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Specificity of Rous Sarcoma Virus Nucleocapsid Protein in Genomic RNA Packaging

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Site-directed mutagenesis has shown that the nucleocapsid (NC) protein of Rous sarcoma virus (RSV) is required for packaging and dimerization of viral RNA. However, it has not been possible to demonstrate, in vivo or in vitro, specific binding of viral RNA sequences by NC. To determine whether specific packaging of viral RNA is mediated by NC in vivo, we have constructed RSV mutants carrying sequences of Moloney murine leukemia virus (MoMuLV). Either the NC coding region alone, the ψ RNA packaging sequence, or both the NC and ψ sequences of MoMuLV were substituted for the corresponding regions of a full-length RSV clone to yield chimeric plasmid pAPrcMNC, pAPrcψM, or pAPrcMψM, respectively. In addition, a mutant of RSV in which the NC is completely deleted was tested as a control. Upon transfection, each of the chimeric mutants produced viral particles containing processed core proteins but were noninfectious. Thus, MoMuLV NC can replace RSV NC functionally in the assembly and release of mature virions but not in infectivity. Surprisingly, the full-deletion mutant showed a strong block in virus release, suggesting that NC is involved in virus assembly. Mutant PrcMNC packaged 50- to 100-fold less RSV RNA than did the wild type; in cotranfection experiments, MoMuLV RNA was preferentially packaged. This result suggests that the specific recognition of viral RNA during virus assembly involves, at least in part, the NC protein.

Rous sarcoma virus (RSV) is a type C retrovirus; assembly of the core structure occurs only during or after virus budding at the plasma membrane. It begins with the formation, under the host cell membrane, of a core complex containing the *gag* and *gag-pol* polyprotein precursors, two 35S viral genomic RNA molecules, and the $tRNA^{Trp}$ primers of reverse transcription. Soon after budding, maturation of the viral particle yields the mature virus core, which contains fully processed *gag* and *gag-pol* proteins, as well as the diploid 70S genomic RNA (4, 5).

What are the elements important for specific viral RNA packaging? The *cis*-acting RNA packaging sequences, recognized by *trans*-acting factors during virus assembly, have been identified and characterized (for reviews, see references 2 and 26). Site-directed mutagenesis of the *gag* gene has shown that the retroviral nucleocapsid (NC) protein is involved in packaging viral RNA into the particles (9, 10, 14, 32, 33). Since virus core maturation occurs after budding, the packaging of viral RNA is performed by the full-length $Pr76^{gag}$ precursor; thus, NC functions as a domain of $Pr76^{gag}$ in packaging viral RNA (36, 46). Recently, sequence-specific binding of the human immunodeficiency virus type 1 (HIV-1) *gag* polyprotein $Pr55^{gag}$ to HIV-1 RNA in vitro has been reported (29), but the domain(s) involved in binding has not been defined. We have also shown that RSV NC is involved in dimerization of the two 35S viral RNAs (9, 33), but this event requires cleavage of the *gag* precursor (36, 46).

Despite the evidence that NC is involved in RNA packaging and dimerization, it remains to be elucidated which protein confers the specificity of recognition of genomic RNA. Retroviral NC protein binds to nucleic acids in vitro but with no apparent sequence specificity (22, 43, 47). Retroviral NC proteins have a conserved pattern of amino acids termed the Cys-His box (7). Mutation of any of the cysteine or histidine residues results in a dramatic decrease in viral RNA packaging and infectivity of the virus in vivo (9, 14). In Moloney murine leukemia virus (MoMuLV) NC, there are specific Cys-His box mutants with a suggestion of altered binding specificity (12, 31). Nevertheless, in light of the in vitro properties of NC, it is probable that the Cys-His box of NC is not sufficient for specific RNA sequence recognition by $Pr76^{gag}$. Thus, the region involved in specific binding may either be another part of the NC protein or another *gag* gene protein. To investigate the role of the NC protein in specific viral RNA packaging, we have analyzed the packaging phenotype of chimeric RSV $Pr76^{gag}$ proteins in which the NC protein was replaced by the sequence of MoMuLV NC. The results obtained suggest that some specificity resides in NC protein, but probably not enough to account for the selectivity seen in vivo. Moreover, our results suggest a new function in viral particle formation for NC, since a deletion mutant was unable to form particles.

MATERIALS AND METHODS

Cell culture. Chicken embryo fibroblasts (CEFs) prepared from SPAFAS eggs (Gs^- and Chf^-; SPAFAS, Inc., Norwich, Conn.) were grown in Dulbecco modified Eagle medium containing 5% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) at 37°C in an atmosphere supplemented with 5% CO_2.

Bacterial strains. *Escherichia coli* DH5α and CJ236 were grown as instructed by the manufacturer of the mutagenesis kit (Bio-Rad Laboratories, Richmond, Calif.). *E. coli* DH5α was rendered competent as previously described (38). Plasmid DNAs were purified from either small or large cultures by the alkaline lysis method and were further purified for transfection by equilibrium density gradient centrifugation in cesium chloride-ethidium bromide (38).

Cloned DNAs. Plasmid pAPrc has already been described

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(33). It contains a nonpermutated copy of the provirus RSV Prague C (PrC) strain. Plasmid pAsPrc is a *Sal*I-*Eco*RV subclone of pAPrc in pBR322 (33) containing the entire *gag* sequence. All mutations were constructed in pBS*gag*, a 773-bp *Pst*I-*Eco*RI fragment cloned in the phagemid vector Bluescribe(+) (Stratagene, San Diego, Calif.). Plasmid pNCA is a nonpermutated copy of the provirus MoMuLV (6) kindly provided by S. P. Goff. Plasmid CM1516 was kindly provided by C. Méric and contains the MoMuLV NC with two *Eco*RI linker insertions (Fig. 1).

Site-directed mutagenesis. The following oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer and purified as previously described (27): EchI (3′-CCAGAGAGAAGCCCCCTTAAGCGGCACCGGG CC-5′) and EchII (3′-CGGGAATTAATACCGTCATCACT TAAGGTTATCTCTCTCCC-5′). The mutant pBS*gag*Ech was constructed as described by Kunkel (21), with minor modifications. Briefly, a single-stranded uracil-containing DNA template was obtained by introducing the phagemid pBS*gag* into *E. coli* CJ236 (*dut ung*) and then infecting the cells with helper phage M13K07 as described by Vieira and Messing (48). Annealing of the oligonucleotides and synthesis of the mutagenic strand were performed as instructed by the manufacturer of mutagenesis kit (Bio-Rad). The resulting double-stranded DNA was introduced into *E. coli* DH5α (*dut*$^+$ *ung*$^+$ RecA$^-$) by the $CaCl_2$ transformation protocol, and the bacteria were grown on plates of Luria-Bertani medium containing ampicillin (100 μg/ml).

Introduction of the mutation was confirmed by the dideoxy-chain termination method of DNA sequencing, using avian myeloblastosis virus reverse transcriptase primed by a synthetic oligonucleotide complementary to the 3′ end of the RSV NC (50).

Construction of a RSV-MoMuLV chimeric plasmid. To introduce the *Eco*RI fragment containing the MoMuLV NC, the *Eco*RI site of plasmid pBS*gag* was mutated by digestion with *Eco*RI, filled in with the Klenow fragment of *E. coli* DNA polymerase I, and blunt-end ligated. The *Nco*I fragment of pBS*gag*Ech was then substituted in this construct to obtain pBS*gag*(-RI)Ech, which was used to delete the RSV NC or to exchange the MoMuLV NC by replacement with the *Eco*RI fragment from plasmid CM1516 (residues 2516 to 2638 of pNCA). The mutant sequences were cloned back into pAsPrc, using the *Nco*I sites to give pAsPrcEch, pAs PrcMNC (murine NC), and pAsPrcDelNC (deletion of NC). To clone the ψ packaging signal of MoMuLV into pAPrc, a *Sac*I-*Nde*I (residues 259 to 1291 of RSV) subclone of pAPrc was cloned in pUC18 to yield plasmid pUClead. A *Sac*I-*Pst*I (residues 418 to 1016) fragment of pNCA, containing the ψ of MoMuLV, was exchanged for the corresponding fragment of pUClead (residues 259 to 267 of RSV) to yield plasmid pUCleadψM. The *Sac*I-*Nde*I fragment from pUCleadψM was then cloned back into pAsPrc and pAsPrcMNC to yield plasmids pAsPrcψM and pAsPrcMψM, respectively. The *Sal*I-*Eco*RV fragments of the pAsPrc constructs were cloned back into pAPrc. To introduce a frameshift into pNCA matrix protein (MA) region, an *Eco*RI-*Sal*I fragment of pNCA (residues 10919 to 4154) was subcloned in pBS+, partially digested with *Pst*I in the presence of ethidium bromide as previously described (33), filled in with Klenow enzyme, and circularized by ligation. The mutated *Eco*RI-*Sal*I fragment was then reintroduced into pNCA (plasmids pNCA25 and pNCA37).

Transfection. CEFs, either freshly prepared or kept frozen in the presence of 15% glycerol, were used after two to seven passages. Transfection and virus infection were performed as described previously (32, 33). Exogenous template reverse transcriptase activity in virions purified from the medium of infected or transfected cells was measured as described previously (32).

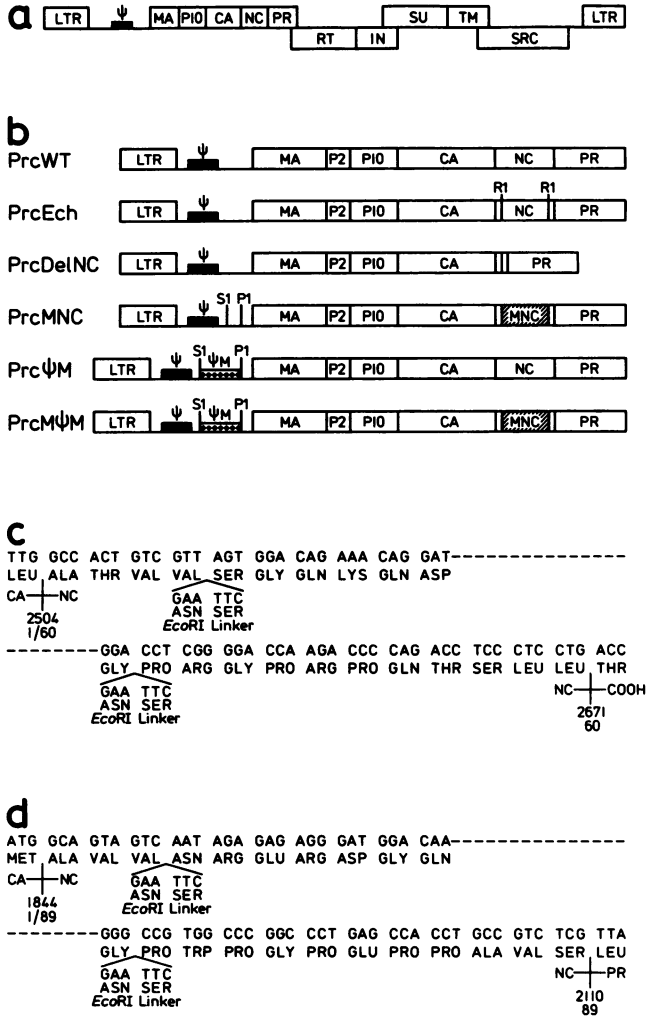

FIG. 1. (a) Complete genome of RSV PrC as a linear provirus. The regions encoding the *gag*, *pol*, *env*, and *src* genes are boxed. LTR, long terminal repeat; RT, reverse transcriptase; IN, integrase; SU, surface protein; TM, transmembrane protein. (b) Enlargement of the leader and *gag* gene of wild-type (PrcWT) and chimeric mutant constructs. The chimeric plasmids were constructed by replacing sequences from the infectious, full-length RSV PrC clone pAPrc, shown at the top, with corresponding MoMuLV sequences as described in Materials and Methods. MoMuLV sequences are shown in shaded boxes marked MNC or ψM. ψM, fragment inserted with the core packaging signal of MoMuLV; black boxes, core ψ sequence of RSV; open boxes, RSV sequences. Restriction sites used in construction of the plasmids: R1, *Eco*RI; S1, *Sac*I; P1, *Pst*I. (c) MoMuLV NC N-terminal and C-terminal amino acid sequences with the two 6-mer *Eco*RI linker insertions (mutant CM1516). NC is the C-terminal protein of MoMuLV Pr65^{gag} polyprotein. (d) RSV NC N-terminal and C-terminal amino acid sequences with the two 6-mer *Eco*RI linker insertions (mutant PrcECH). PR is the C-terminal protein of the RSV Pr76^{gag} polyprotein.

Protein analysis. Viral proteins produced by the transfected or infected cells were analyzed by immunoprecipitation and immunoblotting with polyclonal antibodies against RSV NC (p12), MA (p19), and capsid protein (CA; p27) as

described previously (33). Anti-Rauscher MuLV NC (81S-125) and anti-MoMuLV CA (77S-174) antibodies were obtained from the National Cancer Institute (Bethesda, Md.).

Purification of cellular and viral RNAs. Cellular RNA was purified by guanidinium thiocyanate lysis and centrifugation through cesium chloride as described previously (38). The RNA pellet was digested with RNase-free DNase I to remove contaminating plasmid DNA prior to electrophoresis in agarose-formaldehyde gels. Standard RNA transfer and hybridization protocols were used (38). The viral RNA of virions produced after transfection was purified as follows. Medium from transfected cells grown in two 100-mm culture dishes was harvested every 12 h and stored on ice. Cellular debris was removed by centrifugation at 15,000 × *g* for 10 min at 4°C, and an aliquot was kept for immunoblotting analysis. It was found to be critical not to freeze the harvested medium before RNA extraction. Virus was pelleted through a cushion of 20% sucrose in 1× NTE (100 mM NaCl, 50 mM Tris hydrochloride [pH 7.5], 1 mM EDTA) by centrifugation for 1.5 h at 35,000 rpm (160,000 × *g*) at 4°C in an SW40 rotor (Beckman Instruments, Inc., Fullerton, Calif.). The supernatant was carefully removed by aspiration to avoid contamination of the viral pellet with culture medium. The virions were lysed in a solution containing 100 mM NaCl, 50 mM Tris hydrochloride (pH 7.5), 10 mM EDTA, 1% sodium dodecyl sulfate, 100 μg of proteinase K per ml, and 50 μg of yeast tRNA per ml for 20 min at 37°C. RNA was extracted twice with phenol-chloroform and once with chloroform and then precipitated with 2.5 volumes of ethanol in the presence of 0.3 M sodium acetate (pH 5.2). The pellet was rinsed once with 70% ethanol, dried under vacuum, and digested with 10 μg of RNase-free DNase I (in the presence of human placental RNase inhibitor) per ml for 30 min at 37°C to remove contaminating plasmid DNA. The RNA was again extracted once with phenol-chloroform and once with chloroform and then precipitated with 3 volumes of ethanol in the presence of 0.3 M sodium acetate (pH 5.2). The pellet was rinsed carefully. For slot blot analysis, the RNA from 12 ml of medium was denatured by heat treatment at 68°C for 15 min in buffer (50% formamide, 6% formaldehyde, 1× SSC [0.15 M NaCl plus 0.015 M sodium citrate]), diluted 10- to 100-fold into 20× SSC, and loaded onto nitrocellulose with a slot blotting manifold (Schleicher & Schuell, Inc.). The filter was baked (80°C, 1 h), hybridized with labeled viral DNA, and exposed as described previously (38). To test for the presence of RNA dimer, viral RNA was analyzed by a nondenaturing Northern (RNA) blot procedure as previously described (18, 19).

RESULTS

Construction of MoMuLV-RSV PrC chimeric plasmids. The similarity in genome structure among retroviruses enabled us to exchange the NC proteins of RSV and MoMuLV in order to test whether this protein is functionally conserved between species and whether the specificity of RNA packaging resides entirely in this protein.

The level of amino acid sequence conservation between MoMuLV NC and RSV NC is rather low, except for the Cys-His motifs and for the overall basic properties of the protein. We inserted a restriction enzyme linker between the N- and C-terminal boundaries of each of the proteins by site-directed mutagenesis. RSV and MoMuLV use different strategies to regulate the expression of their reverse transcriptase moieties, (frameshifting and suppression of a nonsense codon, respectively); moreover, there is no protease

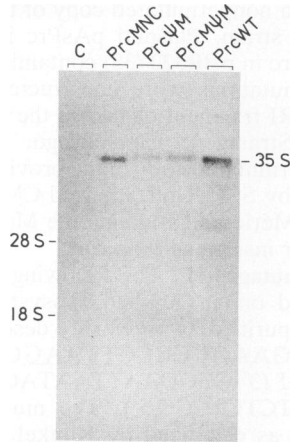

FIG. 2. Steady-state levels of viral RNA transcripts in transfected cells. The analysis was performed 60 h after transfection. Total RNA was extracted, electrophoresed in a denaturing agarose gel, and analyzed by Northern blotting, using a ^{32}P-labeled full-length pAPrC probe. Methylene blue staining of the filter after transfer was used to confirm the equivalence of RNA loading and transfer between samples and to determine the positions of the 18S and 28S rRNAs. Cells were transfected with the mutant DNA indicated above each lane. Lane C, control (cells transfected with no DNA); lane PrcWT, wild-type DNA transfection. 35S represents unspliced viral genomic RNA.

(PR) domain downstream of NC in MoMuLV Pr65^{gag} (Fig. 1). Therefore, the distal linker was inserted away from the C-terminal sequences of MoMuLV NC protein involved in the regulation of the nonsense codon suppression. A linker insertion at position 2636 of MoMuLV NC has been shown not to affect virus infectivity (28). Similarly, we have shown that a linker insertion at position 2047 of RSV NC had no effect on virus infectivity (33). We therefore inserted the C-terminal *Eco*RI linkers in these regions for both proteins (Fig. 1). At the N terminus, we inserted *Eco*RI linkers in a region with similar amino acid sequences in both proteins (Fig. 1) but distant from the viral PR target sequence. Proteolytic cleavage should, therefore, not be disturbed.

The viral core RNA packaging sequence of MoMuLV (ψ sequence) was also inserted both in the wild-type RSV and in the chimera. The sequence necessary for efficient packaging of MoMuLV RNA has been characterized (30) and shown to extend into the *gag* gene (ψ+ sequence) (3). We inserted a fragment which contains the minimally required core sequence described (nucleotides 215 to 355 of MoMuLV RNA), downstream of the packaging signal of RSV, in a region of the leader known not to be crucial for viral infectivity (17) (Fig. 1). The fragment inserted contains both the primer binding site and the splice donor site of MoMuLV RNA, as well as the last 33 nucleotides of U3, R, and U5 sequences (see Materials and Methods). U5 has been shown to enhance MoMuLV RNA packaging (35).

Transcription and translation of the chimeric mutants. Viral mutant DNAs were transfected into CEFs by a DEAE-dextran protocol as described previously (33). Transient expression of the viral sequences was analyzed by Northern blotting of total cellular RNA and hybridization with a full-length RSV probe. The steady-state level of 35S unspliced viral RNA appeared not to be significantly affected for the mutants, although it was lower for mutants containing the murine ψ region (Fig. 2, mutants PrcψM and PrcMψM).

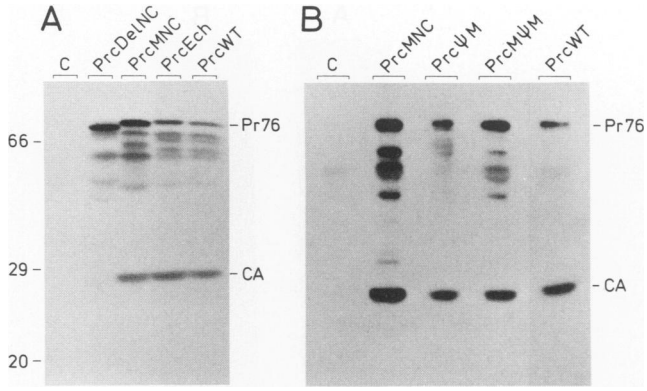

FIG. 3. Intracellular viral proteins of cells transfected with viral mutants. Cell lysates were immunoprecipitated with a polyclonal antibody against CA (p27) and then subjected to protein A-Sepharose adsorption. The eluted proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and detected by immunoblotting with anti-CA (p27) serum and ^{125}I-labeled protein A. Cells were transfected with the mutant DNA indicated above each lane. Lane C, control (cells transfected with no DNA); lane PrcWT, wild-type DNA transfection. Panels A and B represent two separate transfections. Sizes are indicated in kilodaltons.

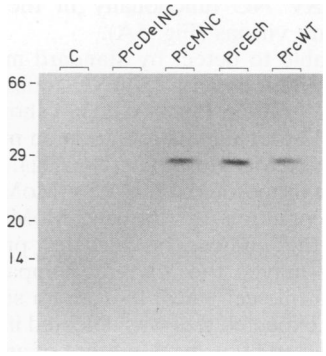

FIG. 4. Analysis of the virion *gag*-encoded proteins. Virions produced by the transfected cells were purified as described in Materials and Methods. Viral proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then immunoblotted with polyclonal antibodies against RSV CA and ^{125}I-labeled protein A. Cells were transfected with the mutant DNA indicated above each lane. Lane C, control (cells transfected with no DNA). Sizes are indicated in kilodaltons.

This lower level could be due to the presence of an additional murine splice donor site in the leaders of these mutants.

We analyzed the viral proteins produced by the mutants in transfected CEFs. Cells from one 100-mm-diameter petri dish were lysed, and the *gag* precursor was immunoprecipitated from the cell lysate with a polyclonal antibody against CA (p27). The viral proteins were eluted, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then immunoblotted with ^{125}I-labeled protein A and polyclonal anti-CA antibody. All of the mutants produced the viral *gag* precursor Pr76^{gag} and its cleavage intermediates (Fig. 3). A slightly smaller precursor was observed with mutants PrcMNC and PrcDelNC, which correspond to the expected sizes of the chimeric construct (74 kDa) and deletion mutant (69 kDa), respectively. Mutants PrcψM and PrcMψM produced precursor proteins size in similar to the wild type, suggesting that translation initiation still occurs at the RSV AUG located in position 380. The presence of a longer leader in these two mutants (an additional 598 nucleotides), containing two small open reading frames of 6 and 4 amino acids, respectively, does not significantly affect the efficiency or the position of translation initiation at the *gag* AUG. Mutant PrcECH also synthesized Pr76^{gag} precursor.

Protein composition of released virions. The effect of the chimeric NC mutants on the production of virus particles and the maturation of viral proteins was investigated as follows. Viral particles released by the transfected cells were purified through a cushion of sucrose and analyzed by Western immunoblotting, using polyclonal anti-RSV NC, anti-MoMuLV NC, and anti-RSV CA sera as described previously (33).

All of the mutants produced virus particles as efficiently as did the wild type except for mutant PrcDelNC, which produced no viral particles (Fig. 4 and 5). This is the first report of a mutation in RSV NC which affects virus particle formation. This result was confirmed by the analysis of intracellular proteins (Fig. 3A); since proteolytic cleavage occurs after virus budding, the presence of mature p27 (CA) protein in cells transfected by PrcECH, PrcMNC, and PrcWT was probably due to particles bound to the cell membrane since, in this experiment, adsorbed virus was not removed by trypsinization. However, fully cleaved CA was not detected after transfection with PrcDelNC, confirming that virus budding does not occur.

Viral NC protein was detected in mutants PrcECH (data not shown) and PrcψM and in the wild-type virus (Fig. 5A). Thus, the addition of the two *Eco*RI linkers does not perturb the maturation or the incorporation into the virion of the protein. With mutants PrcMNC and PrcMψM, viral particles were detected (with anti-CA), showing that MoMuLV NC

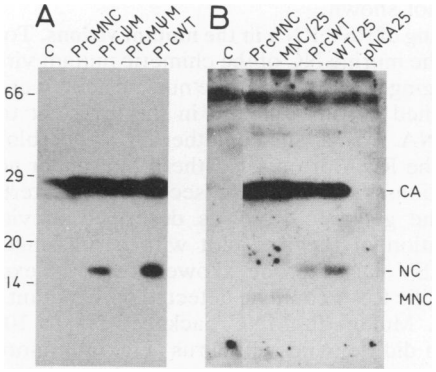

FIG. 5. Analysis of the virion *gag*-encoded proteins. Virions produced by the transfected cells were purified as described in Materials and Methods. Viral proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then immunoblotted with polyclonal antibodies against RSV CA and NC (A) or RSV CA, RSV NC, and MoMuLV NC (B) and ^{125}I-labeled protein A. Cells were transfected with the mutant DNA indicated above each lane as described in Materials and Methods. For cotransfection, PrcMNC or PrcWT and pNCA25 were added in a 5:1 ratio of input DNA (15 μg of PrcWT and 3 μg pNCA25, for example). Lanes C, control (cells transfected with no DNA); lane MNC/25, cotransfection of PrcMNC and pNCA25; lane WT/25, cotransfection of PrcWT and pNCA25). MNC represent the chimeric NC. In the experiment shown in panel B, the conditions were as described previously (31). Sizes are indicated in kilodaltons.

can replace RSV NC functionally in the assembly and release of mature virions (Fig. 5A).

We were unable to detect by standard methods the chimeric NC with either an anti-RSV NC antibody (Fig. 5A) or an anti-MoMuLV NC antibody (data not shown). The size of the protein could preclude its retention on nitrocellulose, as previously shown for MoMuLV NC (31). Moreover, the mixed antigenic properties of this RSV-MoMuLV NC could prevent its recognition by the anti-MoMuLV NC sera. Alternatively, the number of particles produced in our transient assay maybe too low, by comparison with that produced by murine cell lines, to detect a signal. However, a protein of the expected size was detected if more virus was loaded and detected by using a mixture of anti-RSV NC and anti-MoMuLV NC antibodies under more stringent conditions as described previously (31). This may be the chimeric protein, since it was not detected in the wild-type virus. Moreover, the size was that expected for the chimeric MNC protein (7 kDa) (Fig. 5B).

The reverse transcriptase activity of the mutant particles released in our transient assay was determined by the rapid dot assay described in Materials and Methods. Reverse transcriptase activity correlated well with the amount of viral protein released into the medium, as determined by densitometric scanning of the autoradiograph of the immunoblot (using the CA protein as the standard) (data not shown). The amount of CA protein was used to standardize the number of virions in all subsequent experiments.

Infectivity of the virus mutants. To determine whether the viral particles released after transfection were infectious, culture supernatants were added to fresh CEFs in the presence of DEAE-dextran. Replication-competent mutants were detected by immunoblot analysis. Virus CA and NC proteins were detected in the supernatant 4 days after infection of CEFs by wild-type or PrcECH virus (data not shown). Since no viral proteins were detected in the supernatant after infection with PrcψM, PrcMψM, PrcMNC, and PrcDelNC viruses, even after 3 weeks of infection, these data are not shown.

Packaging of RSV RNA in the mutant virions. To determine whether the murine NC of the chimeric mutant virus affected the packaging of RSV RNA, the nucleic acids were extracted from purified virions released in the transient transfection assay. RNA was analyzed either in a slot blot assay to quantify the RNA or on a Northern blot under nondenaturing conditions to maintain the secondary and tertiary structure of the genomic RNA as described previously (33). Hybridization of the slot blot with a full-length random-primed RSV DNA probe showed that, as expected, no RSV-specific RNA could be detected with mutant PrcDelNC (Fig. 6A). Mutant PrcMNC packaged 50- to 100-fold less RNA than did the wild-type virus (Fig. 6A); moreover, this RNA appeared to be poorly structured (see Fig. 8A).

Packaging of MoMuLV RNA by the chimeric mutants. It has been suggested that at least part of the specificity of recognition of viral genomic RNA is determined by the Cys-His box of NC (in MoMuLV) (12, 31). To determine whether PrcMNC would package MoMuLV genomic RNA in preference to RSV RNA, we cotransfected PrcMNC with plasmid pNCA25. This plasmid, derived from the MoMuLV provirus pNCA, has a +1 frameshift introduced at position 741 resulting in the creation of multiple stop codons just after the beginning of the MA coding region. This mutation produces a truncated *gag* protein starting at AUG 618 and ending at nucleotide 759. Thus, as translation of the *gag* proteins stops at nucleotide 759, the protein synthesized

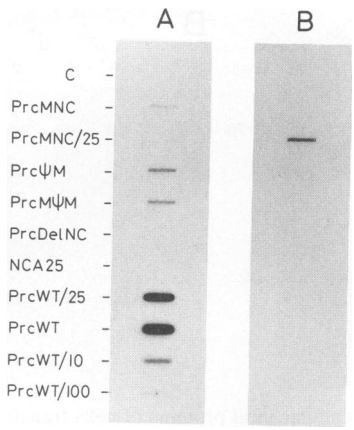

FIG. 6. Slot blot analysis of viral RNA packaged into virion particles. Virions were collected from CEFs transfected with the mutants indicated at the left, and the virion RNA was purified, loaded onto filters, and detected by hybridization. RNA was extracted from an equal number of virion particles, measured by the amount of CA released in the medium. For cotransfection, PrcMNC or PrcWT and pNCA25 were added in a 5:1 ratio of input DNA (15 μg of PrcWT and 3 μg of pNCA25, for example). Row C, control (cells transfected with no DNA); row PrcMNC/25, cotransfection of PrcMNC and pNCA25; rows PrcWT/10 and PrcWT/100, 10- and 100-fold dilutions of the RNA from wild-type DNA transfection; row WT/25, cotransfection of PrcWT and pNCA25. (A) RSV probe; (B) MoMuLV probe.

does not contain any P12, CA, or NC sequences, and the mutant is unable to form particles (Fig. 5B) and therefore to package RNA. No viral RNA was detected in the virus pellet with either an RSV-specific or MoMuLV-specific probe (Fig. 6) in a slot blot assay with this mutant. Thus, upon cotransfection, MoMuLV viral RNA will be packaged only if the *trans*-acting protein which recognizes the ψ packaging sequence is present. Using a MoMuLV-specific probe, we determined that plasmid pNCA25 was transcribed as efficiently as pNCA when transfected alone, whereas when it was cotransfected with PrcMNC or PrcWT, the amount of RNA detected was slightly less than expected (Fig. 7) (in cotransfection, input DNA was only 3 μg, whereas it was 15 μg when the plasmid was used alone). This result could be due to competition for a transcription factor between the RSV and long terminal repeat and the MoMuLV long terminal repeat. Similar results were obtained with a second independently derived clone of pNCA25 (pNCA37; data not shown).

We determined whether the presence of the competitor MoMuLV RNA affected the packaging of RSV RNA by the wild-type and mutant viruses by a nondenaturing Northern blot and slot blot procedure as described above. Cotransfection of wild-type RSV with pNCA25 had no effect on the efficiency of virus particle production (Fig. 5B) and of RSV RNA packaging (Fig. 6A) and dimerization (Fig. 8A). These data show that the truncated *gag* protein produced by pNCA25 does not interfere with virus particle formation. However, a clear inhibition of RSV RNA packaging in mutant PrcMNC resulted when pNCA25 or pNCA37 was cotransfected at a 5:1 ratio of input plasmid DNA (Fig. 6A and 8A).

In a parallel slot blot, viruses produced by the same cotransfection were analyzed for MoMuLV RNA packaging. Mutant PrcMNC packaged some MoMuLV RNA, while the wild-type RSV virus did not (Fig. 6B). However, this RNA

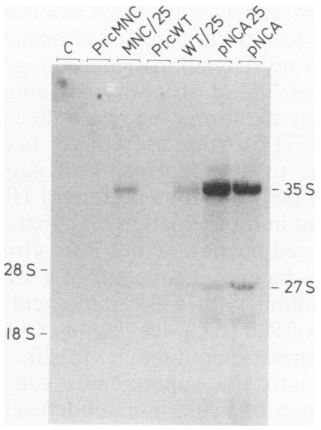

FIG. 7. Steady-state levels of viral RNA transcripts in transfected cells. RNA was isolated, electrophoresed, blotted, and methylene blue stained as described in the legend to Fig. 2 and Materials and Methods. The RNA was hybridized with a ^{32}P-labeled MLV-specific probe (pNCA). The positions of the 27S^{env} and the 35S unspliced genomic RNA are indicated. Cells were transfected with the mutant DNA indicated above each lane. For cotransfection, PrcMNC or PrcWT and pNCA25 were added in a 5:1 ratio of input DNA (15 μg PrcWT and 3 μg pNCA25, for example). Lane C, control (cells transfected with no DNA); lane PrcWT, wild-type DNA transfection; lane MNC/25, cotransfection of PrcMNC and pNCA25; lane WT/25, cotransfection of PrcWT and pNCA25.

appeared to be very degraded (as the RSV RNA which is detected in PrcMNC [Fig. 8A]), suggesting that dimerization is not efficient and the RNA is not fully protected in the RSV particle by the chimeric NC (data not shown).

The presence of the MoMuLV RNA ψ sequence does not enhance RSV RNA packaging by mutant PrcMNC. The results described above suggest that the chimeric NC can package MoMuLV RNA. To further investigate whether ψ and NC interacted, we constructed chimeric mutants having either the MoMuLV ψ sequence or both the MoMuLV NC and ψ sequences in the RSV PrC genome (PrcψM and PrcMψM, respectively). We analyzed the phenotype of the mutants by the same slot blot and nondenaturing Northern blot protocol. With an RSV RNA-specific probe, the control mutant with an RSV NC and MoMuLV ψ (PrcψM) packaged genomic RNA 20- to 30-fold less efficiently than did the wild type (Fig. 6A), and dimerization was inhibited (Fig. 8B). This finding could explain why mutant PrcψM is not infectious, the RNA probably not being able to initiate reverse transcription. Mutant PrcMψM packaged even slightly less RNA (30- to 40-fold) than did mutant PrcψM (Fig. 6A). Comparison between the ability of PrcMNC to package RSV RNA with an avian ψ signal with the ability of PrcMψM (having both a murine ψ signal and NC protein) revealed some improvement in packaging efficiency, confirming the results of the competition experiments (Fig. 6A).

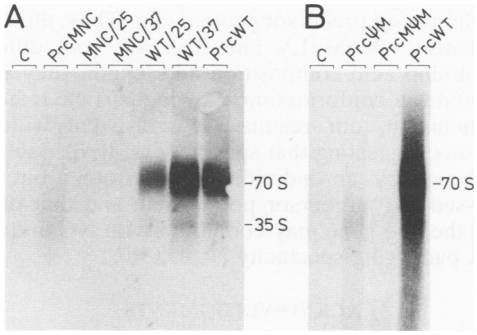

FIG. 8. Viral RNA content of the virions produced in a transient transfection assay. The virions were purified and viral RNA was extracted as described in Materials and Methods. After size fractionation on a nondenaturing 0.8% agarose gel, the RNA was electrotransferred to a nylon membrane and hybridized with a probe specific for RSV RNA (plasmid pAPrc) ^{32}P-labeled by random primer extension. The RNA for each mutant was extracted from equivalent numbers of virions (normalized to the level of CA protein). Cells were transfected with the mutant DNA indicated above each lane. For cotransfection, PrcMNC or PrcWT and pNCA25 or pNCA37 were added in a 5:1 ratio of input DNA (15 μg of PrcWT and 3 μg pNCA25, for example). Lane C, control (cells transfected with no DNA) lane PrcWT, wild-type DNA transfection; lanes MNC/25 and MNC/27 cotransfection of PrcMNC with pNCA25 and pNCA27; lanes WT/25 and WT/37, cotransfection of PrcWT with pNCA25 and pNCA37. 70S indicates the dimerized RSV genomic RNA.

DISCUSSION

The aim of this study was to investigate the role of the NC domain of Pr76^{gag} in virus maturation and specificity of RNA packaging by creating chimeric RSV Pr76^{gag} mutants. The high degree of sequence conservation of the Cys-His box, the overall basic amino acid composition, and the common nucleic acid binding properties of retroviral NCs (7) prompted us to exchange this protein between avian and murine virus and test whether the NC remains functional. Such an approach has already been used successfully for the expression of the PR protein of HIV-1 in a heterologous host virus (MoMuLV) (20).

NC and RNA packaging. Data from in vitro studies showing the nonspecific binding of RNA by isolated NC protein (8, 22, 43) demonstrating that this nonspecific RNA binding can occur even if the cysteine residues are oxidized (15) suggested that NC is involved only in internalization of viral genomic RNA molecules into immature cores by nonspecific binding and that the recognition of viral RNA would not be affected greatly in our construct. However, the phenotype of the mutant PrcMNC was not consistent with this hypothesis, since it shows both a large reduction in packaging efficiency and preferentially binding of a homologous (MoMuLV) RNA.

The sequence of the NC protein encoded by PrcMNC is very similar to the sequence of NC (P10) of MoMuLV (Fig. 1). However, there is a significant difference between the NC proteins of RSV and MoMuLV. The former contains two Cys-His motifs, whereas the latter encodes only one. The C-terminal regions of both proteins are proline rich, and there is a conserved pair of lysines after the proximal box of RSV and the motif of MoMuLV. We have previously demonstrated that the proximal box of RSV NC is more important in RNA packaging and that deletion of the distal box reduced RNA packaging to a degree similar to that observed in PrcMNC (9, 32). Therefore, the phenotype observed for PrcMNC may reflect the necessity of a secondary structure of NC resulting from the interaction of the two Cys-His motifs for the efficient recognition of RSV ψ sequences during virus assembly. The phenotype does not result from amino acid changes introduced by the linker insertions, since these alone have been shown to have no effect on RSV (PrcECH) or MoMuLV (CM1516).

In concordance with the fact that PrcMNC encodes an NC

protein which, at least in primary sequence, resembles the NC of MoMuLV, the data show that MoMuLV RNA is packaged more efficiently than RSV RNA. PrcMNC not only is able to package MoMuLV RNA (wild-type virus is not) but is able to discriminate between MoMuLV and RSV RNAs, since in cotransfection experiments, RSV RNA packaging is further reduced. However, packaging of RSV genomic RNA or MoMuLV genomic RNA is less efficient than for either wild-type RSV or MoMuLV. In the case of packaging of RSV RNA, the complex packaging signal of an avian retrovirus (the 5′ ψ signal [17, 25, 42] and the 115-nucleotide direct repeats bordering the *src* gene of RSV [44]) are recognized less efficiently by the PrcMNC mutant protein. This probably results from the incorrect primary and secondary structure of the Cys-His motif as discussed above but may also involve the overall structure of the *gag* precursor protein, in that the folding of RSV Pr76^{gag} may be inappropriate for the correct presentation of the MoMuLV-like NC. This may also explain the reduced efficiency of MoMuLV RNA packaging by comparison with the wild type. In this respect, it should be noted that in MoMuLV Pr65^{gag}, NC is the C-terminal protein, but in RSV Pr76^{gag}, the C-terminal protein is PR and NC is encoded upstream of PR. However, the data do not exclude the possibility that another protein acts cooperatively with NC as a *trans*-acting specific RNA packaging factor. Moreover, the recent report that for the avian mutant SE21Q1b the decreased specificity of RNA packaging may not be due to mutation of the NC protein (1) is consistent with such a possibility.

Which other protein could be involved in the selection of retroviral RNA in the cell cytoplasm before core assembly? Recently it was shown that in vitro, MA (p15) of bovine leukemia virus binds selectively to its 5′ leader ψ sequence, whereas the NC protein does not exhibit any binding specificity (16). For avian retroviruses, it was claimed that MA interacts with a specific region of the genomic RNA (23, 24), but other results contradict this finding (45). In MoMuLV, P12, encoded between MA and CA, was shown to bind specifically in vitro with the homologous genomic RNA (39–41), but there are no data concerning the in vivo role of this protein in RNA binding.

Since in cotransfection experiments it is not possible to control the level of expression of each RNA within a single cell, we constructed the mutant PrcMψM, which encoded the MoMuLV-like NC and contained the minimal MoMuLV ψ signal (215 to 355) in the leader of RSV. The presence of a splice donor site in this region had little effect on RNA splicing, and the presence of an extra 598 nucleotides to the RSV leader did not affect translation. PrcMψM packaged its genomic RNA with an apparent lower efficiency than PrcMNC packaged MoMuLV RNA (Fig. 6). The addition of the ψ sequence to wild-type virus (mutant PrcψM) reduced the efficiency of packaging of its genomic RNA to less than 5% of the wild-type level. This effect may be due to interference with the packaging signal of RSV, since the leader is short and highly structured.

NC and virus assembly. We constructed a deletion mutant of NC which shows a strong defect in virus particle release. This is the first report of a mutation in the NC coding region of an avian retrovirus affecting virus assembly. In lentiviruses, such as HIV-1 or feline immunodeficiency virus, a role for NC in virus assembly has been suggested from the expression of *gag* protein mutants in baculovirus vector systems. Deletion of the C-terminal p6 region of HIV-1 NC (p16) resulted in an inhibition of particle formation and the accumulation of tubular structures in the cytoplasm (11). However, a later report showed that deletion of p16 resulted in the failure to form viral cores or viruslike particles in the absence of an N-myristylation signal; the presence of either the p9 domain of NC or an N-myristylation sequence was sufficient to allow the formation of corelike particles (37). It was suggested (37) that the discrepancy between these two reports was due to low expression of *gag* in the former. Expression of a p6^{gag} deletion mutant of HIV-1 in mammalian cells resulted in the formation of immature-looking virus particles at the cell membrane but little virus release (13).

Expression by baculovirus vector of a feline immunodeficiency virus mutant with a 34-amino-acid deletion of the C-terminal end of P10 (NC), leaving the Cys-His box intact, resulted in the formation of tubular viruslike particles, which were released into the supernatant, suggesting that the C-terminal domain of feline immunodeficiency virus NC is required for spherical particle formation (34).

On the basis of previous studies of deletion mutants of RSV NC protein in which either the first box, the second box, or the whole central part of the protein has been removed (32, 33), we can conclude that the assembly domain probably lies between the N-terminal part of the protein and the first Cys-His box. In a simian virus 40-based expression vector, used to produce chimeric *gag* protein, a C-terminal deletion of all of the PR and NC coding regions and part of the CA coding region resulted in the synthesis of a truncated *gag* precursor ending at amino acid 418 (mutant ΔBg-Bs) and abolished particle formation in COS-1 cells (49). Thus, in this mutant, the same assembly domain would have been deleted.

The observation that the murine NC is able to rescue the particle release defect seen in mutant PrcDelNC is striking. First, the murine protein is shorter and contains only one Cys-His box. Second, in MoMuLV, NC (p10) is part of a shorter precursor (Pr65^{gag}) without a PR. Deletion of the PR in the RSV Pr76^{gag} precursor does not abolish particle formation but does abolish RNA packaging, and this was suspected to be the result of a change in the conformation of the remaining *gag* precursor protein (36). Thus, although the NC proteins of MoMuLV and RSV are very different in primary amino acid composition and length, they are both able to confer a conformation allowing particle release.

In conclusion, our results are consistent with recent publications suggesting that specific viral RNA packaging is mediated not by an individual *gag* protein but by the unprocessed *gag* precursor polyprotein and that other domains of the *gag* gene may cooperate with NC in determining RNA packaging specificity (1, 29, 36).

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