ^{35}S-labelled cell extracts (3×10^6 cpm per sample) was added, and incubation continued for 4 h at 4°C. After repeated washing with radioimmunoprecipitation buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), the bound material was eluted in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (2 min at 90°C). Proteins were analyzed by SDS-PAGE and autoradiography.

When peptides were included in the immunoprecipitation assay, serial dilutions of the peptides were preincubated with the extracts for 30 min at 4°C before being added to the antibody-coated agarose beads. The procedure was then continued as indicated above.

RESULTS

Identification of P protein sequences required for interaction with NP. We have shown previously that anti-P-specific antibodies coimmunoprecipitate N from extracts of either HRSV-infected cells or transfected cells that coexpress both polypeptides. In addition, as mentioned before, N and P colocalize in cytoplasmic inclusions of either HRSV-infected cells or transfected cells that coexpress both viral products (14). To identify P protein regions that were essential for these interactions, 12 mutants of the P protein gene were generated by PCR mutagenesis and cloned into pGEM-4 vector under the T7 promoter (Fig. 1A). Four mutants contained premature stop codons that truncated the P protein C-terminal end by between 6 and 122 amino acids. Eight further mutants had deletions at the amino-terminal end that eliminated between 6 and 219 amino acids. Expression of the mutant proteins was tested by using the vaccinia virus/T7 system of Fuerst et al. (12). Polypeptides of predicted sizes were detected in all cases by immunoprecipitation of ^{35}S-radiolabelled cell extracts (data not shown).

The capacity of the mutant P proteins to interact with N was tested in HEp-2 cells that were infected with vTF7-3 and then cotransfected with plasmid LN5, which encodes N, and each of the plasmids encoding the mutated P molecules. Complex formation was evaluated by immunofluorescence staining of fixed cells and by coimmunoprecipitation of P and N with specific antibodies. Representative results are presented in Fig. 1B and C.

Expression of the P protein, independently of other HRSV products, induced a diffuse cytoplasmic staining detected with anti-P MAbs (not shown; see reference 14). In contrast, coexpression of P and N led to the formation of cytoplasmic aggregates which localized preferentially around the cell nuclei (Fig. 1B). Immunoprecipitation of cell extracts previously transfected with plasmids LP5 and LN5 revealed the presence of a significant amount of N brought down by anti-P antibodies (Fig. 1C). In contrast, anti-N antibodies brought down only trace (or undetectable) amounts of P. Although there is no definitive explanation for the last result, other groups have also reported that anti-N MAbs coimmunoprecipitated slight amounts of P whereas substantial amounts of N were brought down by MAbs raised against HRSV P protein (36). This situation may be similar to that found in human parainfluenza virus type 1-infected cells, in which case some anti-N MAbs have been shown to release P from N-P nucleocapsid complexes (41). Alternatively, the absence of P protein in the immunoprecipitations done with HRSV anti-N MAbs may indicate the presence of free N protein in the cell extracts that would interfere with the immunoprecipitation of N-P complexes by anti-N MAbs. In any case, it is clear that anti-P antibodies were able to coimmunoprecipitate efficiently N and P.

Removal of the last six amino acids of the P protein (plasmid LP5ΔC6) abolished its capacity to coprecipitate N (Fig. 1C). Concomitantly, the staining of cells cotransfected with LN5 and LP5ΔC6 showed a diffuse cytoplasmic fluorescence (Fig. 1B), indicating the absence of cytoplasmic inclusions. Larger truncations of the P protein C-terminal end behaved similarly to the mutant encoded by LP5ΔC6 (examples in Fig. 1B and C and results summarized in Fig. 1A).

Deletions of up to 91 amino acids at the N-terminal end of the P protein did not inhibit coimmunoprecipitation of N (Fig. 1A). The results obtained with LP5ΔN6, LP5ΔN10, and LP5ΔN20 are shown in Fig. 1C. Whereas an increase in the P protein electrophoretic mobility was observed according to deletion size, the amount of N brought down by anti-P antibodies was essentially the same for all N-terminal mutants of P. In contrast to the immunoprecipitation results, deletions of the first 10 or 14 amino acids of the P protein inhibited the formation of cytoplasmic inclusions with N (results for mutant LP5ΔN10 are shown in Fig. 1B), although larger N-terminal deletions of 20 (Fig. 1B) to 30 amino acids did not have such an effect (Fig. 1A). The reason for these apparently contradictory results is unclear, but they suggest that removal of sequences deleted in LP5ΔN10 and LP5ΔN14 might have induced a conformational change in P which altered its capacity to coaggregate with N, whereas larger deletions restored the

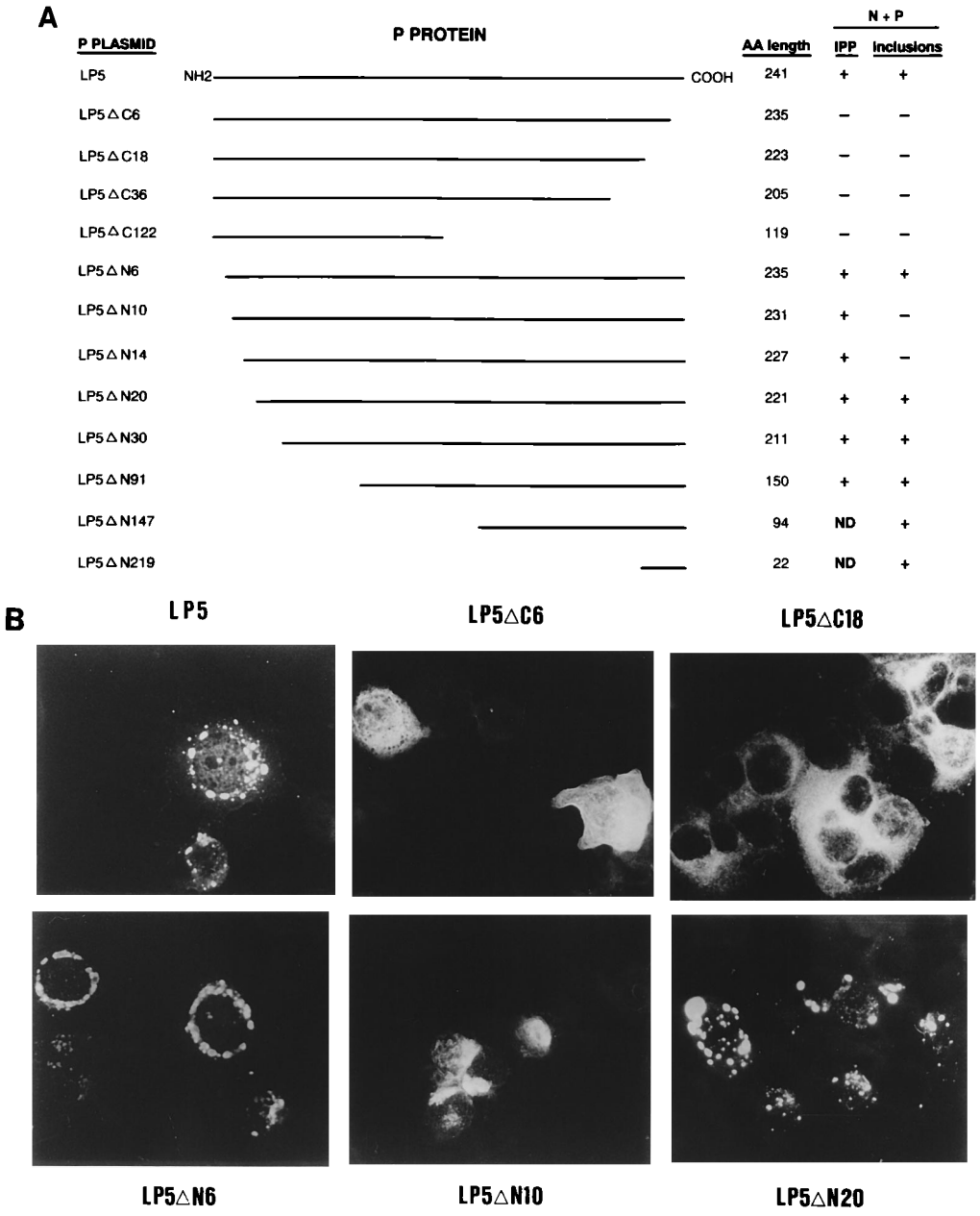


FIG. 1. Interaction of HRSV N with deletion mutant P proteins. (A) Horizontal lines represent either the HRSV Long strain P protein (LP5) or truncated versions of it. Plasmid names refer to the C- or N-terminal locations of P protein truncations. Protein lengths in amino acids (AA) are indicated at right. The different plasmids were tested in the transient expression assay after cotransfection with plasmid LN5 (examples are shown in panels B and C). The two rightmost columns indicate the presence (+) or absence (−) of N in the cell extract immunoprecipitates (IPP) obtained with anti-P antibodies or the formation of N-P cytoplasmic coaggregates (inclusions). ND, not detected. (B) Immunofluorescence staining of HEp-2 cells 24 h after cotransfection with LN5 and each of the plasmids indicated. Cells were stained with a pool of anti-P MAbs (1P, 76P, and 021/2P). (C) Immunoprecipitation of ^{35}S-labelled extracts of HEp-2 cells cotransfected with the indicated plasmids. Extracts were incubated with pools of either anti-N (lanes N; 79N, 42N, and 021/2N), anti-P (lanes P; 1P, 76P, 11P, and 021/2P), or irrelevant (lanes C) antibodies. The bound polypeptides were resolved by SDS-PAGE and visualized by autoradiography. The positions of N and P proteins are indicated at the left.

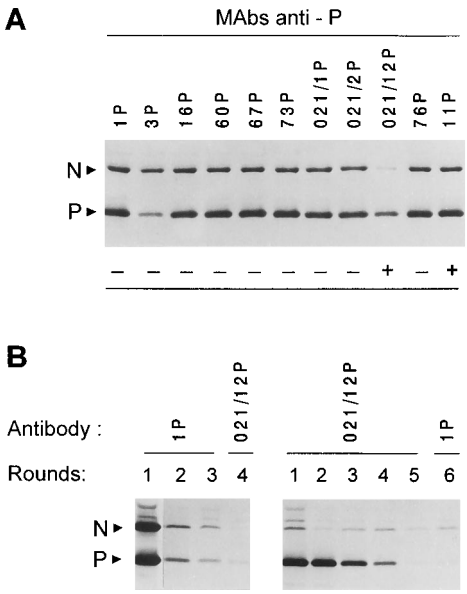


FIG. 2. Immunoprecipitation of infected HEp-2 cell extracts with different anti-P antibodies. HEp-2 cells were infected with HRSV Long strain (1 PFU per cell). Twenty-four hours later, cell cultures were labelled with Tran^{35}S-Label, and extracts were made in lysis buffer. (A) Aliquots of the cell extracts were immunoprecipitated with the anti-P antibodies indicated above the lanes. Proteins were eluted in sample buffer, separated by SDS-PAGE, and visualized by autoradiography. Enzyme-linked immunosorbent assay reactivity of each antibody with a synthetic peptide corresponding to the C-terminal 21 residues of the Long strain P protein is indicated (+ or −) below each lane. (B) Infected cell extracts were immunoprecipitated with antibody 1P or 021/12P, as indicated. After pelleting, the extracts were subjected to repeated rounds of immunoprecipitations (2, 3, etc.) with the indicated antibodies. After washing, the protein A-agarose beads from each round were boiled in sample buffer for 2 min, and the eluted proteins were visualized by SDS-PAGE and autoradiography.

native conformation of the remaining P protein sequence (see Discussion).

The polypeptides encoded by LP5ΔN147 and LP5ΔN219 were unstable and could not be detected under the labelling conditions used for the experiments shown in Fig. 1. Nevertheless, a small number of cells cotransfected with LP5ΔN147 plus LN5, and even a smaller number of those cotransfected with LP5ΔN219 plus LN5, contained cytoplasmic inclusions detected by immunofluorescence. It is likely that these structures were observed only in cells expressing the highest amounts of mutant proteins and that in those cases the interaction with N led to the formation of stable structures that could be detected by immunofluorescence.

In summary, the results in Fig. 1 indicate that sequences at the P protein C-terminal end are essential for interacting with N, as reflected by the absence of both coimmunoprecipitation and formation of cytoplasmic inclusions.

Further evidence for direct interaction of N with the C-terminal end of P was obtained after evaluating the capacity of different anti-P MAbs to coimmunoprecipitate N from extracts of HRSV-infected HEp-2 cells (Fig. 2A). Whereas N was brought down by 10 different anti-P MAbs, it was absent from (or very much reduced in) the immunoprecipitate obtained with antibody 021/12P. This antibody recognized a 21-residue peptide synthesized according to the sequence of the P protein C-terminal end (Long strain [27]). The epitopes of most other antibodies, which did not react with the 21-residue peptide, had been mapped previously toward the N-terminal end of the P protein (13).

To rule out the presence of P molecules uncomplexed to N that were specifically recognized by antibody 021/12P, extracts of HRSV-infected HEp-2 cells were depleted by repeated immunoprecipitation with this antibody (Fig. 2B). Although a faint band of N above background levels was observed after the first immunoprecipitation, it was absent from subsequent immunoprecipitates. In addition, no further P protein could be brought down by antibody 1P from extracts depleted with 021/12P. Similarly, repeated immunoprecipitations of HRSV-infected HEp-2 cell extracts with antibody 1P depleted P molecules bound to N, and no further P protein could be brought down by 021/12P (Fig. 2B). Thus, the last antibody seems to recognize a population of P molecules which is not different from those reacting with other anti-P antibodies. Although long-distance effects of antibody binding cannot be ruled out, the simplest interpretation of the results presented in Fig. 2 is that binding of antibody 021/12P to the C-terminal end of P was displacing N from the N-P complexes. The fact that antibody 11P, which reacted also with the P protein C-terminal peptide, did not have a similar effect may indicate that antibodies 11P and 021/12P react with different epitopes of the P protein C-terminal end. In fact, whereas antibody 11P reacts only with HRSV strains belonging to antigenic group A (16), the epitope recognized by antibody 021/12P is present in strains of the two antigenic groups (A and B) into which HRSV isolates have been subdivided.

Apparent displacement of N from N-P complexes was also observed when a large amount of the P protein C-terminal peptide P221-241 was preincubated with extracts of HRSV-infected cells before immunoprecipitation (Fig. 3). Addition of 1,000 μg of P221-241 reduced the amount of P brought down by antibody 1P to 50% of control values, whereas the amount of N in the same immunoprecipitates was negligible. In contrast, the P protein N-terminal peptide P2-21 had no effect on the amount of either P or N brought down by antibody 76P. The antibodies used for each immunoprecipitation were chosen according to previously reported epitope mapping onto the P protein primary structure (13). Thus, antibody 1P, which recognizes an N-terminally located epitope, was used when the C-terminal peptide was added to the extracts, and antibody

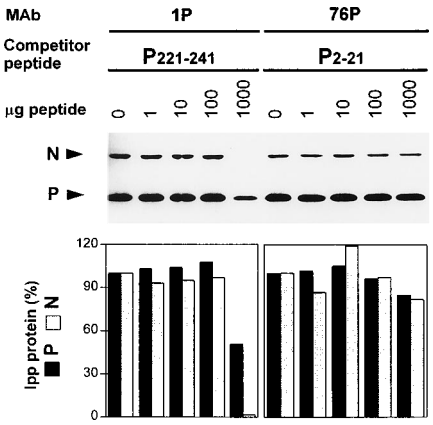


FIG. 3. Effect of P protein peptides upon coimmunoprecipitation of N. The cell extracts and the immunoprecipitation conditions were the same as for Fig. 2 except that the indicated amounts of peptides were added to the extracts and incubated for 30 min at 4°C before being used for immunoprecipitations with the antibodies indicated at the top. The synthesis and characterization of peptides have been reported previously (13). Their names denote residues included in their synthesis, according to the published sequence for the Long strain P protein (27). The bar graph represents quantitations done by densitometry of the N and P bands from the different immunoprecipitates, calculated as percentages of values obtained for each protein in the absence of peptide.

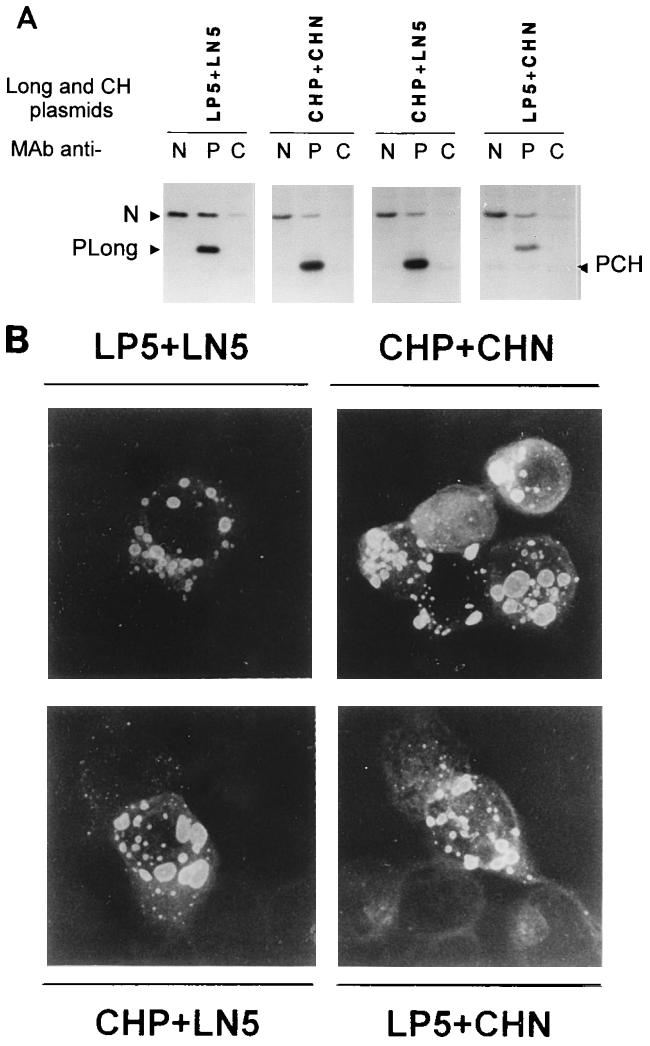
76P, which recognizes a centrally located epitope, was used when the N-terminal peptide was added as competitor. That a large (estimated to be at least 1,000-fold) excess of P221-241 over P was needed to displace N may indicate the presence of multiple conformations of the peptide in aqueous solution, a reduced affinity of the peptide for N compared with the native P, or both.

The results of Fig. 2 and 3 indicate that N could be displaced from N-P complexes by either binding of antibody 021/12P to the C-terminal part of P or interference with peptide P221-241. These data support the notion that the C-terminal end of P interacts directly with N, in agreement with the results for mutants (Fig. 1), but do not rule out the possibility that other P regions are required for such interactions.

The sequence requirements for interaction with N are conserved among the P proteins of HRSV strains belonging to antigenic groups A and B. Thus, when plasmid CHP, encoding the P protein from the CH18537 strain (antigenic group B), was used for cotransfections with LN5, which encodes the N protein from the Long strain (antigenic group A), both polypeptides were immunoprecipitated from cell extracts by anti-P antibodies (Fig. 4A). Similarly, cotransfection of cells with LP5, which encodes the P protein of the Long strain (group A), and CHN, which encodes the N protein of the CH18537 strain (group B), led to the formation of N-P complexes brought down by anti-P antibodies. The interaction of heterologous P and N, as observed in the immunoprecipitation test, was reflected in the formation of cytoplasmic coaggregates detected by immunofluorescence (Fig. 4B). It is worth mentioning that, as reported by others (1, 32, 35), the sequence differences between the P proteins of Long and CH18537, which have the same polypeptide length (25), were reflected in slight differences of their electrophoretic mobilities (Fig. 4A).

Comparison of P protein sequences from the Long and CH18537 strains (25) revealed amino acid differences clustered between residues 58 and 93 (Fig. 4C). Four amino acid differences were also observed within the 21 C-terminal peptides recognized by antibody 021/12P, but these changes did not alter the capacity of P protein to interact with heterologous N.

Since Ser-237 has been shown to be phosphorylated (29) and it is conserved between the P proteins of Long and CH18537 (Fig. 4C), we tested whether the replacement Ser-237→Ala or Ser-237→Thr had any effect in the formation of N-P complexes. The results shown in Fig. 5 demonstrated that P mutants with these replacements immunoprecipitated with N as effectively as the wild type did. In addition, both mutants induced the formation of cytoplasmic inclusions when coexpressed with N (not shown).



C

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LONG      MEKFAPEFHGEDANNRATKFLESIKGKFTSPKDPKKKDSIISVNSTDIEV-  50
CH18537             K           A S                   I

          TKESPITSNSTIINPTNETDDNAGNKPNYQRKPLVSFKEDPIPSDNPFSK- 100
                  GTN   IS A STPEA A  P   D     LT

          LYKETIETFDNNEEESSYSYEEINDQTNDNITARLDRIDEKLSEILGMLH- 150

          TLVVASAGPTSARDGIRDAMVGLREEMIEKIRTEALMTNDRLEAMARLRN- 200
                                          A

          EESEKMAKDTSDEVSLNPTSEKLNNLLEGNDSDNDLSLEDF        - 241
                             K  S   D       D
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FIG. 4. Interaction of P and N from HRSV strains of different antigenic groups. (A) HEp-2 cells were cotransfected with the plasmids indicated above the lanes. LP5 and LN5 encode the P and N proteins, respectively, of the HRSV Long strain (antigenic group A). Similarly, plasmids CHP and CHN encode the P and NP proteins of the HRSV CH18537 strain (antigenic group B). Eighteen hours after transfection, the cells were labelled for 2 h with Tran^{35}S-Label. Extracts were made and immunoprecipitated with the pool of anti-N (lanes N), anti-P (lanes P), or control (lanes C) antibodies indicated in the legend to Fig. 1. PLong and PCH denote the positions of Long and CH18537 P proteins, respectively. (B) HEp-2 cells transfected with the indicated plasmids were fixed 24 h later and stained with the pool of anti-P antibodies. (C) Amino acid sequence of P protein from the Long strain. Amino acid differences (darkened) are shown below for the CH18537 strain P protein.

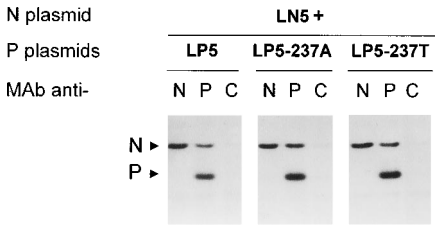

FIG. 5. Coimmunoprecipitation of NP with mutant P proteins containing the replacement Ser-237→Ala (LP5-237A) or Ser-237→Thr (LP5-237Thr). Plasmids encoding the mutant P proteins were generated as indicated in Materials and Methods and used to cotransfect HEp-2 cells with LN5. Plasmid LP5, which encodes the Long P protein, was included as a control. Extracts were made and immunoprecipitated with antibodies as indicated in the legend to Fig. 4.

Identification of N protein sequences required for interaction with P. Using an approach similar to that described above, N deletion mutants were generated to study the sequence requirements for interacting with P (Fig. 6A). Five mutants had stop codons that truncated the N protein C-terminal end by between 6 and 309 amino acids. Another mutant had a deletion that removed the first six N-terminal amino acids of N. The ability of each N mutant to interact with P was evaluated by coimmunoprecipitation and formation of cytoplasmic aggregates after cotransfection of LP5 with plasmids encoding each of the mutated N molecules. Representative results are shown in Fig. 6B and C and summarized in Fig. 6A.

Removal of the last six amino acids of N had no effect in either the formation of cytoplasmic inclusions or coimmunoprecipitation of P and N (compare the results for LP5 plus LN5 and LP5 plus LN5ΔC6 in Fig. 6B). Deletion of the last 39 amino acids of N did not affect its capacity to immunoprecipitate with P, but the formation of inclusion bodies was inhibited. Larger C-terminal deletions of N generated proteins that were detectable by immunofluorescence staining of transfected cells but not by immunoprecipitation with anti-N antibodies; nevertheless, the diffuse staining pattern observed after cotransfection of LP5 with either LN5ΔC58, LN5ΔC277, or LN5ΔC309 indicated the absence of cytoplasmic coaggregates of P and N.

Deletion of the N-terminal six amino acids of N did not ablate its capacity to coaggregate with P (Fig. 6B); however, the complex between the proteins encoded by LP5 and LN5ΔN6 seemed to be dissociated under the conditions of the immunoprecipitation test (Fig. 6C). In summary, large segments of the N protein are apparently required to form stable complexes with P. Further refinement of the regions involved in those interactions awaits analysis of other N mutants. Nevertheless, as shown in Fig. 4, the capacity of N to interact with P is conserved across HRSV antigenic groups.

DISCUSSION

The interaction of P and N proteins is a common feature of paramyxoviruses, such as Sendai virus (8, 38, 40) or measles virus (23), and of rhabdoviruses, such as vesicular stomatitis virus (9, 26, 28) or rabies virus (4, 11). In all cases, sequences located at the P protein C-terminal end are essential for interacting with N. However, other P protein regions may also be required for efficient N binding. In the case of rabies virus, two independent N binding sites are located within the N- and C-terminal P protein sequences (4). In Sendai virus, two separate C-terminally located P protein regions combine to form a single N binding domain (39). Recently, a third N-terminal domain of Sendai virus P protein which is required for interacting with soluble N (N^0) has been identified (8).

In analogy to those studies, we have found that the C-terminal end of HRSV P protein is essential for immunoprecipitation with N when both proteins are expressed in transfected cells. Removal of only six amino acids at the P protein C-terminal end abrogated its capacity to precipitate with N. Although it is possible that the deleted sequences compromised the conformation of the P protein and consequently its interaction with N, further evidence indicated a direct involvement of the P protein C-terminal end in this interaction. Both a MAb that recognized a C-terminally located epitope of P (021/12P) and a 21-residue peptide that represented the C-terminal end of P could displaced N from N-P complexes. These results, however, do not rule out the possibility that other regions of the P molecule participate in the interaction with N.

There is not, however, a strict requirement of all HRSV P protein C-terminal residues for interacting with N. Replacement of Ser-237 by either Ala or Thr did not inhibit N binding to P. Ser-237 is a major casein kinase II-mediated phosphorylation site which is apparently required for transcription but not for binding to nucleocapsids (30). In addition, P proteins of either antigenic group A or B viruses were able to interact with heterologous N, despite having four amino acid changes within the C-terminal 21 residues. There is 90% conservation of P protein sequences between the Long (group A) and CH18537 (group B) strains, but most sequence changes are clustered in a highly divergent region located between amino acids 58 and 93 (25). This sequence organization suggests a modular organization of the P molecule with two conserved domains separated by a short divergent region (25). The results in Fig. 1 indicate that a P molecule lacking the first 91 amino acids was metabolically stable and could interact efficiently with N, lending support to the proposed modular organization of P.

The sequence requirements of N to interact with P are ill defined in most nonsegmented negative-stranded RNA viruses. Large regions of vesicular stomatitis virus (44) and Sendai virus (18) N proteins have been shown to be essential for interacting with P. Although our analysis of HRSV N mutants is still very limited, the results in Fig. 6 indicate that large segments of N are required for interacting with P. The capacity of N to interact with P is maintained across the HRSV antigenic group boundary. Sequence changes between N proteins of both antigenic groups, which have 96% sequence identity, are scattered throughout the entire protein length (391 amino acids) (24).

As previously reported, coexpression of HRSV N and P proteins induces the formation of cytoplasmic aggregates that resemble the inclusion bodies found in infected cells (14). The inclusions of HRSV-infected cells contain RNA sequences of both positive and negative polarity (data not shown) and likely represent aggregates of viral nucleocapsids. We have not investigated whether the aggregates found in transfected cells coexpressing P and N contain RNA. Nevertheless, it is likely that, as in the case of vesicular stomatitis virus (28) or Sendai virus N (3), HRSV N would bind unspecific RNA to form nucleocapsid-like structures.

Whereas in measles virus the expression of N is sufficient to induce the formation of nucleocapsid-like aggregates, independent of other viral proteins (43), in HRSV, as in rabies virus (4), both N and P are required simultaneously for the formation of cytoplasmic coaggregates. The protein sequence requirements observed for coaggregation of HRSV P and N were similar to those found for coimmunoprecipitation of the two proteins. However, some differences were observed between

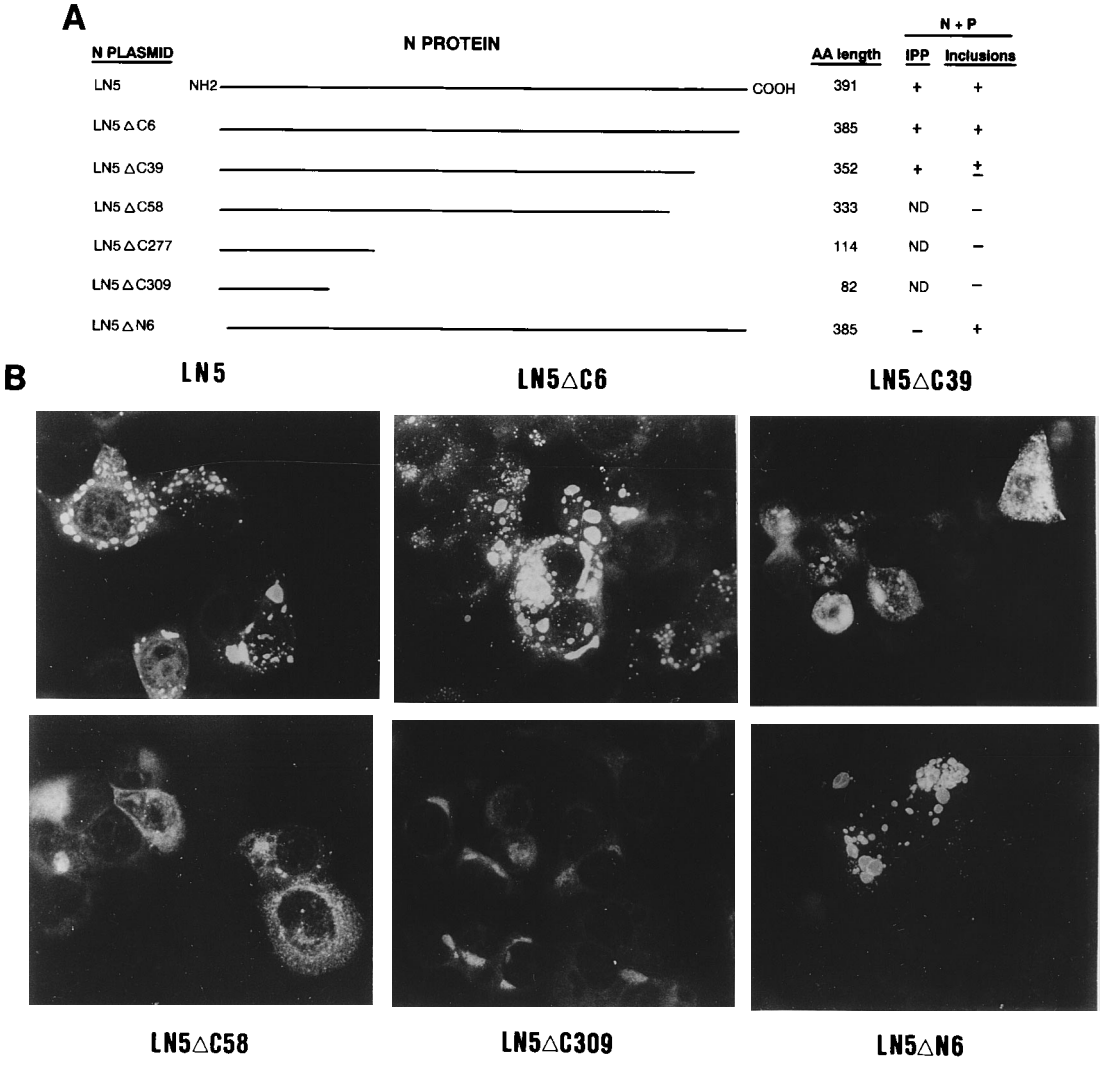

FIG. 6. Interaction of HRSV P with deletion mutant N proteins. Nomenclature, symbols, and abbreviations are as in the legend to Fig. 1. (A) Structure of Long strain N protein and deletion mutants. (B) Immunofluorescence staining of HEp-2 cells cotransfected with LP5 and each of the plasmids indicated. Cells were stained with a panel of anti-N antibodies (79N, 42N and 021/2N). (C) Immunoprecipitation of ^{35}S-labelled extracts of HEp-2 cells with the indicated antibodies.

the two assays. For instance, whereas P mutants encoded by LP5ΔN10 and LP5ΔN14 precipitated with N, either mutant failed to induce coaggregation of N. In a reciprocal manner, the N mutant encoded by LN5ΔN6 aggregated with P but did not precipitate with this protein. Thus, it is likely that the formation of stable N-P complexes involve subtle interactions of multiple regions of both protein components.

Although the relevance of cytoplasmic inclusions for the HRSV infectious cycle remains to be elucidated, these structures are present from the earliest postinfection time at which their protein components are observed in the infected cell (not shown). Thus, the formation of microscopic RNP aggregates is apparently an intrinsic property of their components rather than a consequence of the high constituent concentrations reached at late stages of the infectious cycle. The availability of assays for in vitro RNA synthesis (22, 30) and in vivo rescue of synthetic defective genomes (7, 45) should allow an evaluation of functional aspects of N-P complex formation and coaggregation with respect to the HRSV infectious cycle.

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